Original Article

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Dentistry Section

Cymbopogon Citratus (Lemongrass Oil) Oral Sprays as Inhibitors of Mutans Streptococci Biofilm Formation

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ABSTRACT

Introduction: Dental caries that occurs in young children or Early Childhood Caries (ECC) remains highly prevalent worldwide. *Streptococcus mutans* is not only one of the pioneer groups in plaque formation but is also crucial for its continuous development. It is important to prevent the presence of Mutans Streptococci (MS) for more effective prevention of dental caries.

Aim: To evaluate the susceptibility of *Streptococcus mutans* (ATCC 25175), *Streptococcus sobrinus* (ATCC 6715) and three *Streptococcus mutans* clinical isolates from Thai children to three oral spray formulations (2%L38, 4%L40, 6%L42) of *Cymbopogon citratus* extracted oil, and evaluate the inhibitory effect of each formulation on growth and biofilm formation of each strain.

Materials and Methods: *S. mutans* clinical isolations were obtained from children (12 males, 5 females aged 3.5-10-years-old) who came to the Paediatric Dental Clinic, Faculty of Dentistry, Chulalongkorn University, Bangkok. The inhibitory effect of each formulation on growth and biofilm formation was performed using a 96-well polystyrene plate biofilm assay. The inhibition effect was further analysed using fluorescent dyes (SYTO9 and propidium iodide) and Confocal Laser Scanning Microscopy (CLSM). All experiments were performed in triplicate. Data was expressed as mean±SD. For the determination of live/dead cells at the excitation wavelength of 485 nm, the green emission at 530 nm (live cells) and the red emission at 630 nm (dead cells) were measured and their ratio was calculated. The Kruskal-Wallis Test (Bonferroni and Dunn Test) was used to compare the experimental and control groups (significance at 95%).

Results: All oral spray formulations showed excellent antibacterial activity. Results showed that 3 and 6 hours. preformed biofilms of all strains were sensitive to all formulations (100%) when compared to the controls. Biofilm formation of all strains was inhibited (100%) when compared to the controls by all formulations at different concentrations. Six percent (L42) oil formulations showed the maximum inhibition effect, followed by 4% (L40) and 2% (L38), respectively. Results from CLSM analysis of *S. mutans* (ATCC 25175) showed that 6% (L42) oil formulations gave the maximum inhibition effect, followed by 4% (L40) and 2% (L38), respectively. For *S. sobrinus* (ATCC 6715), 2% (L38) oil formulations showed the maximum inhibition effect, followed by 4% (L40) and 6% (L42). For all *S. mutans* clinical isolates, all formulations inhibited biofilm effectively and were not significantly different.

Conclusion: In this study, all oral spray formulations from *Cymbopogon citratus* essential oil showed excellent antibacterial activity and they might be an alternative inexpensive natural medication to prevent dental caries in the near future.

Keywords: Biofilm, Confocal laser scanning electron microscopy, Essential oils, Lemon grass, *S. mutans*, *S. sobrinus*

INTRODUCTION

Dental caries is an infectious disease that results from the dissolution of tooth mineral by acids derived from bacterial fermentation of sucrose and other dietary carbohydrates [1]. It is a multi-factorial disease that includes cariogenic microorganisms, substrate and a susceptible tooth/host. Microorganisms are one of the major components in dental caries progression. High risk caries children are colonised early in life by cariogenic bacteria transmitted from caregivers or other family members through salivary contact [1,2].

Early childhood caries is a destructive, debilitating and the most common chronic childhood disease that affect the young children. Not life-threatening but it affects normal health and well-being of the child [2]. Though dental caries prevalence has reduced worldwide, ECC prevalence is still high and is currently a WHO concern [2,3, 4]. Severe ECC as the name indicates is a severe, aggressive and debilitating form of this disease with ramifications.

Even though in Thailand, caries prevalence has been reported to be decreasing, it is still in a rate of high prevalence [5,6]. The consequences of ECC includes a higher risk of new carious lesions in both primary and permanent dentitions, hospitalisations, high treatment costs and diminished oral health-related quality of life [2].

Biofilm or dental plaque is a complex microbial community composed of numerous aggregated microorganisms that attach to a surface and are surrounded by an extracellular polymeric matrix [7,8]. The acidogenic bacterial species *Streptococcus mutans*, in combination with various lactobacilli, is recognised to be involved in cariogenic processes [9]. Over time, its attributed role changes from that of the pathogen (specific plaque hypothesis) to enhancer (active role) and/or indicator (passive role) in a sugar-triggered cariogenic vicious circle (extended caries ecological hypothesis) [8,9].

Epidemiological studies have consistently demonstrated that MS, which include *Streptococcus mutans* and *Streptococcus sobrinus,* are the primary cariogenic microorganisms associated with dental caries [10–13]. It was not found only in the high risk caries group but also in low risk or cavities-free groups [14,15]. Children who were either colonised early by MS or presented with persistently higher levels of MS experienced significantly more ECC [16,17]. Although *S. sobrinus* is less prevalent than *S. mutans*, studies have suggested that the presence of both species in dental plaque is positively correlated with ECC development [16,17]. MS is not only one of the pioneer groups in plaque formation but is also crucial for

its continuous development. It is important to prevent the presence of MS for more effective prevention of dental caries.

Essential Oil (EO) has been reported for its effectiveness against bacteria embedded within the biofilm [18-21]. Lemongrass oil (*Cymbopogon citratus*) is a volatile oil obtained from lemongrass leaves. It is widely used in the perfume and cosmetic industries. It is mainly composed of citral, a natural mixture of geranial and neral and some myrcene, geraniol and geranyl acetate, which is a potential natural biocide for use as a disinfectant [22]. In this study, we developed three oral spray formulations, 2% L38, 4% L40, and 6% L42, to test. The efficiency of these oral spray formulations on the inhibition of biofilm growth and formation, along with a susceptibility test of *S. mutans* clinical isolations, should be further investigated before starting future clinical trials.

Confocal Laser Scanning Microscopy (CLSM) has been used to evaluate bacterial viability in numerous experiments. Fluorescence dyes are applied on the biofilm to differentiate live bacteria from dead bacteria, allowing the bacteria to be distinguished according to cytoplasmic membrane permeability. Moreover, 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 [23-25]. CLSM can capture a series of image-scans showing changes in the viability of the bacterial cells over time, making the real-time visualisation of death of the microorganisms possible.

The objectives of this study were to: 1) Evaluate the susceptibility of MS and three clinical isolations from Thai children to different oral spray formulations of *Cymbopogon citratus* essential oil; 2) Evaluate the inhibitory effect of each oil formulation on biofilm growth and formation of each strain using a 96 well-polystyrene plate biofilm assay; and 3) Analyse the inhibition effect on biofilm using fluorescent dyes and CLSM.

MATERIALS AND METHODS

Essential Oil Preparation

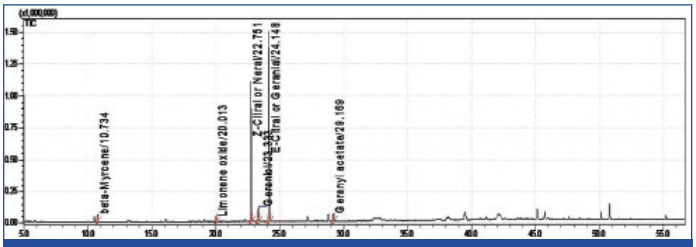
The *Cymbopogon citratus* (Lemongrass Oil) were purchased from Thai-China Flavours and Fragrances Co., Ltd., (510/3-4 Soi Ngamwongwan 25, Ngamwongwan RD., Ngamwongwan RD, Muang, Nonthaburi 11000). The components of the essential oils were analysed using GC-MS (Auxs) as described previously [26].

Main components in *Cymbopogon citratus* oil are E-Citral or Geranial (53%) and Z-Citral or Neral (38%), respectively [Table/Fig-1]. Three oral spray formulations (6%, 4%, and 2%) were prepared.

Subject Selection and *S. Mutans* Clinical Isolation

S. mutans clinical isolations were obtained from child subjects. This cross-sectional study was approved by the Ethical Human Research Committee, Faculty of Dentistry Chulalongkorn University, Bangkok Thailand (ethical approval number: 42/2010) [26]. Consents were signed from all child parents/legal guardians. All subjects were free to withdraw from the study at any time. *S. mutans* clinical isolations were obtained from 17 children (12 males, 5 females aged 3.5-10-years-old) who came to the Paediatric Dental Clinic, Faculty of Dentistry, Chulalongkorn University, Bangkok., For convenience sampling during the duration of the study within five months, after having obtained written and oral consents, oral examination was performed based on World Health Organisation definition. DMFT/dmft scores were recorded. In brief, stimulated saliva was collected and pooled in an equal proportion, and then cultured on MSA agar (Difco Laboratories, Detroit, MI) containing bacitracin (0.2 U/mL, Sigma Chemical Co., St Louis, MO), 0.001% (v/v) tellurite solution (Becton, Dickinson and Co., Sparks, MD) and 15% (w/v) sucrose (SigmaChemical Co.,). Subjects who had any systemic disease, were taking any kind of antibiotics, had professional fluoride application or any dental treatment within three months prior to the sample collection period were excluded. This was a once only collection of plaque samples so that participating in this study would not affect the subject's regular dental treatment. Ten colonies resembling *S. mutans* from each subject were then transferred to Brain Heart Infusion (BHI) broth and incubated at 37°C for 48 hours in an anaerobic jar. DNA samples from 280 isolates were extracted by washing and boiling for 10 minutes with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0)22. All isolates were then confirmed to be *S. mutans* by biochemical analyses. Two standard strains (*Streptococcus mutans* ATCC 25175 and *Streptococcus sobrinus* ATCC 6715) were cultured on BHI agar or broth. For standard strains (*S. mutans* ATCC 25175, *S. sobrinus* ATCC 6715), they were cultured on BHI agar or broth and incubated for 48 hours at 37°C, supplemented with 5% CO_2 for 18 hours.

Peak	Retention Time (min)	Base peak (m/z)	Area (%)	Components
1	10.734	93	2.13	β-Myrcene
2	20.013	44	1.40	Limonene oxide
3	22.751	41	38.13	Z-Citral or Neral
4	23.333	69	3.66	Geraniol
5	24.148	69	52.88	E-Citral or Geranial
6	29.169	69	1.80	Geranyl acetate



[Table/Fig-1]: Chemical constituents of *Cymbopogon citratus* (Lemongrass Oil) by GC-MS and GC chromatogram of *Cymbopogon citratus* (Lemongrass Oil).

Bacteria and Culture Conditions

A -20ºC aliquot stocks of *S. mutans* (ATCC 25175) were grown on BHI and incubated for 48 hours at 37ºC and supplemented with 5% CO_2. After that, 3-5 colonies were picked and suspended in THB broth (Todd-Hewitt broth, Difco, USA) and incubated for 24 hours. Aliquot stocks (-20ºC) out of a total of three *S. mutans* clinical isolations (N006, N029, N113) were grown as described above.

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

MIC and MBC were carried out using the Microbroth Dilution method, as described previously [27]. In brief, the oral spray solution was diluted in a series of two fold dilutions (ranging from 200 µg/mL to 1.5625 µg/mL) in Tryptic Soy Broth (TSB). Freshly grown bacterial suspensions in TSB were adjusted to approximately 10^6 CFU/mL. For MIC determination, the researcher added 100 µL of seeded broth to 8 wells and added 100 µL of test agent. The first test tube contained 100 µL of the seeded broth for eight wells. The solution of the test agent (100 µL) was added to the first tube to bring the volume to 200 µL. One hundred µL of this was transferred to the next tube and the process was repeated giving a two-fold serial dilution. The tubes were incubated at 37°C for 24 hours. The first assay well, with no apparent growth of the microorganism, contained the lowest dilution of the test essential oil and represented the MIC. The bactericidal effect was assessed by MBC determination. Samples were removed from the tubes that showed no turbidity and were dropped onto TSA plates. After incubation 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 oral spray formulations on 3 and 6-hour-old biofilms and to determine whether different strains had different susceptibilities to the bactericidal activity of

each formulation, a biofilm susceptibility test was performed. All strains were diluted with THB broth supplemented with 10% (w/v) sucrose. The starting cell density was 10^8 CFU/mL. The researcher transferred 2 mL of diluted cells into a new tube and incubated it for 3 and 6 hours. After incubation, planktonic cells were removed by washing with sterile water three times. Then, 2 mL of tested oral spray formulation were added into the same tube. After one minute this was washed out and ten 2×2 mm sterile beads and 2 mL of normal saline solution added, with one minute of vortex. 20 mL of the solution were pipetted and dropped on BHI agar. The numbers of CFU were obtained by plate counting. All experiments were done in triplicate. 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 96 and 24-Well Plate for Biofilm Inhibition Assay

All plates in this study were coated with saliva to create an oral environment atmosphere. Stimulated pooled saliva was obtained from five volunteers by omitting from tooth brushing for more than 6 hours. and then chewing paraffin to collect the saliva. The pooled saliva was centrifuged and diluted to 1:10 with PBS buffer and filtered (filter paper, Millipore, USA). Each well containing 100 µL of diluted saliva was incubated at 37ºC for 24 hours and then the saliva rinsed off.

Biofilm Inhibition Assay

This assay was to analyse the inhibition effect of oil formulations on biofilm formation. All strains grew in THB for 24 hours. and were then diluted with 5% (w/v) sucrose to the cell density of 10^6 CFU/mL. Each well contained 200 µL of solution. The researcher added oral spray formulations in each concentration with a pipet and mixed. All experiments were done in triplicate. After 18 hours of incubation, growth was measured using a micro-plate reader at the optimal density of 595 nm (Microplate Spectrophotometer, Biotek, USA). Then they were stained with 100 µL of crystal violet 0.05% (w/v) for 15 minutes, rinsed with water and placed in room temperature for one hour. Biofilm formation was read by adding 150 µL/well of 90% ethanol and put on a shaker at slow speed in order to remove all planktonic cells. A calculation of the mean percent inhibition of replicate tests and the IC50 was determined as a concentration of samples which inhibited 50% of biofilm formation in comparison to the control.

Fluorescence Staining and Confocal Laser Scanning Microscope (CLSM)

The researcher diluted all strains in BHI broth with 5% (w/v) sucrose to the cell density of 10^8 CFU/mL, then pipetted it into the 24-well polystyrene plate. Each well contained 600 µL and was incubated under the same conditions for 24 hours. After 24 hours, we pipetted and discarded all solution from each well and rinsed with PBS three times to remove non adherent bacteria on the cover glass. A 600 µL of each oral spray formulation was pipetted into each well and left it for five minutes. After five minutes, we pipetted and discarded all solution from each well and rinsed with PBS three times. We then added 600 µL PBS into each well to prevent biofilm dryness, And pipetted 3 µL of 1:10 dilutedSYTO-9 and propidium iodide (L7012 LIVE/DEAD® BacLight. A Bacterial Viability Kit *for microscopy and quantitative assays* (Invitrogen, USA) was put into each tested well and mixed with a pipette gently not to disturb the biofilm. This was stored in the dark for 15 minutes, then analysed under a confocal laser scanning microscope (CLSM, IX83ZDC, Olympus). The researcher adjusted the filter and lens of the fluorescent confocal electron microscope to the following parameters: live cell fluorescent imaging system IX83ZDC to standardise. Lens 60x green narrow 48%, PJ 46%, and PH 36.6% was used.

STATISTICAL ANALYSIS

All experiments were performed in triplicate. Data was expressed as mean±SD. For the determination of live/dead cells at the excitation wavelength of 485 nm, the green emission at 530 nm (live cells) and the red emission at 630 nm (dead cells) were measured and their ratio calculated. Each well was measured three times. Mean and standard deviations were calculated, and the Kruskal-Wallis Test (Bonferroni and Dunn Test) was used to compare experimental and control groups (significance at 95%).

RESULTS

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

The MIC and MBC of *Cymbopogon citratus* oral sprays were in the range of 0.125-3 µg/mL and 0.25-6 mg/mL, respectively [Table/Fig-2]. Although the MIC and MBC varied among tested bacteria, the MIC in most cases was equivalent to the MBC, indicating a bactericidal action in the oil.

Bacterial strains	2% (L38) (µg/mL)		4% (L40) (µg/mL)		6% (L42) (µg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC
S. mutans (ATCC 25175)	0.5	0.5	1	1	1.5	3
N006	0.5	0.5	1	1	3	6
N029	0.25	0.5	0.125	0.25	1.5	6
N113	0.5	0.5	0.125	0.25	1.5	3
S. sobrinus (ATCC 6715)	0.5	0.5	1	1	3	6

[Table/Fig-2]: Minimum Inhibitory Concentration (MIC) and Minimum Bactricidal Concentration (MBC) of *Cymbopogon citratus* oral spray formulations on the inhibition of two standard strains and three clinical isolates.

Biofilm Susceptibility Test

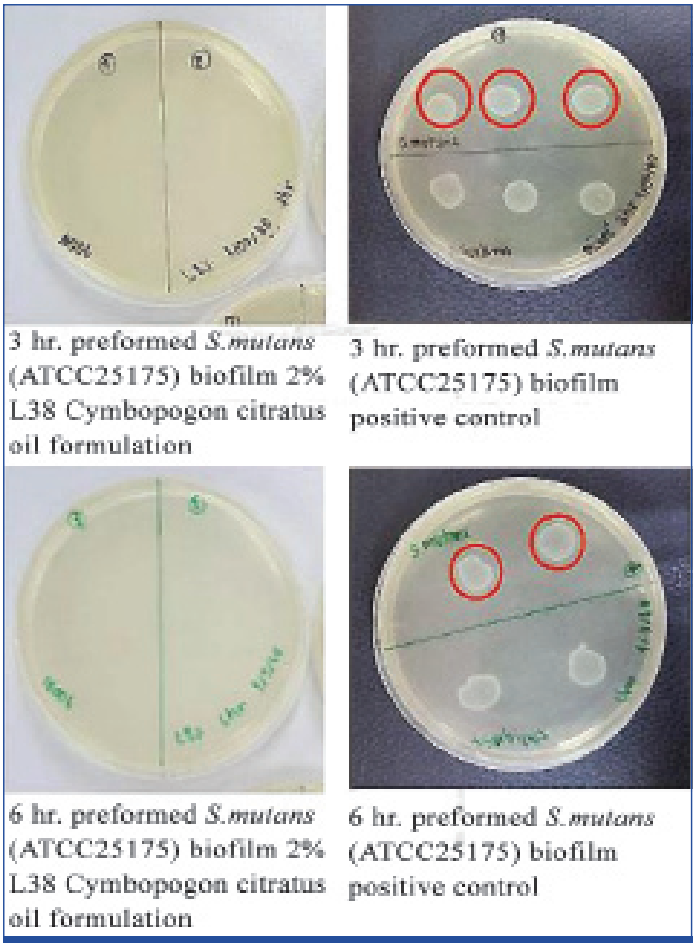
All strains of bacteria tested in this study were sensitive to all of the oral spray formulations (≥99.9%) when compared to the controls. All spray formulations had an inhibitory effect on 3 and 6 hour biofilm formation (100%) of all strains, compared to the control [Table/Fig-3,4].

Bacterial strains	Minimum amount of oral spray to inhibit biofilm formation (µL)		
	2% (L38)	4% (L40)	6% (L42)
S. mutans (ATCC 25175)	6.25	3.125	1.56
N006	3.125	3.125	1.56
N029	6.25	1.56	1.56
N113	6.25	3.125	1.56
S. sobrinus (ATCC 6715)	6.25	1.56	1.56

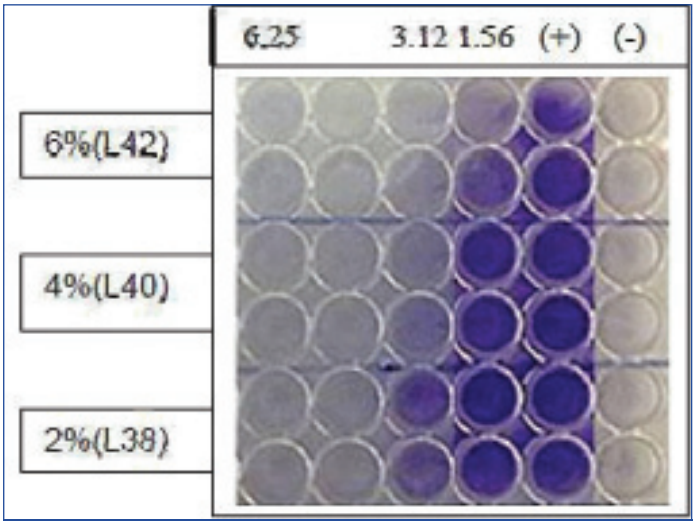
[Table/Fig-3]: Minimum amount of *Cymbopogon citratus* oral spray to inhibit biofilm formation (µL) of two standard strains and three clinical isolates.

Biofilm Inhibition Assay

Most of the oil formulations had an inhibitory effect on biofilm formation (≥50.0%) compared to the control when stained with crystal violet solution 0.05% (w/v) At 10^8 CFU/mL the dose-dependent form showed an inhibitory effect. A 2% (L38) spray formulation inhibited growth and biofilm formation of *S. mutans* (ATCC 25175), *S. mutans* clinical isolates N029, N113 and *S. sobrinus* (ATCC 6715) at a concentration of 3.125 µL. For *S. mutans* clinical isolates N006 at a concentration of 1.56 µL. 4% (L40), spray formulation inhibited growth and biofilm formation of *S. mutans* (ATCC 25175), and of *S. mutans* clinical isolates N006 at a concentration of 3.125 µL. For *S. mutans* clinical isolates N029, N113 and *S. sobrinus* (ATCC 6715) at a concentration



[Table/Fig-4]: Example of 3 hour and 6 hour preformed biofilms by *S. mutans* (ATCC 25175), which was inhibited by 2% (L38) *Cymbopogon citratus* oral spray. All spray formulations had an inhibitory effect on 3 hour and 6 hour biofilm formations (100%) of all strains.

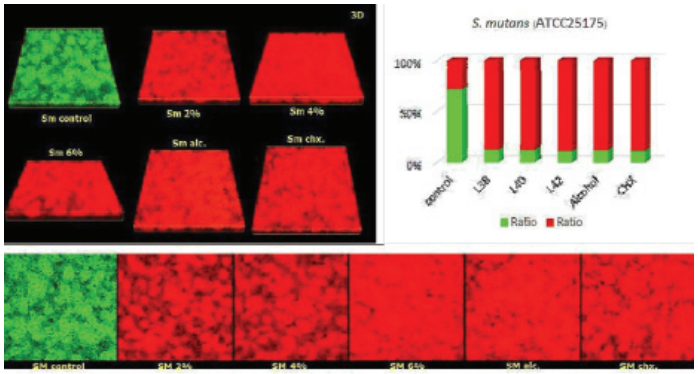


[Table/Fig-5]: *S. sobrinus* ATCC 6715 biofilm formation after staining with crystal violet solution 0.05% (w/v). Three *Cymbopogon citratus* oral spray formulations were added into wells containing cell inoculums of 108 CFU/mL and incubated for 24 hours. A 6% (L42) oil formulations showed maximum inhibition effect followed by 4%(L40) and 2%(L38).

of 1.56 µL. A 6% (L42), spray formulation inhibited growth and biofilm formation of all strains at a concentration of 1.56 µL. Higher concentrations of low percentage oral spray was needed to inhibit growth and biofilm formation [Table/Fig-5].

Saliva-coated 24-well Plate and Biofilm Assay and Fluorescence Staining and CLSM

For *S. mutans* (ATCC 25175), 6% (L42) showed the highest inhibition rate, which gave the lowest percentage of live cells (11.28±2.90%), followed by 4% (L40)(11.56±0.82%), and 2% (L38)



Chemical	n	Mean Rank
Ratio neg	6	33.50
form2	6	18.08
form4	6	16.33
form6	6	13.67
alc	6	13.33
chx	6	16.08
Total	36	

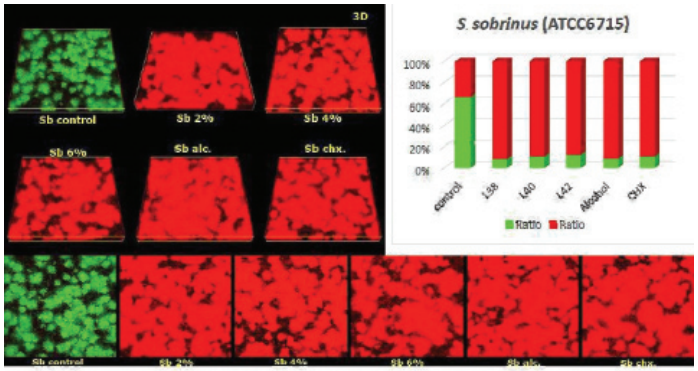
Test Statistics[ab]	
Peak	Base peak
Chi-square	15.449
Df	5
Asymp.	0.009
Sig.	

a. Kruskal Wallis Test
b.Grouping Variable: Chemical

[Table/Fig-6]: A 24 hour. *S. mutans* (ATCC 25175) biofilm formation. Positive control: Live cells were stained and exhibited green fluorescent under CLSM, while dead cells exhibited red fluorescent under CLSM. Negative controls were 0.12% Cholorhexidine and 95% ethanol. Graph represents percent reduction of *S. mutans* biofilm by each spray formula.

(12.18±2.08%), respectively [Table/Fig-6]. All formulae showed significantly (p=0.005) different inhibition rates compared to controls in the Kruskal-Wallis Test. When compared between each formula, there was no significant difference among the three formulations in inhibition of *S. mutans* (ATCC 25175).

For *S. sobrinus* (ATCC 6715), 2% (L38) showed the highest inhibition rate, which gave the lowest percent of live cells (8.19±0.69%) followed by 4% (L40)(10.64±1.05%) and 6% (L42)(12.23±2.00%), respectively [Table/Fig-7]. All formulae showed significantly (p=0.005)



Chemical	n	Mean Rank
Ratio neg	6	33.50
form2	6	6.17
form4	6	19.83
form6	6	24.83
alc	6	8.17
chx	6	18.50
Total	36	

Test Statistics[ab]	
Peak	Base peak
Chi-square	28.442
Df	5
Asymp.	0.000
Sig.	

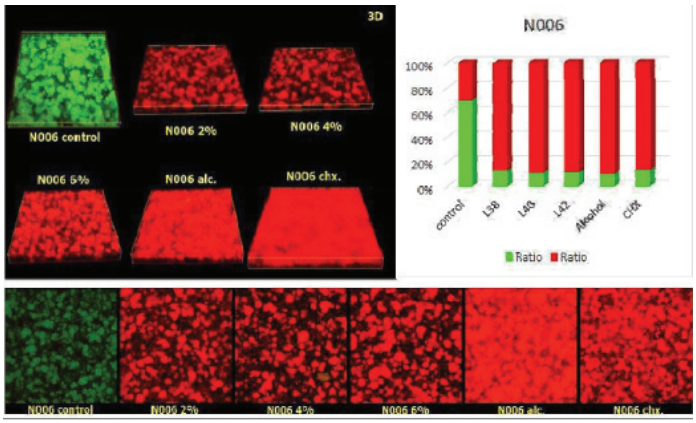
a. Kruskal Wallis Test
b.Grouping Variable: Chemical

[Table/Fig-7]: A 24 hour *S. sobrinus* (ATCC 6715) biofilm formation. Positive control: Live cells were stained and exhibited green fluorescent, while dead cells exhibited red fluorescent under CLSM. Negative controls were 0.12% Cholorhexidine and 95% ethanol. Graph represents percent reduction of *S. sobrinus* biofilm by each spray formula.

different inhibition rates compared to the controls. When compared between each formula, there was no significant difference among

the three formulations in inhibition of *S. sobrinus* (ATCC 6715). When compared between each formula, 2% (L38) showed a significantly higher biofilm inhibition rate (p=0.013) when compared to 4% (L40) and 6% (L42) at the significance level of 0.05.

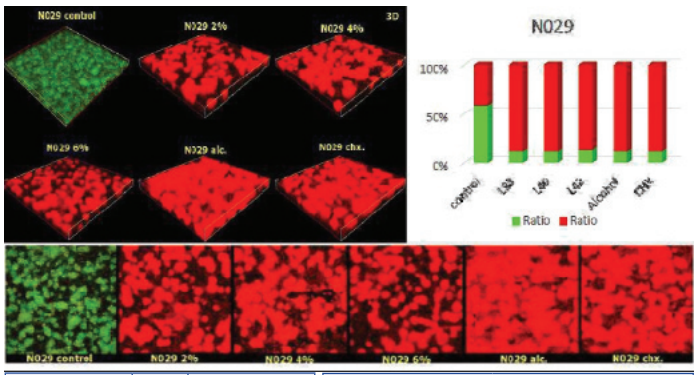
For *S. mutans* clinical isolate N006, 4% (L40) showed the highest inhibition rate, which gave the lowest percent of live cells (11.16±1.14%), followed by 6% (L42)(11.50±2.57%) and 2% (L38) (12.31±1.82%), respectively [Table/Fig-8]. All formulae showed significantly (p=0.005) different inhibition rates compared to controls in the Kruskal-Wallis Test. When compared between each formula, there was no significant difference among the three formulations in inhibition of clinical isolate N006.



Chemical	n	Mean Rank
Ratio neg	6	33.50
form2	6	18.83
form4	6	13.17
form6	6	15.17
alc	6	8.67
chx	6	21.67
Total	36	

Test Statistics[ab]	
Peak	Base peak
Chi-square	20.075
Df	5
Asymp.	0.001
Sig.	
a. Kruskal Wallis Test b.Grouping Variable: Chemical	

[Table/Fig-8]: A 24 hour. *S. mutans* clinical isolate N006 biofilm formation. Positive control: Live cells were stained and exhibited green fluorescent while dead cells exhibited red fluorescent under CLSM. Negative controls were 0.12% Cholorhexidine and 95% ethanol. Graph represent percent reduction of *S. mutans* clinical isolate N006 biofilm by each spray formula.



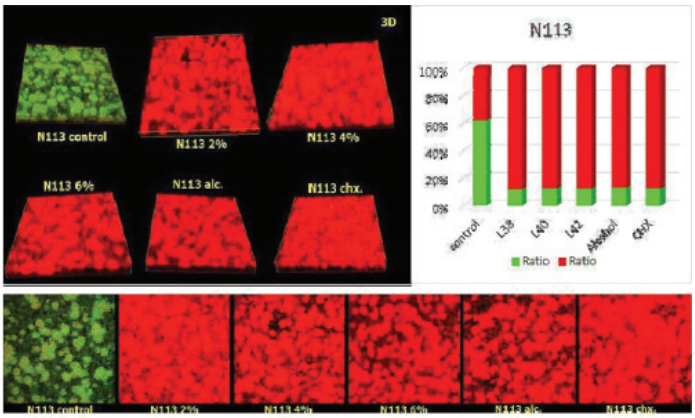
Chemical	n	Mean Rank
Ratio neg	6	33.50
form2	6	13.33
form4	6	13.67
form6	6	21.00
alc	6	14.50
chx	6	15.00
Total	36	

Test Statistics[ab]	
Peak	Base peak
Chi-square	16.733
Df	5
Asymp.	0.005
Sig.	
a. Kruskal Wallis Test b.Grouping Variable: Chemical	

[Table/Fig-9]: A 24 hour. *S. mutans* clinical isolate N029 biofilm formation. Positive control: Live cells were stained and exhibited green fluorescent while dead cells exhibited red fluorescent under CLSM. Negative controls were 0.12% Cholorhexidine and 95% ethanol. Graph represent percent reduction of *S. mutans* clinical isolate N029 biofilm by each spray formula.

For *S. mutans* clinical isolate N029, 4% (L40) showed the highest inhibition rate, which gave the lowest percent of live cells (11.10±1.47%), followed by 2% (L38)(11.45±2.81%) and 6% (L42) (12.98±2.42%), respectively [Table/Fig-9]. All formulae showed significantly (p=0.005) different inhibition rates compared to controls in the Kruskal-Wallis Test. When compared between each formula, there was no significant difference among the three formulations in inhibition of clinical isolate N029.

For *S. mutans* clinical isolate N113, 2% (L38) showed the highest inhibition rate, which gave the lowest percent of live cells (11.71±1.34%) followed by 6% (L42) (11.97±2.15%) and 4% (L40) (12.31±1.22%), respectively [Table/Fig-10]. All formulae showed significantly (p=0.005) different inhibition rates compared to controls in the Kruskal-Wallis Test. When compared between each formula, there was no significant difference among the three formulations in inhibition of clinical isolate N113.



Chemical	n	Mean Rank
Ratio neg	6	33.50
form2	6	12.50
form4	6	17.08
form6	6	12.92
alc	6	19.00
chx	6	16.00
Total	36	

Test Statistics[ab]	
Peak	Base peak
Chi-square	16.255
Df	5
Asymp.	0.006
Sig.	
a. Kruskal Wallis Test b.Grouping Variable: Chemical	

[Table/Fig-10]: A 24 hour. *S. mutans* clinical isolate N113 biofilm formation. Positive control: Live cells were stained and exhibited green fluorescent while dead cells exhibited red fluorescent under CLSM. Negative controls were 0.12% Cholorhexidine and 95% ethanol. Graph represent percent reduction of *S. mutans* clinical isolate N113 biofilm by each spray formula.

DISCUSSION

It has been well established that *S. mutans* is the major etiological agent in dental caries [6]. Plant-derived essential oil are ideal for use in oral care products, including toothpastes, mouthwashes, spray and gels, because they are non-toxic and have antiseptic properties [28]. This investigation of Thai herbal oral spray formulations used an oil extraction from *Cymbopogon citratus* against dental caries, allowing for the possibility of lower costs to prevent dental caries in the Thai population. Chlorhexidine has been ranked as a gold standard among antibacterial agents effective in reducing oral Biofilm [26,29]. Nevertheless, it is an antiseptic agent which effectively kills bacteria by penetrating into a cell's cytoplasm and lyses cell components [27]. Previous reports have shown that bacteria might develop a resistant mechanism if used for a long time. Most researchers are trying to use herbal extractions as an alternative.

Cymbopogon citratus, or lemongrass, is a tropical plant that is easy to find in Thailand. The oil extraction of lemongrass has antioxidant anti-inflammatory effects which can be used in a variety of medicines. This work had demonstrated an antimicrobial efficacy in mono-specie of bacterium before stepping into a multi-species study. Single organism biofilms are advantageous in examining the

mechanisms and actions of therapeutic agents on the interested specie. From a previous study by Manvitha K et al., they found that a *Cymbopogon citratus* oil extraction demonstrated anti-bacterial, fungal, protozoan, and other medical benefits such as being anti-inflammatory, anti-cancer, anti-oxidant, etc., [30]. Another study by Tofiño RA et al., tested the antibacterial effect of *Cymbopogon citratus* oil extraction against *S. mutans* (ATCC 35668) *in vitro* using MBEC-High-Through put screening (HTP) assay, their results showed that oil extraction at 0.1 mg/dL inhibited *S. mutans* at 95.4% and at 0.01 mg/dL inhibited *S. mutans* at 93.1% [31]. Also, study by Oliveira MAC et al., tested the antimicrobial activity *Cymbopogon citratus* oil against *Actinomyces naeslundii*, *Lactobacillus acidophilus*, *S. gordonii*, *S. mitis*, *S. mutans*, *S. sanguinis* and *S. sobrinus*. Results showed an inhibitory effect on all tested species. *Cymbopogon citratus* oil (10XMIC) reduced the number of viable cells of lactobacilli and streptococci biofilms ($p<0.05$). In addition, the essential oil inhibited adhesion of caries-related polymicrobial biofilm to dental enamel ($p<0.01$) [32]. Another study by Rego CB et al., investigated the effect of *Cymbopogon citratus* oil on *S. mutans* biofilm developed in hydroxyapatite discs surface. The biofilms of *S. mutans* were developed on the discs for five days and immersed daily in the three groups which were *Cymbopogon citratus* oil, BHI broth and control. Then biofilms were counted for CFU. The susceptibility test was indicating inhibition of microorganisms and the MIC value was 0.04 mg/mL. As for biofilm results, it decreased the bacterial growth in *Cymbopogon citratus* oil group when compared to control [33]. In this study, all oral spray formulations showed excellent antibacterial activity. Results showed that 3 and 6 hours preformed biofilm of all strains were sensitive to all formulations (100%) when compared to the controls. Biofilm formation of all strains was inhibited (100%) when compared to the controls by all formulations at different concentrations. Six percent (L42) oil formulations showed maximum inhibition effect, followed by 4% (L40) and 2% (L38), respectively. There were three different stages of biofilm formation, with accumulations at 0-5 hours active accumulation at 4-20 hours and a slower accumulation with a plateau-phase at 28 hours. The 3 hours biofilm was in the first stage, whereas the 6 hour. biofilm was in the second stage. The 6 hours biofilm was more complex and resistant because of a rapid increase in adherence [4]. This study demonstrated exceptional inhibition effects of all formulations, even in 6 hours preformed biofilm.

This study also tested the inhibitory effect on biofilm growth and formation of MS and three *S. mutans* clinical isolates. Results showed that all oral spray formulations exhibited an inhibitory effect on biofilm formation. The 6% (L42) formula reduced cell growth and inhibited biofilm formation at a cell density of 10^6 CFU/mL at all concentrations. The 4% (L40) was found to inhibit biofilm formation at 10^6 CFU/mL of *S. mutans* (ATCC 25175), and clinical isolate N006 at concentrations of 3.125 and 1.56 µL, for clinical isolates N029, N113 and *S. sobrinus* (ATCC 6715). The 2% (L38) inhibited biofilm formation at 10^6 CFU/mL at all concentrations of 3.125 µL, except for N006, which inhibited it at 1.56 µL.

In this study, biofilm was stained with fluorescence dye which penetrated through the cell cytoplasmic membrane. Live cells will exhibit green colour (SYTO9) and dead cells will exhibit red colour (propidium iodide). CLSM was used to evaluate bacterial viability in biofilms [31]. Fluorescence dyes were applied on the biofilm to differentiate live and dead bacteria, allowing the bacteria to be distinguished according to cytoplasmic membrane permeability [31]. Furthermore, CLSM can capture a series of image-scans showing changes in the viability of the bacterial cell over time, making the visualisation of real-time death of microorganisms possible [19]. Results indicated that all formulations showed significantly ($p=0.005$) different inhibition rates compared to the controls, and when compared between each formula, there was no significant difference among the three formulations in inhibition of all strains tested, except *S.sobrinus* (ATCC 6715). There are many possibilities in showing the various susceptibilities, such as the different genotypes of *S. mutans* strains, the components of each oil formulation, which might result in a different gene expression and lead to different aciduric and biofilm formation properties [15].

When evaluating 3D images with CLSM, all formulations were able to penetrate the biofilm layer to kill bacteria in the deepest layer and in all directions of biofilm structure. This result was better than our previous result using *Citrus hystrix* (makrut oil) oral sprays [23]. This result is similar to a study by Benjamin S et al., which studied the effectiveness of various dental products, including mouthwashes containing essential oils and dentifrice slurries of toothpastes using a CLSM camera which found that a mouthwash with a mixture of essential oils could penetrate the biofilm on all layers and in all directions and killed bacteria in biofilm in the first 30 seconds. The main antimicrobial agents in extracted *Cymbopogon citratus* oil are α-citral (geranial), β-citral (neral), linalool, myrcene, Passenger α-citral (geranial), β-citral (neral), and linalool [34,35]. Myrcene is a substance in the essential oil which has an antibacterial and lipophilic property which can be absorbed through the cell via the lipid-bilayer and damage the cell membrane [36]. In addition, studies of the properties of citral (geranial) have been shown to reduce ATP within the bacterial cell, resulting in pH changes in cells and hyperpolarisation [37]. From previous study tested the cytotoxic effect of *Cymbopogon citratus* oil against a human keratinocyte (HaCaT) cell line was in the range of 0.25 to 25 mg mL-1. After exposure to 0.25 mg mL-1, the cell viability rate was 98% which did not affect the cell viability of 100% which showed low cytotoxicity to human keratinocytes.

LIMITATION

S. mutans biofilm was developed as single-species plaque under the laboratory environment. This *in vitro* pre-established bacterial biofilm may not represent the actual oral environment of multi-species dental plaque with structural complexity and dynamic condition developed in oral cavity. Different responses of dental plaque to these oral spray essential oils in the toxicity aspect should be observed further in the future before starting the clinical study.

CONCLUSION

In conclusion, three oral spray formulations using an oil extraction of *Cymbopogon citratus* effectively inhibited biofilm formation and biofilm susceptibility. Oral sprays showed significant inhibition results even at a high cell density of bacteria. These formulations demonstrated satisfying results and need further clinical studies to verify its efficacy against ECC.

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