

Basil Essential Oil (*Ocimum basilicum* L.) Activities on *Streptococcus mutans* Growth, Biofilm Formation and Degradation and its Stability in Micro-Emulsion Mouthwash Formula

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ABSTRACT

This study was aimed to investigate the effect of basil essential oil (*Ocimum basilicum* L.) on *Streptococcus mutans* growth, biofilm formation and degradation and examine its stability in micro-emulsion mouthwash formula. Antibacterial, antibiofilm and biodegradation activities of basil essential oil were conducted using microtiter broth dilution method. Commercially available "X" mouthwash was used as positive control. The percentage of inhibition or degradation was calculated based on optical density measurement. Micro-emulsion mouthwash of basil essential oil was tested on antibacterial activity. Stability testing was conducted using accelerated storage condition. In this study, basil essential oil exhibited antibacterial activity against *Streptococcus mutans* with IC₉₀ of 0.23 %, biofilm formation inhibition with IC₅₀ of 0.68%, and biofilm degradation by 32.15% at 1.00%. The antibacterial activity of basil essential oil within its micro-emulsion mouthwash formula was maintained at 100% after 3 months storage at room temperature and sustained at 99.62% at accelerated storage condition. The most common compounds detected by gas chromatography-mass spectrometry were geranial (43.74%) and neral (31.19%). This study has demonstrated that basil essential oil exhibited antibacterial and antibiofilm activity. The activity appears to be stable after three month storage at accelerated storage conditions suggested that basil essential oil is potential to function as active ingredient in antibacterial and antibiofilm product formulation.

Keywords: Basil essential oil, *Ocimum basilicum* L., antibacterial, antibiofilm, stability, micro-emulsion

INTRODUCTION

Basil essential oil (*Ocimum basilicum* L.) has been used traditionally as a natural therapeutic agent such as asthma, headache, and cough. Several studies demonstrated that basil essential oil exhibited various biological activities such as antioxidant¹⁻⁴, anticonvulsant and hypnotic activities⁵, antiplasmodial activity⁶, antiviral⁷ and antiinflammatory as well as antiproliferative activities¹. These effects may be attributed from its pharmaceutical potential of its active phyto-chemicals within the essential oil such as methyl eugenol, methyl chavicol, geranial, neral, α -terpineol, and β -caryophyllene^{1,2,8}. The chemical composition of basil essential oils differs according to the varieties⁹⁻¹¹.

The antibacterial activities of basil essential oil have been reported by several researchers^{2,8,12}. Beside its antimicrobial activities against most of Gram positive and negative bacteria, basil essential oils were found to be active against fungi and mold^{8,13}. The antibacterial potency of basil essential oil as antibacterial agents suggest its potential activity against *Streptococcus mutans*, which is the main cause of dental caries¹⁴. Bacteria *S. mutans* which attach to tooth surface could excrete structure or matrix called slime layer which increases further attachment of bacteria on the tooth surface. This then creates the formation of microbial community that embedded in

extracellular slime layer, known as dental biofilm or plaque. *S. mutans* survives in the acidic condition so that reduced pH level results in demineralization and cavity of the tooth, and development of dental caries^{15,16}. The gold standard antiseptic mouthwashes containing chlorhexidine have been used to prevent and control the forming of plaque and dental caries. However, this compound is reported to have side effects in which chlorhexidine could induce tooth pigmentation, taste alteration and formation of supragingival calculus¹⁷. Previous studies reported the potency of essential oils to function as antibiofilm agents^{18,19}. This study aims to investigate the basil essential oil activity on inhibition of *S. mutans* growth, inhibition biofilm formation, and degradation of existing biofilm as well as examine its stability in micro-emulsion mouthwash formula.

MATERIALS AND METHODS

Plant materials and isolation of essential oils

The *Ocimum basilicum* L. var. *citratum* Back. plants were collected from Yogyakarta, Indonesia. The essential oils were extracted from the fresh leaves using hydro-distillation for 4 hours. The essential oils were dehydrated using anhydrous sodium sulfate, filtered, and stored in sealed vials at -4^o C until used for GC-MS analysis and biological activity testing.

Table 1: The percentage inhibition of basil essential oil on *S. mutans* growth, biofilm formation, and degradation of biofilm.

Concentration of basil essential oil (% (v/v))	% inhibition of bacteria growth	% inhibition of biofilm formation	% degradation of biofilm
0.06	54.13±4.40	19.57±0.62	0
0.12	68.65±3.44	23.50±1.44	0
0.25	92.58±2.01	33.26±5.28	19.98±13.72
0.50	93.21±0.29	46.71±2.19	24.52±11.19
1.00	95.71±0.99	61.81±3.42	32.15±13.70

Values are mean ± standard deviation, n = 3.

Table 2: Chemical composition of basil essential oil.

Compounds	Retention time	% of compounds
Methylheptenone	11.754	2.41
Cyclopentane	14.733	0.37
Linalool	15.033	7.03
Trans- α -bergamotene	15.925	0.66
β -caryophyllene	16.233	1.20
Neral	17.183	31.19
Geranial	17.825	43.74
β -selinene	18.392	1.84
Nerol	18.608	6.93
Geraniol	19.133	4.62
Total	-	100

Analysis of basil essential oil chemical composition

The basil essential oil composition was evaluated using GC-MS method in Laboratory of Organic Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada. The analysis was carried out on GC-MS Shimadzu QP 2010 S equipped with Rastek RXi-5MS column (30 m x 0.25 mm i.d., 0.25 μ m film thickness). The GC conditions were as follows: Helium was a carrier gas at 0.51 mL/min flow rate. The injector temperature was 215°C, injection size 0.2 μ L, split ratio 1:158.4. Temperature program started from 60°C, hold for 5 min, increased 10°C up to 205°C, and hold for 20 min. Interface temperature was 215°C. Detector temperature was 250°C. MS were taken at 70 eV in the electronic ionization (EI), with mass scan range 28-600 m/z.

Basil essential oil constituents were identified based on gas chromatogram and mass spectrum. The gas chromatogram showed component peaks with retention time. Each peak had mass spectrum which was compared to authentic mass spectral data from Wiley 229 library. The mass spectral data with highest similarity index was considered as the name and structure of component. The relative amounts of individual components were calculated based on peak area.

Antibacterial assay

S. mutans was cultured in nutrient broth media. To determine antibacterial activities, serial dilutions of basil essential oil were prepared in nutrient broth media. The assay was conducted in 96 well conventional sterile polystyrene microplates. The testing mixtures contained 10 μ L of *S. mutans* (6 X 10⁸ CFU/mL) and 190 μ L of essential oil in media (final concentration of 1 – 0.06% v/v). A mixture of 10 μ L of *S. mutans* (6 X 10⁸ CFU/mL) and 190 μ L of media were used as control and a mixture of 10 μ L of *S. mutans* (6 X 10⁸ CFU/mL) and 190 μ L 4% tween

80 in media were used as vehicle control. A mixture of 10 μ L of *S. mutans* (6 X 10⁸ CFU/mL) and 190 μ L of commercially available mouthwash “X” in media were used as positive comparison. The microplates were incubated for 24 hour at 37°C. The optical density of each well in microplate was read at 595 nm in Benchmark microplate reader (Bio-Rad, USA). The percentage of bacterial growth inhibition (GI) was calculated using following formula:

$GI\% = [(C_{Abs} - T_{Abs}) / C_{Abs}] \times 100$; Where C_{Abs} is the absorbance of the control treatment and T_{Abs} is the absorbance of samples treated with different concentration of basil essential oils.

The 90% inhibitory concentrations (IC₉₀) against *S. mutans* was determined by probit analysis.

Inhibition of biofilm formation

The IC₅₀ is determined by the ability to prevent biofilm formation by 50%. The effect of basil essential oil in inhibiting the formation of biofilm was conducted by modified microdilution method²⁰. The essential oil concentrations were prepared in BHI media containing 2% sucrose. *S. mutans* culture was standardised to a cell density of 1.5 x 10⁸ CFU/mL, equal to Standard McFarland 0.5. The testing mixtures were prepared by adding 75 μ L of media containing essential oil (final concentration of 0.06 – 1% (v/v)), 25 μ L of bacterial suspension on 96 well round bottom polystyrene microplate. Similar composition was used as control without addition of essential oil. Vehicle control was prepared by adding 75 μ L of media containing 4% tween 80, 25 μ L of bacterial suspension. Commercially available “X” mouthwash was used as positive comparison. After incubation for 24 hour at 37°C, the microplates were rinsed gently using PBS. The biofilm formation was stained with 0.1% crystal violet for 15 minutes at room temperature. After rinsing, the stained biofilm was dissolved using ethanol and left for 15 minutes. Ethanol was transferred to new 96-well flat bottom polystyrene microplates for optical density reading at 595 nm. The percentage of biofilm inhibition (BI) was calculated using following formula:

$BI\% = [1 - (S_{Abs} / C_{Abs})] \times 100$; Where C_{Abs} is the absorbance of the control treatment and S_{Abs} is the absorbance of samples treated with different concentration of basil essential oils.

The IC₅₀ is determined by calculating the concentration of essential oil which is able to inhibit the formation of biofilm by 50% using probit analysis.

Biofilm degradation testing

Table 3: Physical stability of micro-emulsion mouthwash containing 1% of basil essential oil

Time of storage (moth)	Storage conditions	Samples	Parameters				
			Color	Turbidity	Sedimentation	Odor	pH
0	RT & 40°C, 75% RH (climatic chamber)	Micro-emulsion containing basil essential oil	-	-	-	Typical basil essential oil and mint	6
		Vehicle control	medium	-	-	Mint	6
		Commercial mouthwash	"X"	-	-	-	Mint
3	Room Temperature	Micro-emulsion containing basil essential oil	-	-	-	Typical basil essential oil and mint	6
		Vehicle control	medium	+	-	Mint	5
		Commercial mouthwash	"X"	-	-	-	Mint
3	40°C, 75% RH (climatic chamber)	Micro-emulsion containing basil essential oil	-	+	-	Typical basil essential oil and mint	5
		Vehicle control	medium	+	-	Mint	5
		Commercial mouthwash	"X"	-	-	-	Mint

+ = positive indicated changes in color and turbidity

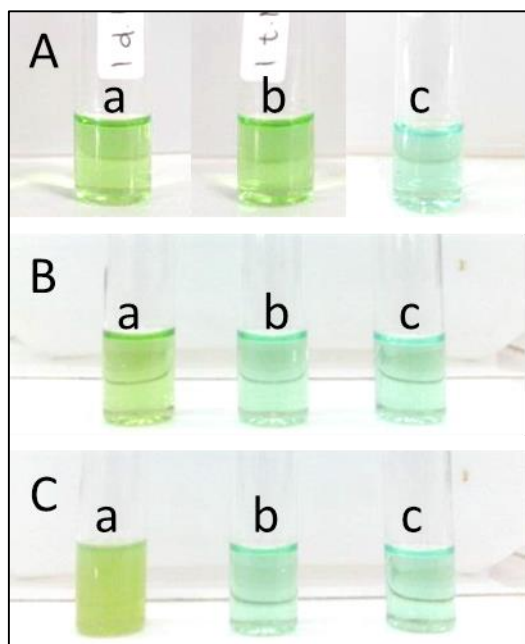


Figure 1: Physical stability characteristics of micro-emulsion mouthwash containing basil essential oil after 3 month storage.

To test the effect of basil essential oil on biofilm adherence, 96-well round bottom polystyrene microplates were inoculated with 75 μ L of BHI + 2% sucrose and 25 μ L of bacterial suspension (1.5×10^8 CFU/mL) and incubated for 24 h at 37°C. After incubation, microplates were rinsed, 200 μ L of a serial concentration of basil essential oil in BHI+ 2% sucrose media (final concentration of 0.06 – 1.00% v/v) were added. Microplates were further

incubated for 24 h at 37°C, rinsed gently using PBS and the biofilm was stained with 0.1% crystal violet for 15 minutes. After washing with tap water, the biofilm was dissolved using ethanol and left for 15 minutes prior to transfer to new 96-well flat bottom polystyrene microplates for optical density reading at 595 nm. BHI media + 2% sucrose without essential oil was used as control. The BHI media + 2% sucrose containing 4% tween 80 was used as vehicle control and the BHI media + 2% sucrose containing "X" mouthwash was used as positive comparison. The percentage of biofilm degradation (BD) was calculated using following formula: $BD\% = [1 - (S_{Abs} / C_{Abs})] \times 100$; Where C_{Abs} is the absorbance of the control treatment and S_{Abs} is the absorbance of samples treated with different concentration of basil essential oils.

The IC_{50} is determined by calculating the concentration of essential oil which is able to degrade biofilm adherence by 50% using probit analysis.

Micro-emulsion mouthwash formulation

The micro-emulsion mouthwash containing 1% basil essential oil was prepared according to previously published [35], which contain basil essential oil, tween 80, glycerin, peppermint oil, Na-Benzoate, Na-saccharine, green coloring agent and H₂O. Basil essential oil, peppermint oil and tween 80 were mixed to obtain oil phase. Glycerin, Na-Benzoate solution, Na-saccharine solution and H₂O were mixed to obtain water phase. The water phase was added to oil phase and immediately mixed to homogenous. An adequate amount of green coloring agent was added and H₂O was added to reach final volume. The formula was kept in glass bottles.

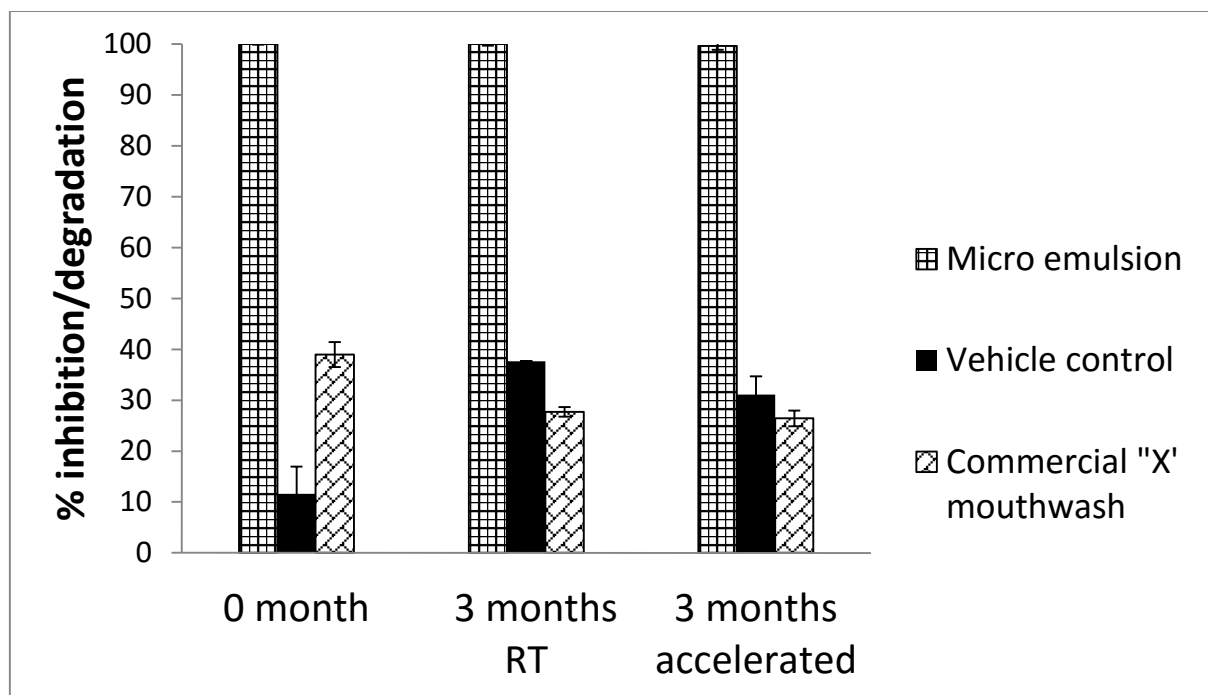


Figure 2: Antibacterial activities of micro-emulsion mouthwash containing 1% basil essential oil after 3 month storage.

Stability and antibacterial testing of basil essential oil micro-emulsion formula

The micro-emulsion mouthwash containing 1% basil essential oil was kept on climatic chamber (Binder, Germany) at 40 °C; 75% RH for three months. The same formula was also kept at room temperature for three months. Physical parameters such as color, turbidity, precipitation, odor and pH were evaluated before and after three months storage at accelerated storage condition and at room temperature. The antibacterial activity was also tested by adding 10 µl of *S. mutans* suspension (6×10^8 CFU/mL), 10 µl of micro-emulsion mouthwash containing basil essential oil formula and 180 µl of BHI media + 2% sucrose on 96 well flat polystyrene microplates. The same mixtures without addition of mouthwash were used as control. A mixture of 10 µl of *S. mutans* suspension (6×10^8 CFU/mL), 10 µl of micro-emulsion mouthwash without basil essential oil and 180 µl of BHI media + 2% sucrose was used as vehicle control. A mixture of 10 µl of *S. mutans* suspension (6×10^8 CFU/mL), 10 µl of commercially available "X" mouthwash and 180 µl of BHI media + 2% sucrose was used positive comparison. The microplates were incubated for 24 hour at 37°C. The optical density of each well in microplate was read at 595 nm in Benchmark microplate reader (Bio-Rad, USA). The percentage of bacterial growth inhibition (GI) was calculated using following formula:

$GI\% = [(C_{Abs} - T_{Abs}) / C_{Abs}] \times 100$; Where C_{Abs} is the absorbance of the control treatment and T_{Abs} is the absorbance of samples.

Statistical analysis

The results were expressed as the means of three samples \pm the standard deviation (SD) and all experiments were conducted at least two times. Differences between means were analyzed with a One-Way ANOVA, followed by

multiple comparison analysis using Bonferroni method on SPSS 16 software with level of significance of $p < 0.05$.

RESULTS

The basil essential oil inhibited the growth of S. mutans, prevented the formation of biofilm and degraded the biofilm adherence.

Basil essential oil at the concentration of 0.06% inhibited the growth of *S. mutans* by $54.13 \pm 4.40\%$. The percentage of inhibition increases by doubling the concentration of essential oil to 0.12% ($68.65 \pm 3.44\%$). The percentage of inhibition starts to levels up when the essential oil was given from 2.5 to 1% to reach maximum inhibition of 95.71 ± 0.99 at 1% concentration of essential oil (Table 1). The IC_{90} of the basil essential oil against *S. mutant* was found to be 0.23%. Examination on biofilm formation showed that basil essential oil prevented the formation of biofilm at all concentrations tested with IC_{50} value of 0.68%. The basil essential oil started to degrade the formation of biofilm at the concentration of 0.25% and at the concentration of 1% reached $32.15 \pm 13.70\%$ level of degradation.

The chemical constituents of basil essential oil

Table 2 lists the chemical components of basil essential oils. A total of 16 components of basil essential oil were identified by GC-MS used in this study. The main constituents of the two were geranial (43.74%) and neral (31.19) followed by linalool (7.03%) and nerol (6.93%). The other components constituted less than 5% each of the total concentration. The chemical constituents of basil essential oil used in this study were categorized into cluster 8 which mainly contain geranial and neral⁹.

The antibacterial activity of the micro-emulsion mouthwash containing basil essential oil was maintained after three months storage, with few physical parameters change

The mouth wash formula containing 1% basil essential oil did not change its color, odor, pH and turbidity after three months storage at room temperature (Table 3; Fig. 1). However, storage at accelerated condition changes the turbidity and the pH. Although other parameters appeared to be stable, the color of vehicles medium changes its color. The pH also decreased to 5. On the other hands, physical characteristics of commercially available "X" mouthwash were stable at all conditions, except that the pH decreased from 5 to 4.

The micro-emulsion mouthwash containing basil essential oil (a) was prepared and the physical characteristics were evaluated at the time of mouthwash was formulated (A); and then stored at room temperature/RT (B) and accelerated storage condition (C). Evaluation of the physical characteristics were conducted again after three month storage. Solvent control (b); commercially "X" mouthwash as positive comparison (c).

Storing the formula either at room temperature or at accelerated storage condition for three months did not affect the ability of micro-emulsion in inhibiting the growth of *S. mutans*. The mouthwash containing 1% basil essential oil is still able to inhibit the growth of bacteria by $99.62 \pm 0.72\%$ after storage in climatic chamber for three months (Fig. 2). The vehicle medium, however, exhibited increased antibacterial activity after storing for three months at all conditions. On the other hand, the ability of commercially available "X" mouthwash in inhibiting the growth of *S. mutans* decreased. It is interesting to note that the antibacterial activity of micro-emulsion containing 1% basil essential oil is better than that of "X" mouthwash. The micro-emulsion mouthwash containing 1% basil essential oil was prepared and the antibacterial activities against *S. mutans* were determined at time 0 (month). Following three month storage at room temperature/RT and accelerated storage condition, the antibacterial activities were examined again as described in materials and methods. The results are conducted in triplicates and represent of at least two independent experiments.

DISCUSSION

The antibacterial and antifungal activities of several essential oils have been reported by a number of studies against several oral pathogens²¹⁻²⁴. *Ocimum basilicum* L. is a well-known culinary herb used as traditional medicine. Previous studies showed that essential oil of *O. basilicum* L. possess some biological activities¹⁻⁷. Its antimicrobial potency against some pathogenic microbes^{2,25,26}, suggests its potential to be developed as antimicrobial agents against *S. mutans*, one of predominant pathogenic microbes grown in tooth surface^{19,27,28}. In this study we found that basil essential oil inhibited the growth of *S. mutans* and prevented the formation of biofilm. The major components within the basil essential oil used in this study are geranial (43.74%) and neral (31.19%) followed by linalool (7.03%) and nerol (6.93%). Geranial is identified as *E*-isomer of citral whilst neral is its *Z*-isomer, known as citral A and citral B, respectively²⁹. Citral has been reported to inhibit the mixed biofilm formation of foodborn *Staphylococcus aureus* and *Salmonella*

*enteritidis*³⁰ and *Candida albicans* biofilms³¹. Neral and geranial are monoterpene aldehydes. Aldehydes are known to possess antimicrobial activity. Aldehyde group conjugated to a carbon with double bond has high electronegative which could potentiate its antibacterial activity. Electronegative compounds act as antibacterial agents through interference in biological processes involving electron transfer and react with vital nitrogen components such as proteins and nucleic acids, and therefore inhibit bacterial growth³². In addition, the other two compounds (linalool and nerol) are terpenoids which are known to possess antibacterial activities including those with extracellular polysaccharides³². Terpenoids are compounds which are able to degrade biofilm and this may associate with its ability to penetrate the extracellular polysaccharides or slime layer that protecting bacteria and thus degrading the biofilm³³. These data support our finding regarding the ability of basil essential oil in inhibiting the formation of *S. mutans* biofilm as well as its degradation. In this study we also found that the dose required to prevent biofilm formation is higher than that of planktonic bacterial growth and even higher in degrading the preformed biofilm. This may be explained by the fact that pre-established biofilms have greater resistance to external agents³⁴.

Previous study reported that 1% basil essential oil in the form of micro-emulsion mouthwash inhibited the growth of *S. mutans*, inhibited the formation of biofilm and degraded biofilm adherence³⁵. Attempts to examine its stability in inhibiting the growth of *S. mutans* in the micro-emulsion formula after storing in room temperature and accelerated storage condition for three months were conducted. In this study, we found that although some physical characteristics change during the storage, the mouthwash was still able to inhibit the growth of planktonic *S. mutans*. The inhibition persisted after three month storage at room temperature and at accelerated storage conditions suggesting the stability of bioactive compounds in inducing the effect. These data altogether suggest the potential of basil essential oil in affecting biofilm formation and degradation. Better formulation, however, is needed to obtain better physical characteristic stability.

CONCLUSION

This study demonstrates the ability of basil essential oil in inhibiting the growth of *S. mutans*, preventing biofilm formation and degrading the preformed biofilm. Considering the implication of this microbe in inducing dental plaque, further development of basil essential oil as an alternative therapeutic strategy against biofilm-related oral diseases is promising.

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