

THE ANTIMICROBIAL AND ANTIBIOFILM POTENTIAL OF SWEET BASIL ESSENTIAL OIL ON *Streptococcus mutans* AND *Staphylococcus aureus*

NURHAYATI MOHAMAD ZAIN^{1*}, NABILAH MOHD AMIN²,
FATIN ATHIRAHUSNA NORDIN², JUNAIDAH MAHMUD² and INDAH MOHD AMIN¹

¹Centre of Preclinical Science Studies, Faculty of Dentistry, University Teknologi MARA, Sungai Buloh Campus,
Jalan Hospital, 47000, Sungai Buloh, Selangor, Malaysia

²Faculty of Dentistry, University Teknologi MARA, Sungai Buloh Campus, Jalan Hospital,
47000, Sungai Buloh, Selangor, Malaysia

*E-mail: nurhayati8370@uitm.edu.my

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ABSTRACT

The antimicrobial and antibiofilm activities of sweet basil essential oil (SBEO) against oral microorganisms, *Streptococcus mutans* (*S. mutans*) and *Staphylococcus aureus* (*S. aureus*) were determined in this study. The antimicrobial activities were evaluated using the disk diffusion method (DDM), where Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were tested against both bacteria. Different formulations of SBEO (microemulsion, emulsion, water) were tested for biofilm dispersion assay on 24 h of preformed biofilm. Commercial chlorhexidine (CHX) 0.12% w/v was used as a positive control. The scanning electron microscope (SEM) was used to observe the changes on the treated surface. The data were analyzed using SPSS Version 27.0. A Kruskal-Wallis test followed by a Post-hoc Mann-Whitney U test was applied, and the level of significance was set at $p < 0.05$. All formulations of SBEO showed antimicrobial activities against the tested microorganisms. Exposure to 2.5% (v/v) microemulsion for two min exhibited 42.56% and 32.10% ($p < 0.001$) of biofilm dispersion for *S. mutans* and *S. aureus*, respectively. The SEM micrographs revealed the number of microorganisms on the treated group surface reduced compared to the negative controls. SBEO exerts an antimicrobial and antibiofilm effect on *S. mutans* and *S. aureus*. This finding suggests that the SBEO microemulsion has the potential to control planktonic *S. mutans* and *S. aureus* and their biofilm formation in the oral environment.

Key words: Antibiofilm activity, *Staphylococcus aureus*, *Streptococcus mutans*, sweet basil essential oil

INTRODUCTION

The oral microbiota is one of the most complex microbial communities in the human body, with about 700 different species of microorganisms (Lamont *et al.*, 2018). Oral diseases encompass caries, periodontitis, angular cheilitis, and denture stomatitis that can occur at different anatomic locations of the oral cavity such as the tooth surface, gingiva, and deeper oral tissues. Among the reported pathogenic bacteria in the oral environment, Gram-positive bacteria are *Streptococcus mutans* (*S. mutans*) and *Staphylococcus aureus* (*S. aureus*). The significant virulence factors of *S. mutans* are the ability to synthesize extracellular polysaccharides (EPS) for biofilm formation on the tooth and dental prosthesis, they are also highly acidogenic and aciduric. The production of acids acidifies the local environment's pH (pH up to 2.5), which can damage tooth structures and further

cause caries (Sheng & Marquis, 2007; Flemming & Wingender, 2010). *S. aureus* is a common commensal of the skin and nasal passages and can also be found in the oral cavity. *S. aureus* secretes EPS and forms a complex structure of biofilm that provides a protected environment. Other virulence factors include a wide variety of toxins that can be divided into three major groups—pore-forming toxins (PFTs), exfoliative toxins (ETs), and superantigens (SAGs) (Oliveira *et al.*, 2018). The diseases caused by *S. aureus* mostly involve soft tissue and bone, such as angular cheilitis and parotitis (Smith *et al.*, 2001), acute dentoalveolar abscess (Bahl *et al.*, 2014), dental implant infection (Salvi *et al.*, 2008), and denture stomatitis (Garbaze *et al.*, 2019).

Chlorhexidine gluconate (CHX) is a gold standard for antimicrobial therapy and is widely prescribed by dental practitioners as an antiseptic mouthwash (Brookes *et al.*, 2020) to prevent accumulation and reduce the preformed bacterial biofilm. However, among the side effects of CHX are tooth staining (Van

* To whom correspondence should be addressed

Strydonck, 2012), antimicrobial resistance (Kampf, 2016), and the potential to cause Type IV and Type I hypersensitivity reactions accompanied by severe anaphylaxis (Pemberton & Gibson, 2012).

In recent years, numerous studies had been conducted to identify compounds that can be used as an adjunct to the current approach to managing oral biofilm, especially for patients sensitive to CHX. One of the compounds is essential oil due to its effective therapeutic activity against pathogenic biofilms (Wang *et al.*, 2016; Scotti *et al.*, 2018). The sweet basil leaves essential oil (SBEO) used in this study has shown antimicrobial, antifungal, anti-inflammatory, anti-cancer, and analgesic properties (Beier *et al.*, 2014; Jin *et al.*, 2020). Consequently, this study aimed to determine the antimicrobial and biofilm dispersion activity of water-based, emulsion, and microemulsion of SBEO against preformed biofilm of *S. mutans* and *S. aureus*.

MATERIALS AND METHODS

Sweet basil essential oil (SBEO)

The essential oil extracted from sweet basil (*Ocimum basilicum*) leaves, also known as '*kemangi*' was purchased from Plant Therapy (ID, USA). The manufacturer provided the certificate of analysis for the gas chromatography (GC) profile of the SBEO. Three major components reported were linalool (48.7%), 1,8-Cineole (9.6%), and eugenol (6%).

Preparation of standard suspension (0.5 McFarland)

The strains used in this study, *S. mutans* ATCC 700610 and *S. aureus* ATCC CRM-6538, were obtained from the stock culture of Research Lab 1, Faculty of Dentistry, UiTM. The microorganisms were cultured in a Brain Heart Infusion (BHI) broth. The 16-hr cultures were diluted in the broths at a density adjusted to 0.5 McFarland turbidity, equivalent to 1.5×10^8 colony-forming units per milliliter (CFU/mL).

Disk diffusion susceptibility test

The antimicrobial activity was determined using the disk diffusion method (CLSI, 2012) with 98% (v/v) SBEO and 0.12% CHX (w/v) as a positive control. Fifty microlitres of standard microorganism suspension were pipetted onto the agar and streaked using a sterile cotton swab over the entire agar surface.

Then, twenty microlitres of SBEO were pipetted and impregnated into a sterile 4mm blank disk. The same volume was used for the positive (0.12% CHX), and negative (deionized water) controls. All impregnated disks were thoroughly dried in an incubator at 45 °C before being placed on the agar's surface one at a time using forceps and gently pressed to ensure complete contact with the agar surface. The plates were incubated at 37 °C for 24 h. Following incubation, the zone inhibition sizes were measured to the nearest millimeter using a caliper. All experiments were done in triplicates and repeated three times.

Preparation of water-based, emulsion, and microemulsion of SBEO.

The preparation of water-based, emulsion, and microemulsion of SBEO was conducted following the method described by Valizadeh *et al.* (2018) with minor modifications. The initial concentration prepared was to achieve a final concentration of 40% volume per volume (v/v) for water-based, emulsion, and microemulsion. Table 1 shows the percentage (v/v) of SBEO, Tween 80, and distilled water used. The emulsion and microemulsion were prepared using a low-energy method with a magnetic stirrer. The SBEO (oil phase) was mixed with Tween 80 (surfactant agent) and distilled water (water phase), and the mixtures were stirred at 800 revolutions per min (rpm) for 90 min.

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

The MIC and MBC were conducted according to the methods described in Clinical and Laboratory Standard Institute (CLSI) 2015 with minor modifications. The water-based suspension, emulsion, and microemulsion of SBEO were diluted in the BHI broth in a two-fold serial dilution ranging from 0.625 to 20%. The *S. mutans* and *S. aureus* (15 µL) with a final concentration of 1×10^6 CFU/mL were added to each well and incubated for 24 h at 37 °C. For MBC, 20 µL of the strains from wells with no growth was pipetted onto the agar and incubated for another 24 h. The MBC was the lowest compound concentration that showed no growth or fewer than three colonies of bacteria or fungi to obtain approximately 99.0 – 99.5% killing activity (CLSI, 2015). All experiments were done in triplicates and repeated three times.

Table 1. Composition of SBEO formulation. The initial working stock concentration was 40% for all formulations

Formulation	Oil (% v/v)	Surfactant Tween 80 (% v/v)	Distilled water (% v/v)
Emulsion	40	5	55
Microemulsion	40	10	50
Water	40	0	60

Biofilm eradication assay (crystal violet assay)

The manufacturer recommended a topical application of SBEO not exceeding 3.3%. Based on this recommendation, the concentrations used for the biofilm eradication assay were tested at 1.25% and 2.5%. The biofilm eradication was quantified by the crystal violet method described by Polonio *et al.* (2001) with minor modifications. The 5 mm glass beads (Merck KGaA, Darmstadt, Germany) served as a substrate for the biofilm, and all beads were used only once. Before the experiment, the beads were washed with soap solution, rinsed in double-distilled water (ddH₂O), incubated overnight in 80% isopropanol, and thoroughly cleaned in ddH₂O. The beads were autoclaved and oven-dried. For the crystal violet (CV) assay, one bead was placed in each well of a 24-well microplate. Then, each well was pipetted with 1 mL of *S. mutans* suspension (1.5×10^6 CFU/mL). Consequently, the microplate was incubated at 37 °C for 24 h to allow biofilm formation.

After 24 h, the spent media was discarded using a pipette, new media was carefully added, and the plate was returned to the incubator. After 24 h, the biofilm-coated beads were rinsed to remove any non-adherent bacteria. Beads with adhered bacteria were placed in sterile phosphate-buffered saline (PBS) of 0.1 Molar (M) and pH seven as the negative control, 0.12% CHX as the positive control, Tween 80 as vehicle control, and different formulations of SBEO at 1.25% (v/v) and 2.5% (v/v) (water, emulsion & microemulsion). The plates were put on an orbital shaker for two min at 100 rpm to provide shear forces. After incubation, the beads were subjected to crystal violet staining. One milliliter of 1% weight per volume (w/v) crystal violet was added, and the beads were further incubated at room temperature for ten min. After incubation, the non-bound dye was thoroughly washed from the wells with deionized water and then dried at 37 °C. Bound crystal violet was dissolved by adding 1 mL of acetic acid (33%) and incubated for five min at room temperature. The absorbance (optical density, OD) levels of the dissolved dye were measured at a wavelength of 600 nm using an optical density reader. The same procedures were repeated for *S. aureus*.

The percentage of dispersion was calculated using the following equation:

$$\text{Cells/biofilm dispersion percentage} = \frac{\text{OD negative control} - \text{OD test}}{\text{OD negative control}} \times 100$$

Scanning electron microscope (SEM)

Based on previous results, the efficacy of biofilm eradication was the highest for 2.5% (v/v) microemulsion in dispersing *S. mutans* and *S. aureus* biofilm upon short exposure. Therefore, the assay was repeated for only 2.5% (v/v) microemulsion, negative control, and positive control to obtain the beads for SEM viewing. The treated glass beads were processed according to the method described by Rahim and Thurairajah (2011). The beads were observed for cell population at 10,000× magnification through a Quanta™ Feg 450 scanning electron microscope (Netherlands).

Statistical analysis

The IBM SPSS Statistic Windows Version 27.0 (Armonk, NY: IBM Corp., 2020) was used for statistical analysis. Data were expressed as mean and standard deviation (SD) by computational analysis from the experiments with the non-parametric Kruskal-Wallis test and post-hoc Mann-Whitney analysis test. The results for *p*-values of 0.05 or less are considered statistically significant.

RESULTS**Disk diffusion susceptibility test**

The SBEO used in the study showed antimicrobial susceptibility towards *S. mutans* and *S. aureus*. The mean ± SD zone of inhibition was 32.94 ± 4.89 mm and 32.64 ± 5.77 mm for *S. mutans* and *S. aureus*, respectively (Table 2).

Preparation of water, emulsion, and microemulsion of SBEO

Different formulations of SBEO were used in this study. The emulsion and microemulsion were considered stable when creaming, sedimentation or

Table 2. The antimicrobial susceptibility test of *S. mutans* and *S. aureus*

	<i>S. mutans</i>		<i>S. aureus</i>	
	SBEO (98 % v/v)	0.12% CHX (w/v)	SBEO (98 % v/v)	0.12% CHX (w/v)
Zone of inhibition (mm ± SD)	32.94 ± 4.89)	19.10 ± 2.09	32.80 ± 5.29	14.25 ± 2.10

Data are presented as mean values of three replications ± standard deviation.

disproportionation did not occur within 24 h.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of SBEO against *S. mutans* and *S. aureus* ranging from 2.5% to 20% (v/v) are tabulated in Table 3.

Biofilm eradication assay

Based on the biofilm eradication assay (Figure 1), the 1.25% (v/v) dispersion activity was between 1.43% - 14.35%. On the other hand, all formulations of SBEO at 2.5% (v/v) significantly reduced the viability of biofilm cells compared to the negative control, with microemulsion showing the highest dispersion

activity. The viability of biofilm cells was reduced approximately to $42.56 \pm 4\%$ and $35.10 \pm 1.89\%$ ($p < 0.001$) for *S. aureus* and *S. mutans*, respectively. Interestingly, the eradication activity of 2.5% (v/v) SBEO microemulsion against *S. mutans* and *S. aureus* was higher than the effect of 0.12% CHX ($p \leq 0.05$).

Scanning electron microscope analysis

The SEM micrographs (Figure 2 & Figure 3) showed that the two-min exposure of 2.5% SBEO microemulsion to the preformed biofilm of *S. mutans* and *S. aureus* had reduced the population of adhered bacteria on the treated surface compared to the negative controls.

Table 3: Results of MIC and MBC different formulations of SBEO

	<i>S. mutans</i>		<i>S. aureus</i>	
	MIC (% v/v)	MBC (% v/v)	MIC (% v/v)	MBC (% v/v)
Water-based	10 ^a	20 ^a	10 ^a	20 ^a
Emulsion	2.5 ^b	10 ^b	5 ^b	10 ^b
Microemulsion	2.5 ^b	5 ^c	2.5 ^c	5 ^c

Values within the same column followed by different superscripts are significantly different at $p < 0.05$

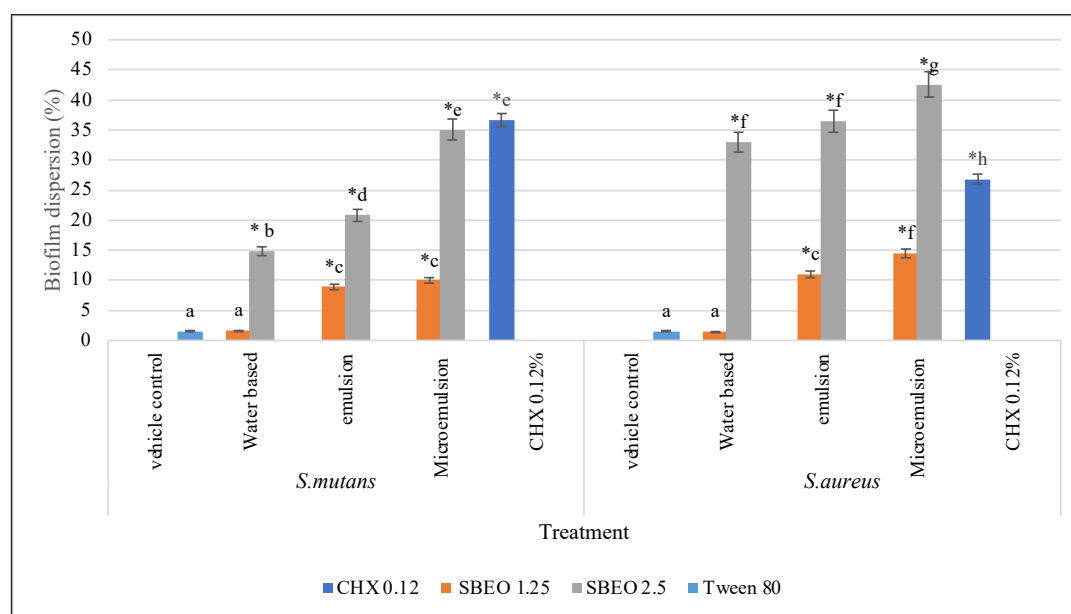


Fig. 1. Percentage of biofilm dispersion for *S. mutans* and *S. aureus* after treatment with different formulations of SBEO at 1.25% and 2.5%, vehicle control (Tween 80) and positive control (Chlorhexidine 0.12%). The overall means SD percentage of biofilm dispersion is presented in a bar graph. * indicates significant differences compared between the treatment groups and the negative control group ($p > 0.05$).

Data are the average of three replications \pm standard deviation.

Different superscripts indicate significant differences in each value.

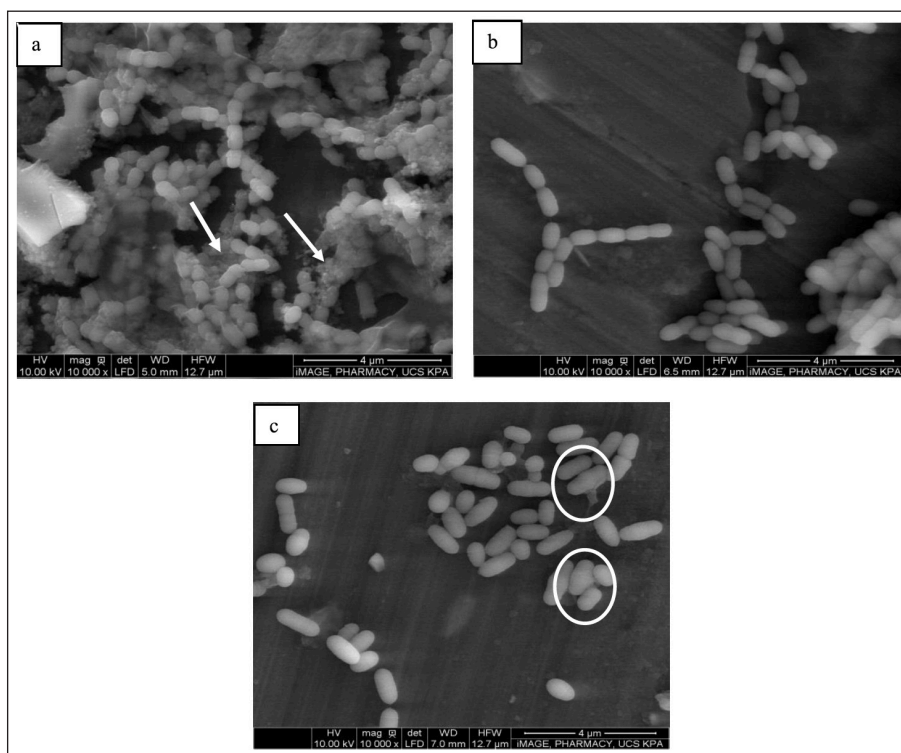


Fig. 2. Scanning electron micrograph of *S. mutans* (10,000× magnification): (a) Sterile distilled water treated group (negative control) *S. mutans* cells were elliptical and regular with a smooth intact surface and presence of EPS (white arrow), (b) Treated cells with SBEO at 2.5% were found to be significantly reduced in numbers and clearance of EPS compared to the negative control, (c) Cells treated with CHX 0.12% showed single isolated cells and few swollen cells (white circle).

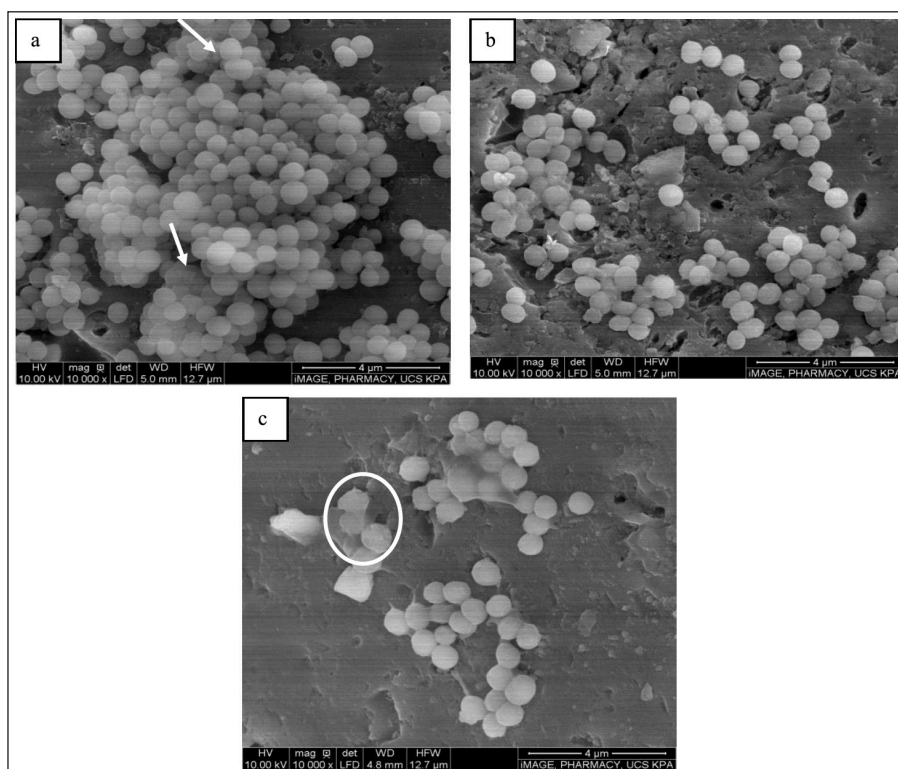


Fig. 3. Scanning electron micrograph of *S. aureus*. (10,000× magnification): (a) Sterile distilled water treated group (negative control) show biofilm-forming cells and presence of EPS matrix (white arrow), (b) Treated cells with SLEO at 2.5% showed a reduced number of bacterial cells on the surface when comparing with a, (c) Treated cells with CHX 0.12% showed morphological alteration/wrinkled cells (white circle).

DISCUSSION

The growing interest in the exploitation of natural products as an alternative treatment for oral diseases has driven the scientific community to explore numerous plant-based products such as extracts, compounds, and essential oils with antimicrobial and antibiofilm properties (Wiwattanarattanabut *et al.*, 2017; Manconi *et al.*, 2018; Aires *et al.*, 2021). However, most of the work did not investigate the short exposure effects of the products, which is an important parameter to be considered. In this study, pre-established (24 h) biofilms of *S. mutans* and *S. aureus* were used, and the biofilm eradication effects of SBEO within two min to mimic the short exposure of mouthwash in clinical applications were observed.

This study evaluated the antimicrobial and antibiofilm activities of SBEO against *S. mutans* and *S. aureus*, pathogenic microorganisms usually found in the oral cavity. The antimicrobial susceptibility test done through the disc diffusion method showed that *S. mutans* and *S. aureus* were susceptible to the SBEO with a zone of inhibition of more than 20 mm. The MIC and MBC data supported the antimicrobial activity. The antimicrobial activity of SBEO was also reported against pathogenic bacteria *Bacillus cereus*, *B. subtilis*, *B. megaterium*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Shigella boydii*, *S. dysenteriae*, *Vibrio parahaemolyticus*, *V. mimicus*, and *Salmonella typhi* (Hossain *et al.*, 2010).

The results showed the potential ability of SBEO as a biofilm dispersion agent. No studies have tested the short exposure effect of SBEO against preformed biofilms of *S. mutans* and *S. aureus*. The microemulsion dispersed a higher number of cells adhered on the surface (>35%), reducing the microorganisms' remaining population on the surface. The findings in this study suggest that SBEO is potentially beneficial in dispersing biofilm accumulation on the surface hardly reached by mechanical brushings, such as interproximal surfaces, behind the braces' wire, and around the brackets. Clinically, oral diseases are usually more pronounced in interproximal areas, and the efficacy of brushing in removing accumulated plaque in that areas is only 42% (Slot *et al.*, 2012).

The efficacy of microemulsion obtained from this study also supports the results from previous studies that suggested that the smaller oil droplets of the microemulsion provided a better penetration as antibacterial agents into bacterial cells and damaged bacterial cell walls (Valizadeh *et al.*, 2018). Additionally, the surfactants present in the SBEO microemulsion formulations had decreased the hydrophobicity of bacterial cell surfaces, leading to the release of DNA and RNA to extracellular spaces.

It was suggested that essential oil's bioactivities

might depend on the main components or major constituents that make up the oil (Juliani *et al.*, 2002; Hyldgaard *et al.*, 2012). The SBEO used in this study was a mixture of more than 50 compounds, and the major compounds were linalool (48.7%), 1,8-Cineole (9.6%), and eugenol (6%). Bassole *et al.* (2012) reported that the antimicrobial activity of sweet basil was highly attributed to eugenol (19%) and linalool (54%) content, and both compounds exhibited a synergistic effect. Preformed biofilms are difficult to eradicate by conventional antimicrobial therapy. In this study, the matrix of extracellular polymeric substance (EPS) became degraded in SBEO-treated groups as observed on the SEM micrograph. The possible mechanism of action was due to the activity of linalool, the major component in SBEO. This result is in agreement with the study of Astuti *et al.*, 2016, who showed that linalool penetrated the extracellular polysaccharides that protect bacteria and degrade biofilm formation by biofilm-associated bacteria. Previously, linalool was reported to exhibit significant antifungal activity for *C. tropicalis* strains and had moderate activity against the *S. aureus* species, and killed 99% of the inoculum within 7.5 min of treatment (Dias *et al.*, 2017). Determination of the potent single bioactive compound could contribute to the commercial value in drug development because the variety, season of cultivation, and soil type could have significant effects on the quantitative profile of the whole essential oil obtained from the same plant species (Rapposelli *et al.*, 2015; Murarikova *et al.*, 2017).

Due to short exposure, we postulate the activity of active dispersion by the external trigger (SBEO). The SEM results revealed the consequences of exposure to the SBEO, such as reduced cell adherence. In this study, SEM analysis is used as qualitative analysis to support the effectiveness of SBEO to reduce cell adherence of cells. The SEM micrographs of the control group depicted clusters of bacterial cells surrounded by the EPS matrix. The small molecule of microemulsion provides a better diffusion rate of the SBEO in the matrix, thus initiating the EPS degradation that protects the biofilm.

A single microorganism for each test used in this investigation may not reflect the actual oral environment of multispecies microorganisms. Therefore, future studies should include co-cultures of *S. mutans* and *S. aureus* or multispecies biofilm to imitate the oral microbiome. Future studies should also include the long-term effectiveness, stability of microemulsion, and cytotoxicity involving oral cells such as human oral keratinocytes.

CONCLUSION

In conclusion, SBEO exhibited antimicrobial and

antibiofilm activities against oral microorganisms *S. mutans* and *S. aureus*. The microemulsion preparation of SBEO could potentially be used to manage biofilm-associated infections caused by *S. mutans* and *S. aureus* as a single causative microorganism or co-infection. These findings could also pave the way for further microbial and molecular research to learn more about SBEO's potential as an alternative treatment for oral diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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