The Stability and Antibacterial Properties of Citronella and Lemon Oil Mouthwashes on Oral Biofilm

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Abstract

Combination of citronella and lemon oil may be used as an antibacterial mouthwash. The objective of this study is to develop mouthwashes using citronella and lemon oil, assess their sensory properties, stability, and investigate their antibacterial activity against Streptococcus sanguinis and Porphyromonas gingivalis.

The formulation of each mouthwash was achieved through the application of a solubilization process. Three mouthwash formulations were developed using varying quantities of citronella and lemon oil, denoted as F I, F II, and F III. The evaluation of the concentration levels encompassed an assessment of the organoleptic characteristics, stability, and anti-bacterial activities. An ultraviolet-visible spectrophotometer with a wavelength of 650 nm was used to evaluate the transmittance value. A particle size analyzer (PSA) was used to evaluate the diameter particle size of the formula. An antibacterial susceptibility assay using agar dilution methods was undertaken to evaluate the inhibitory activities of different formulas against the tested bacteria. Analysis of variance and Kruskal-Wallis tests were used to compare the stability value and inhibitory effect.

The formula that was obtained exhibited a characteristic aroma reminiscent of citronella and possessed a pleasant taste on F I, while exhibiting a slightly bitter taste on F II and F III. The qualities remained unchanged during the duration of storage. All formulas tested on transmittance and the PSA tester did not show any statistical difference in 30 days. This means that all formulas were unaltered physically for up to 30 days of storage at room temperature. However, formula I showed the best characteristics. All formulas were tested against S. sanguinis and P. gingivalis but were not statistically different compared to the with other formulas ($p<0,05$). However, F I showed the smallest number of colony forming units.

Formula I (contains of 1% citronella oil and 2.77% lemon oil) is displayed the best characterization and stability when stored at room temperature for 30 days and exhibited inhibitory growth effects against S. sanguinis dan P. gingivalis on in vitro study.

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Introduction

Gingivitis is an inflammatory condition of the gingiva. Gingivitis is the most common of periodontal diseases[1]. It is a mild periodontal disease that causes redness and swelling (inflammation) of the gingiva[2]. It is caused by the interaction between the host immune response and biofilms of pathogenic microorganisms in dental plaque[3]. Bacteria present in oral biofilms are responsible for the most prevalent diseases of gingivitis[1]. Untreated gingivitis can develop to further periodontal diseases such as periodontitis, pocket formation, and tooth loss[4]. Over one billion cases of severe periodontal diseases are thought to exist worldwide, affecting approximately 19% of the adult population. Beginning in late adolescence, the prevalence of severe periodontal disease peaks at the age of 55 and continues to increase until old age[5].

The main risk factor for periodontal disease is poor oral hygiene[5]. The control of oral biofilms becomes essential in the prevention of

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these diseases. The prevention of periodontal diseases is based on supragingival biofilm control, by mechanical and or chemical oral hygiene products, that can limit the onset of gingivitis. Therapeutic mouthwashes have been recognized as a crucial element for lowering oral biofilm[6]. Numerous active ingredients of mouthwashes have been evaluated such as chlorhexidine (CHX), cetylpyridium chloride (CPC), povidone-iodine (PVP-I) as antibacterial agent[7,8] and mouthwashes containing PVP-I, CPC. The gold standard is CHX mouthwashes. Chronic use of CHX can induce changes in the sense of taste, causing staining on the tooth surface[7–10]. Other studies have also reported the side effects of using CHX such as ulceration, salivary glands swelling, and allergic reactions[9,10].

Therefore, novel, environmentally friendly, economical compounds that may be incorporated into everyday treatment solutions are needed, such as mouthwashes that are simple to use and can access confined regions of the mouth. At present, consumers are more interested in utilizing natural products because of their safety and health benefits[11]. Cymbopogon winterianus is indigenous to tropical and subtropical regions of Asia, India and Indonesia. The essential oil derived from Cymbopogon winterianus, commonly referred to as Java citronella oil[12]. Lemon (Citrus limon) is an essential medicinal plant of the Rutaceae family that originated in tropical and subtropical Southeast Asia[13]. Citronella oil and lemon oil have demonstrated antibacterial activities on Gram-positive and Gram-negative bacteria[14–19]. Streptococcus sanguinis (S. sanguinis) and Porphyromonas gingivalis (P. gingivalis) are bacteria that are often affected by periodontal tissue damage[20,21]. Biofilm formation management is the key to preventing periodontal disease. The purpose of the present study was to develop a polyherbal mouthwash using combination of citronella and lemon oils that can be optimized for controlling periodontal pathogen, by evaluating the organoleptic test, stability properties and antibacterial effects against Streptococcus sanguinis and Porphyromonas gingivalis.

Materials and methods

Characterization

Citronella oil (*Cymbopogon winterianus*) and lemon oils (*Citrus limon*) were obtained from Indesso Aroma Corporation (Indonesia). Oil characterization was performed by assessing visual appearance and analysis of component. Analysis of component compounds using GC-MS gas chromatography-mass spectrometry (Thermo Scientific Trace 1310, Italy. The chromatograph had an HP-5MS UI capillary column (30 m x 0.25 mm, ID x 0.25µm film thickness). The temperature of the column was maintained at 50°C for 2 min before being raised to 280°C at a rate of 5°C per min. The injector was heated to 300°C, and helium (He) was used as the carrier gas at a flow rate of 50 mL/min and split ratio of 1:50. The relative essential oil constituents were indicated as a percentage of the oil's peak area[18,22].

Formulation of citronella and lemon oil mouthwashes

The formulated mouthwashes contain citronella oil, lemon oil, and tween 80 dissolved in water. The mixture of some citronella and lemon oil were stirred magnetic stirrer, then add tween 80 while stirring continuously and add aquadest while stirring continuously. The emulsion was stirred for 24 h at room temperature while added colorants and sweeteners. The formulas of the mouthwashes are summarized in Table 1.

Ingredients	Quantity (mL)		
	Formula I (F I)	Formula II (F II)	Formula III (F III)
Citronella oil	0.50 (1%)	5,50 (11%)	2,07 (4,13%)
Lemon oil	1.38 (2.77%)	0,5 (1%)	0,5 (1%)
Tween 80 (surfactant)	4.62 (9.23%)	5.50 (11%)	3,93 (7.83%)
Sorbitol (sweetener)	15	15	15
Coloring agent (orange)	1 drop	1 drop	1 drop
Water	Up to 50	Up to 50	Up to 50

Table 1. Formulation of mouthwashes.

Evaluation of mouthwashes stability

Samples were bottled and stored at room temperature for 30 days. Their organoleptic properties such as color, odor, and flavor were evaluated[23].

Transmittance measurement

The percentage of transmittance was calculated as a determinant of optical clarity. The transmittance measurement of the formula was performed using a UV/Vis spectrophotometer (UV-1800 Shimadzu, Japan) with a wavelength of 650 nm and distilled water was used as blank[24].

Particle size distribution

Particle size distribution was evaluated

using Particle Size Analyzer (Malvern Zetasizer ZS, UK). The sample solution was prepared on the cuvette and place into a particle size analyzer for size reading.

Antibacterial methods

The agar dilution method was used to test the antibacterial activity of the mouthwash's formula. Bacterial strain used in this study were *S. sanguinis* ATCC 10556 and *P. gingivalis* ATCC 33277.

S. sanguinis preparation

The bacterial suspension turbidity using 0.5 McFarland standard or the equivalent of 1.5×10^8 CFU/mL. To examine antibacterial effect of mouthwash formula, 10 µl bacterial suspension of *S. sanguinis* was inoculated with 80 µl brain heart infusion (BHI) broth and 10 µl mouthwashes formula (I, II and III). Dilute to 10^5 using sterile distilled water. The cell count was determined by plating 100 µl on a mueller hinton agar (MHA). After 24 h of incubation at 37°C, colony forming unit (CFU) were measured using a colony counter[25]. All experiments were conducted in triplicates.

P. gingivalis preparation

The bacterial suspension turbidity using 0.5 McFarland standard or the equivalent of 1.5×10^8 CFU/mL. To examine antibacterial effect of mouthwash formula, 10 µl bacterial suspension of *P. gingivalis* was inoculated with 80 µl BHI broth and 10 µl mouthwashes formula (I, II and III). Dilute to 10^1 using sterile distilled water. The cell count was determined by plating 100 µl on a blood agar. After 8 d of incubation at 37°C under anaerobic conditions, CFU were measured using a colony counter. All experiments were conducted in triplicates.

Statistical analysis

The results were expressed as means ± standard deviations (SDs) for triplicate at least. Analysis of variance was used to compare the means of the results of all parameters except transmittance stability test of F II was use Kruskal-Wallis. All data analysis was performed using the IBM SPSS statistics. Results were considered significant at $p< 0.05$.

Results

Characterization results

Visual appearance of essential oils was pale yellow (lemon oils) and yellow (citronella oils) (Figure 1). The results of aroma trapping with GC-MS analysis revealed that the essential oils of from *Citrus limon* contained 30.89% D-Limonene as the main compound and essential oils of *Cymbopogon winterianus* contained 17.30% citronellol as the main compound (Figure 2 and Table 2).

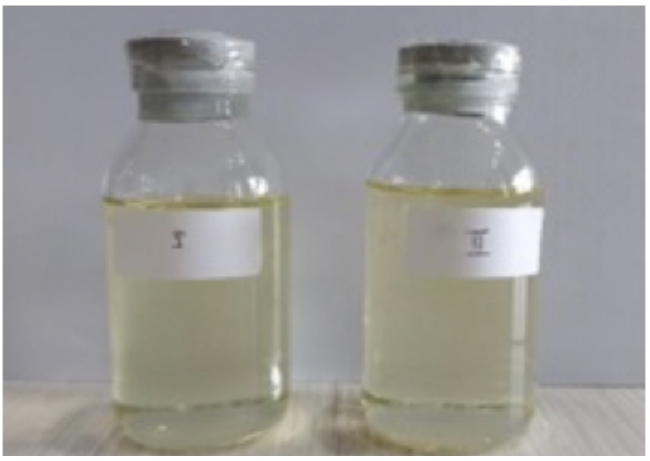

Figure 1. Visual appearance of lemon oils (I) and citronella oils (II).

Essential oils	Compound	Retention time (min)	%
Lemon oil	D-limonene	9.51	30.89
	ß- pinene	8.00	20.11
	γ-terpinene	10.66	14.79
	alpha pinene	6.80	6.76
	citral	16.47	6.35
	2,6-octadienal	15.64	4,42
	geranyl acetate	18.97	4.10
	ß-bisabolene	22.61	1.91
	terpineol	14.26	1.02
	linalool	11.69	1
Citronella oil	citronellol	15.42	17.30
	geraniol	16.23	17.07
	citronellal	13.12	15.97
	D-limonene	9.49	6.52
	linalool	11.63	6.23
	citronellyl butyrate	18.74	4.07
	germacrene D	22.03	3.42
	muurolene	21.92	3.10
	geranyl acetate	19.51	2.84
	α-cadinol	25.87	2.50

Table 2. Main compounds* of lemon and citronella oil.

* The minor compounds have not been shown.

Evaluation of mouthwash stability

The organoleptic of mouthwashes formula including odor, color, flavor, and visual appearance were evaluated. The organoleptic test results of the formula are provided in Figure 3 and Table 3. Visually, all formulas presented a consistent color and citronella fragrance that was unaltered for up to 30 days of storage at room

temperature. Compared with the other formulas, F I have the most acceptable flavor (Figure 3 and Table 3).

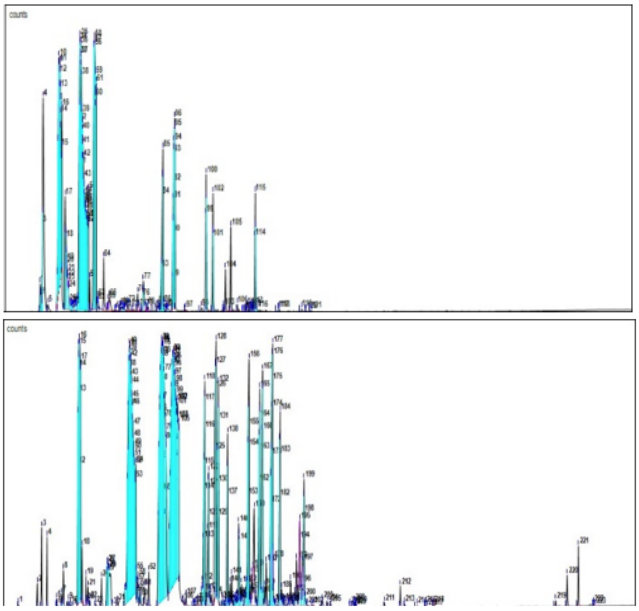

Figure 2. GC-MS chromatograms of citrus limon (a) and citronella oil (b).

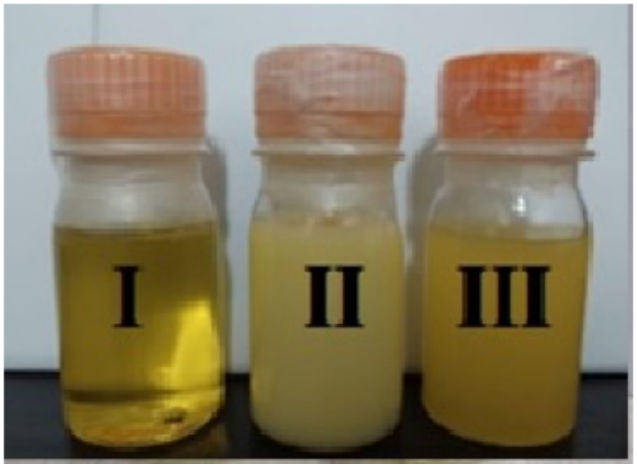

Figure 3. Appearance of mouthwashes formulas I, II, and III.

Mouthwashes	Parameters			
	Visual appearance	Odor	Flavor	
F I	Clearly orange	Citronella fragrance	Fresh	
F II	Milky orange	Citronella fragrance	Fresh bitter	and slightly
F III	Orange	Citronella fragrance	Fresh bitter	and slightly

Table 3. Organoleptic test results.

The results of the transmittance stability test for 30 days were not significantly different among the formulas (Table 4). This showed that all formulas have stable transmittance values during storage at room temperature. Formula I had the highest transmittance value. This demonstrates the clarity of F I than the other formulas.

Mouthwashes	Time (weeks)				
	0	1	2	3	4
F I	81.12±5.27	83.64±6.35	79.43±7.69	81.98±7.69	82.13±9.38
F II*	42.11±6.01	42.19±6.33	43.16±5.58	43.76±7.21	41.75±7.56
F III	12.86±1.96	12.19±1.28	13.52±2.40	13.22±1.94	12.75±2.12

Table 4. Transmittance.
The mean ± standard deviation in the same were not significantly different (p<0,05), *Kruskall-Wallis test.

As part of the stability test, the diameter of the size particles of the different formulations was measured over time. Formula I had the smallest particles among the formulas. The particle size stability test for 30 days also showed not significantly different results among the three formulas, indicating that the formulas were stable during storage at room temperature (Table 5).

Mouthwashes	Time (Weeks)				
	0	1	2	3	4
F I	216.85±22.34	213.30±42.06	216.21±16.49	209.62±31.93	220,20±44,82
F II	307.98±10.47	325.30±44.29	269.55±20.57	310.64±54.07	307,87±40,00
F III	466.80±63.41	425.60±70.86	443.04±37.35	46.73±21.50	494,29±100,32

Table 5. Diameter size of the particles
Antibacterial activity.
The mean ± standard deviation in the same were not significantly different (p<0,05).

The potential antibacterial activity of the formula was determined against *S. sanguinis* and *P. gingivalis*. All formulas could inhibit the growth of these microorganisms. Formula I was highly effective, and it did not show statistical difference with the other formulas. Data are reported as means± SD. Data were analyzed by analysis of variance (Figure 4).

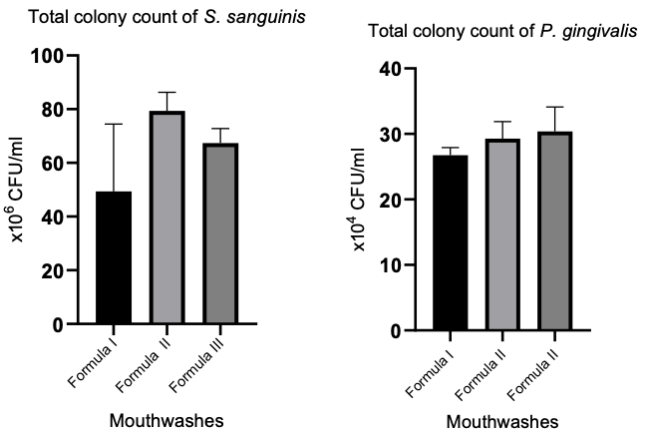

Figure 4. Antibacterial effect against S. sanguinis and P. gingivalis.

Discussion

Mouthwashes are developed to reduce biofilm growth and control and prevent gingivitis and unpleasant odors. The addition of antimicrobial substances such as sodium fluoride, and essential oils generally enhances their effect[7,26]. Chlorhexidine is currently the antibacterial gold standard, despite its disadvantages, such as tooth discoloration and altered taste perception[9,10]. Thus, new substances with strong antimicrobial and protective effects than CHX are desired. In this study, citronella oil and lemon oil were used as active ingredients, and they are known to have antibacterial activity. To ensure their quality, characterization was carried out and test results are shown in Figure 1,2 and Table 2. The results of the GC-MS analysis showed *three major* compounds of lemon oils are D-limonene 30.89%, ß-pinene 20.11% and γ-terpinene 14.79%. D-limonene as the largest compounds is consistent with the results of previous studies[18]. The analysis of citronella oils revealed the presence of three primary compounds: citronellol 17.30%, geraniol 17.07%, and citronellal 15.97%. These findings align closely with the results reported in previous studies[27]. The percentage composition of the essential oils detected was found different from those in previous studies [18,22,27–29]. These could be due to the different environmental factors such as weather, season, or geography[29,30].

Vision, hearing, touch, taste, and scent are the main human senses involved in organoleptic assessment. In general, sensory properties are categorized into appearance, flavor, and texture. Appearance refers to the totality of a substance or object's visible characteristics. There is a common association between appearance and colour. Flavor is the combination of olfactory, gustatory, and trigeminal sensations experienced during sampling. This definition acknowledges the interaction between the sense of smell, perceived via the nostrils, and the sense of taste, perceived via the tongue[31]. The results of the organoleptic assessment of the formula are provided in the Table 3. Visually, all formulas presented a consistent color, which were unaltered for up to 30 days of storage at room temperature. The emulsion is in the form of a liquid with the characteristic aroma of citronella, with a different colour gradient for each formula. The yellowish color is produced from coloring agent. Formula I also tastes fresher than F II and F III, because the citronella oil content in formula I is lower. The high citronella oil content will generate bitter taste. The visual characterization of F I presented clear appearance compared with the other formulas; this is influenced by the transmittance value as well as the diameter particle size of F I.

The screening was conducted to determine the stability of the emulsion produced by looking at the transmittance assessment results. The percent transmittance test was performed to determine the clarity of the emulsion. The clarity of the emulsion is one of the parameters of perfect dispersion. Based on the results in Table 4, F I has the highest transmittance value among the other formulas and produces a visually clear dispersion with a transmittance value of >80%. This indicates that emulsion contains small particles. The percentage of the transmittance value that is close to 100% indicates that the size of the emulsion particles is so small and transparent visually[32]. The results of the particle size distribution analysis using PSA obtained the particle size of F I approximately 200 nm, which is smaller than the other formulas (Table 5).

The antibacterial activity test used two types of bacteria, which are Gram-positive (*S. sanguinis*) and Gram-negative (*P. gingivalis*). *S. sanguinis* is an abundant commensal bacterium in the oral biofilm that facilitates the attachment of pathogenic bacteria associated with inflammation in periodontal tissues[20]. *P. gingivalis* is a Gram-negative oral anaerobe implicated in periodontitis pathogenesis[21]. In the antibacterial activity testing, F I had the highest inhibition on Gram-positive and Gram-negative bacteria compared with other formulas. Particle size has a strong effect on dissolution and absorption of drug. The smaller particle size increases the surface area and consequently higher dissolution rate. Micronizing the particle size improves dissolution and solubility[33,34]. Another research has demonstrated that Gram-positive are more sensitive than their Gram-negative bacteria[35,36]. In this study showed Gram-positive bacteria exhibited a higher level of resistance compared to the Gram-negative bacteria towards the examined formulations of mouthwashes. It has been proposed that the variation in sensitivity to antimicrobial agents

between Gram-positive and Gram-negative bacteria may be ascribed to the structural and compositional characteristics of their cell envelope, which includes the cytoplasmic membrane, outer membrane, and cell wall[37]. Gram-positive bacteria lack the outer membran, but the cell wall is formed by a thicker peptidoglycan layer that confers rigidity to cells and makes it difficult to penetrate by antimicrobials. This characteristic can be one explanation to the lower activity of essential oil constituents against the Gram-positive bacteria[38].

Volatile compounds in citronella oil and lemon oil also had antibacterial activity. A previous study showed citral and limonene compounds have antibacterial activity against several Gram-positive and Gram-negative bacteria[39]. The presence of citral and limonene compounds has the potential to induce changes in membrane permeability, resulting in significant alterations to the surface properties and morphological characteristics. Consequently, this could lead to a decrease in the capacity of pathogens to adhere to host surfaces[40]. The study conducted by[38] also showed the antibacterial activity of citronellol and citronellal against Gram-negative and Gram-positive bacteria. Both compounds disrupt the bacterial membrane by interacting with the cell surface.

The limitations of study include the short time for the stability test, i.e., only 30 days, and only two species of oral bacteria were analyzed in the antibacterial test, which might not show the full effect of mouthwashes on all periodontal pathogenic bacteria. Further long-term studies are warranted the stability of mouthwashes and antibacterial test with other oral bacteria species.

Conclusions

Within the limitations of the study, the result may indicate that formula I is the best formula. Formula I (contains of 1% citronella oil and 2.77% lemon oil) has the best characteristics compared with other formulas based on the organoleptic test, particle size, percent of transmittance and antibacterial activity. However, clinical use of the Formula I mouthwashes must be explored in future research.

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Declaration of Interest

There are no competing interests to declare.

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