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Formulation and Antibacterial Activity Test of Betel Leaf (*Piper Betle* L.) Essential Oil Mouthwash Against *Streptococcus mutans*

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ABSTRACT: Dental caries is a prevalent oral health issue in Indonesia, largely caused by *Streptococcus mutans*. While *Piper betle* L. essential oil possesses known antibacterial properties, its utilization in a stable mouthwash formulation requires scientific validation. Objective: This study aimed to formulate *Piper betle* L. essential oil into a mouthwash emulsion and evaluate its antibacterial activity against *S. mutans*. Essential oil was obtained via steam distillation and formulated into mouthwashes at concentrations of 1%, 2%, and 4% (v/v) using Tween 80 as an emulsifier. Antibacterial activity was assessed using the well-diffusion method against *S. mutans* (0.5 McFarland standard) in independent triplicate wells (n=3). Vehicle control (without essential oil) and Povidone-Iodine 10% (positive control) were used for comparison. Data were analyzed using One-Way ANOVA followed by Tukey HSD post-hoc test ($p<0.05$). The formulation containing 4% essential oil exhibited the strongest antibacterial activity among the test groups, with an inhibition zone of 12.66 ± 1.76 mm, which was significantly higher than the vehicle control (9.29 ± 2.67 mm; $p < 0.05$). The positive control showed the highest inhibition (18.03 + 1.16 mm). Physical evaluation confirmed that all formulations remained stable during 7 days of storage at room temperature (27 ± 2°C), with no specific control of relative humidity, with pH values (6.46–6.82) and specific gravity (1.03–1.04 g/mL) within physiological limits. *Piper betle* L. essential oil mouthwash is physically stable and effective against *S. mutans* in a concentration-dependent manner, suggesting its promising potential as a natural alternative for oral hygiene maintenance.

Keywords: Antibacterial; essential oil; mouthwash; *Piper betle* L; *Streptococcus mutans*

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INTRODUCTION

Infectious diseases caused by bacteria are a major health problem in developing countries, including Indonesia. One of the most common infections is oral disease, including dental caries, periodontal disease, and soft-tissue infections. These infections have a high prevalence and have a significant impact on people's quality of life. According to WHO (2025), approximately 3.7 billion people worldwide experience oral disease, with the greatest burden in low- and middle-income countries.

In Indonesia, the Basic Health Research/Rikesda (2018) found that the prevalence of dental caries among children aged 5-6 years was 57.6%. About 14% of them have oral disorders such as gingivitis and abscesses. Periodontal disease is also a common health problem, affecting around 20-50% of the global population. It is associated with several risk factors such as smoking, poor oral hygiene, diabetes, and age. Oral infections not only cause pain and discomfort but can also trigger systemic complications such as heart disease and diabetes, even increasing the risk of death (Sharma & Ranga, 2019).

One of the main bacteria that causes dental caries is *Streptococcus mutans*. This Gram-positive bacterium can synthesize insoluble glucan and produce lactic acid via homofermentation. These activities allow *Streptococcus mutans* to form a strong biofilm on the tooth surface, making it more virulent and acidogenic than other Streptococcus species (Zhang et al., 2021). Therefore, controlling *Streptococcus mutans* is an important focus in the prevention of dental caries.

The utilization of natural materials as antibacterial alternatives continues to grow. One plant that has been widely studied is betel leaf. These leaves contain bioactive compounds, such as eugenol, cinnamaldehyde, and flavonoids, known to have antibacterial activity. The mechanism of action includes damage to bacterial cell walls and membranes, as well as protein denaturation (Hermanto et al., 2023). Research by Sujono et al. (2019) showed that betel leaf essential oil exhibits increasing inhibition with concentration, with effectiveness observed in the 20-40% range.

Various studies have revealed the antibacterial potential of betel leaves against pathogenic microorganisms. Research by Nasution & Daulay (2022) showed that toothpaste formulations containing red betel leaf (*Piper ornatum* N.E.Br) and green betel leaf (*Piper betle* L.) extracts effectively inhibited the growth of *Staphylococcus aureus*. However, the study focused on non-oral bacteria and did not examine effectiveness against *Streptococcus mutans*, the main caries-causing bacteria.

In addition, the research by Sujono et al. (2019) uses essential oils in pure form and has not developed pharmaceutical preparations, such as mouthwash. Mouthwash is a more practical and relevant dosage form for oral health care.

Thus, there is still a research gap regarding the effectiveness of betel leaf essential oil in liquid dosage forms, such as mouthwash, against *Streptococcus mutans*. Few studies have specifically evaluated the optimal concentration, antibacterial activity, and potential clinical applications of such formulations. In addition, there is limited literature linking in vitro laboratory test results to the development of practical, community-applicable phytopharmaceutical products. Using mouthwash as part of an oral hygiene routine is effective in reducing plaque and gingivitis, especially when combined with regular brushing.

Research by Hermanto et al. (2023) showed that mouthwash containing essential oils may provide additional benefits in reducing plaque and gingivitis, especially when used in conjunction with dental floss.

However, the direct use of pure essential oil remains impractical due to its pronounced irritant potential and limited solubility in saliva. Consequently, developing a formulation capable of effectively emulsifying the oil into an aqueous medium is essential to improve patient acceptability and enhance bioavailability. To date, evidence on the formulation of Indonesian *Piper betle* essential oil into a stable mouthwash emulsion, as well as its specific inhibitory activity against *Streptococcus mutans* compared with an appropriate vehicle control, remains scarce. This study addresses this gap by developing a scientifically standardized herbal mouthwash formulation. Based on this background, this study aims to assess the antibacterial activity and inhibition of mouthwash preparations containing betel leaf essential oil against *Streptococcus mutans* bacteria in vitro.

METHODS

Materials

Fresh betel leaves were obtained from Muntilan Market, Magelang and distilled using the steam-water distillation method to produce essential oil. The test bacteria used were *Streptococcus mutans* obtained from the UGM Microbiology Laboratory. Other materials included BHI agar, povidone-iodine 10% (positive control), and various components of the mouthwash formula, such as tween 80 (Merck), sorbitol (Merck), peppermint oil (T&T Chemicals), Na-benzoate (Smart-Lab), and sterile distilled water (OneMed).

Betel leaf essential oil was formulated into mouthwash preparations at 1, 2, and 4% with additional components or excipients, as shown in **Table 1**.

Table 1. Formula of Betel Leaf Mouthwash

Composition	Formula (%)			
	F1	F2	F3	Vehicle Control
Essential Oil (v/v)	1 %	2 %	4 %	-
Na-Benzoate	0.1 g	0.1 g	0.1 g	0.1 g
Sorbitol	15 ml	15 ml	15 ml	15 ml
Peppermint	0.03 g	0.03 g	0.03 g	0.03 g
Menthol	0.02 g	0.02 g	0.02 g	0.02 g
Tartrazine	Qs	qs	qs	qs
Tween 80	3 g	3 g	3 g	3 g
Citric Acid	0.01 g	0.01 g	0.01 g	0.01 g
Na Citrate	0.03 g	0.03 g	0.03 g	0.03 g
Aquadest	Ad 100	Ad 100	Ad 100	Ad 100

Mouthwash Formulation Procedure

The mouthwash was prepared by first dissolving the *Piper betle* essential oil, peppermint oil, and menthol in Tween 80 to form the oil phase. In contrast, sodium benzoate, citric acid, sodium citrate, sorbitol, and tartrazine were separately dissolved in distilled water to create the aqueous phase.

To ensure stable emulsification, the aqueous phase was added dropwise to the oil phase under continuous stirring using a magnetic stirrer at 500 rpm for 15 minutes at room temperature (25°C). Finally, the volume was adjusted to 100 mL with sterile distilled water, and the mixture was stirred for an additional 5 minutes to achieve homogeneity. A vehicle control was prepared using the same procedure, omitting the essential oil, to serve as a baseline comparator.

Vehicle Control Preparation

To verify that the antibacterial activity was attributed to *Piper betle* essential oil rather than to the emulsifiers or excipients, a vehicle control was prepared. This formulation contained all the same components as the treatment groups (Tween 80, sorbitol, peppermint oil, menthol, and preservatives), but without *Piper betle* essential oil. This replaced the conventional negative control to evaluate the effect of the base matrix specifically.

Bacterial Preparation and Standardization

The *Streptococcus mutans* strain was rejuvenated on Brain Heart Infusion (BHI) agar slants for 24 hours at 37°C. Colonies were suspended in sterile physiological saline (0.9% NaCl). Turbidity was visually adjusted to match the 0.5 McFarland standard (approximately 1.5 × 10^8 CFU/mL) following standard protocols (Weinstein, 2020)**.** This standardized suspension was used immediately for the antibacterial assay.

Antibacterial Activity Testing

The antibacterial activity test was conducted using the well-diffusion method against *Streptococcus mutans* on BHI agar. Each mouthwash sample, up to 45 µL, was added to a well (6 mm in diameter), and the mixture was incubated at 37°C for 24 hours. The zone of inhibition was measured using a digital calliper, and the average of three repetitions was calculated. The experiment was performed in independent triplicates (n=3) to ensure reproducibility, and the mean diameter was calculated for statistical analysis.

Evaluation of Mouthwash Preparations

The evaluation comprised organoleptic assessment (color, odor, and taste), clarity, specific gravity, and pH measurements to assess the physical stability and overall quality of the formulation during 7 days of storage at room temperature (27 ± 2°C).

Data analysis

Inhibition zone diameter data were analyzed using a one-way ANOVA to determine differences between groups, followed by a Tukey HSD post hoc test if significant differences were detected. The significance level was set at $p<0.05$.

RESULT AND DISCUSSION

Plant Identification

Macroscopic identification of the betel leaf showed an ovoid to oval leaf shape with a pointed tip and a heart-shaped base. Microscopic examination, as listed in **Figure 1**, showed the presence of typical fragments, including epidermis with oil cells, transport bundles with ladder-shaped thickening, and anisocytic stomata, as described in the Indonesian Herbal Pharmacopoeia (Kemenkes, 2017).

Yield and Characteristics of Essential Oil

From the distillation of 7.5 kg of betel leaves, 25 mL of essential oil was obtained, yielding 0.33% (v/b). The produced essential oil has a refractive index of 1.507, indicating its relatively high purity. This result shows a significant difference compared to the research by Sujono et al. (2019), which reported a yield of 0.14% and a refractive index of

1.34, which may be due to differences in extraction methods, raw material conditions, or plant geographical variations.

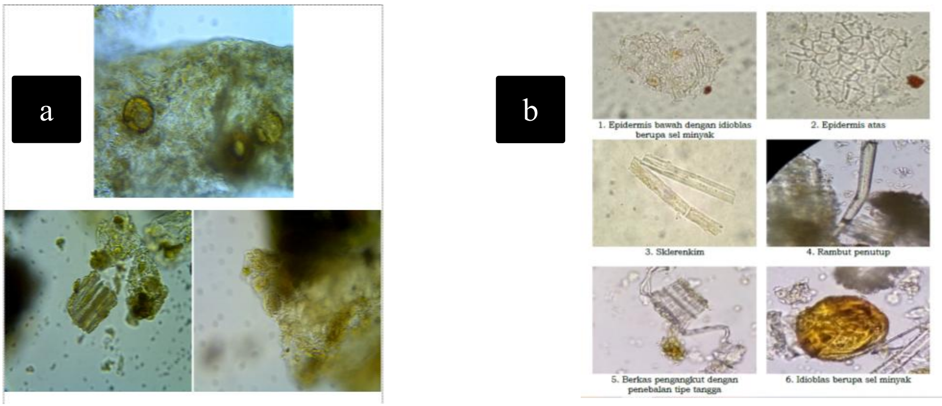

Figure 1. Microscopic identification of betel leaf. (a) observation/experiment results in this study, (b) adapted from the Indonesian Herbal Pharmacopoeia (Ministry of Health, 2017)

Mouthwash Preparation and Characteristics

The mouthwash was prepared in three concentrations of essential oil: 1%, 2%, and 4%. The formula composition includes ingredients such as menthol, peppermint oil, sorbitol, tween 80, sodium benzoate, citric acid, and sodium citrate. Tween 80 is the result of the condensation of oleate from sorbitol and its anhydride with ethylene oxide. Tween is used as an emulsifier, 5-10% of the oil phase (Adiningsih, 2019). Tween 80 used in this formula helps dissolve essential oils in water. The sweetener used in this study was sorbitol. The choice of sweetener is because sorbitol has advantages, namely, it lacks carbonyl groups in its chain. Sorbitol is less reactive and does not cause acid formation in dental plaque, so it acts as a safe sweetener against *Streptococcus mutans* (Soesilo et al., 2006).

The safety profile of each excipient in the formulation was evaluated in accordance with both Indonesian regulatory standards and recent international toxicological evidence. Sodium benzoate (0.1% w/v) and tartrazine (q.s.) are permitted food additives under BPOM regulation (BPOM RI, 2021), indicating that both compounds are acceptable for oral exposure when used within regulated limits. Recent evaluations by the European Food Safety Authority (EFSA) reaffirm that sodium benzoate and its related benzoates do not exhibit genotoxic, carcinogenic, or reproductive toxicity at exposure levels below the established acceptable daily intake (ADI) (EFSA, 2016). Similarly, international assessments by EFSA and JECFA over the past decade maintain an ADI of 0–10 mg/kg body weight/day for tartrazine, with no evidence of mutagenicity at the regulated doses. Considering that the mouthwash is used topically and expectorated, resulting in substantially lower systemic exposure than dietary intake, the toxicological risk from both additives remains low.

Additionally, the essential oil concentration was limited to 4% v/v to reduce the potential for mucosal irritation associated with phenolic compounds in *Piper betle*. At the same time, emulsification with Tween 80 minimizes direct epithelial contact and enhances tolerability. Taken together, the formulation aligns with national regulatory guidance and contemporary international toxicology data, supporting its suitability for short-term topical oral use.

Organoleptic

Organoleptic tests showed that all formulas had a green color and a distinctive betel and mint flavor, as shown in **Table 2**. This is in line with the organoleptic description of betel in the Indonesian Herbal Pharmacopoeia (Kemenkes, 2017). The distinctive smell of betel comes from the eugenol compounds in betel leaf essential oil. According to Guo et al. (2024) the Eugenol content has a fairly strong odor. Meanwhile, the content of avicol compounds gives betel a distinctive smell (Lukito et al., 2024). The mint flavor in the mouthwash formulation comes from peppermint and menthol additives.

Table 2. Organoleptics of mouthwash preparations

Formula	Color	Smell	Taste
Formula 1 (1%)	Green	Typical betel nut	Mint
Formula 2 (2%)	Green	Concentrated betel characteristic	Mint
Formula 3 (4%)	Green	Very strong betel nut flavor	Mint

Clarity

Visual differences can be seen in **Figure 2**, where there are differences between the vehicle control and three mouthwash formulas containing betel essential oil with graded concentrations. The vehicle control appeared clear and bright green without turbidity, indicating the absence of essential oil or emulsification process. Formula 1, containing 1% essential oil, began to show mild turbidity. In comparison, formula 2 containing 2% essential oil appeared more turbid, with a whitish-green color, indicating a greater number of oil droplets dispersed in the aqueous phase. Formula 3, with a 4% essential oil concentration, showed the highest turbidity and a whitish, opalescent color, indicating strong emulsification and possible aggregation of oil droplets.

The increase in turbidity between formulas follows the basic theory of emulsification, in which a higher amount of essential oil results in more oil droplets dispersing in the system, leading to stronger light scattering and a more turbid visual appearance (Linke & Drusch, 2016). Tween 80, a non-ionic surfactant, plays an important role in forming and stabilizing oil-in-water (O/W) emulsions by lowering the interfacial tension between oil and water and by forming a protective film on oil droplets (Jiang et al., 2022). As the oil concentration increases, the surfactant's capacity to stabilize the entire droplet may become limited, leading to increased system size and turbidity. This visualization supports the idea that the emulsion system is formed and physically stable; however, at high concentrations,, it can approach its limits of stability

pH

The pH values of the three mouthwash formulas produced in this study ranged from 6.46 to 6.82. This range is still within the physiological pH range of saliva (5.6-7.0), indicating that the formulas are relatively safe and do not cause irritation of the oral cavity. These results are in line with a study by Lee et al. (2021), which reported that most natural-based mouthwashes have a pH between 3.45-6.75, and that preparations with a pH above 4.0 are less likely to cause enamel erosion than highly acidic formulas (< 4.0). Therefore, the formula in this study can be considered safe and compliant with applicable herbal formulation standards, supported by empirical data and valid industry references.

Physical Stability Evaluation

The mouthwash's physical stability was assessed organoleptically, and pH and specific gravity were monitored during storage. While rheological properties (viscosity) and microscopic droplet size were not quantified in this study, visual monitoring revealed no signs of phase separation, creaming, flocculation, or sedimentation in any of the formulations. This macroscopic stability indicates that the emulsion system was adequately stabilized by Tween 80, which effectively reduced interfacial tension and maintained the essential oil in the aqueous phase, thereby creating a kinetically stable system throughout the study. All formulations maintain pH values during storage.

The specific gravity of all mouthwash formulas in this study ranged from 1.03 to 1.04 g/mL and did not differ significantly from that of the control formula. This indicates that the preparation has good physical stability and does not change during storage. These results are in line with research by Venâncio et al. (2015), which reported that herbal mouthwash made from *Libidibia ferrea* had a stable specific gravity between 1.03-1.04 g/mL for 60 days, without sediment or other physical changes. In addition, research by Ardini & Mulatasih (2020) who formulated mouthwash from essential oil of Cinnamon (*Cinnamomum burmannii*) and betel leaf (*Piper* betel) the addition of stabilizers contributed to good stability in both specific gravity and viscosity such as Tween 80 and glycerin, with a density close to water (~1.0 g/mL) and still showed antibacterial activity against *Streptococcus mutans*. Based on these two studies, it can be concluded that the specific gravity values of the formulas in this study remain within a reasonable range and reflect physical stability equivalent to that of previously proven herbal formulations.

Figure 2. Betel leaf essential oil mouthwash (a) vehicle control, (b) Formula 1 (1%), (c) Formula 2 (2%), (d) Formula 3 (4%)

Antibacterial Activity

The antibacterial inhibition test results showed that the three mouthwash formulas containing betel leaf essential oil inhibited the growth of *Streptococcus mutans,* with varying inhibition zones. As presented in **Table 3**, the formula with 4% essential oil concentration (Formula 3) produced the greatest inhibition, which was 12.66 ± 1.76 mm, followed by Formula 2 (2%) at 11.34 ± 1.49 mm, and Formula 1 (1%) at 10.45 ± 2.02 mm. For comparison, the positive control with povidone-iodine produced an inhibition zone of 18.03 ± 1.16 mm, while the vehicle control (without essential oil) showed an inhibition zone of only 9.29 ± 2.67 mm.

Table 3. Diameter of inhibition of betel leaf essential oil mouthwash

Formula	Inhibition (mm)
Formula 1	10.45 ± 2.02^{ab}
Formula 2	11.34 ± 1.49^{bc}
Formula 3	12.66 ± 1.76^{c}
Positive Control	18.03 ± 1.16^{d}
Vehicle Control	9.29 ± 2.67^{a}

Values are expressed as Mean ± SD (n=3). Different superscript letters (a-d) within the same column indicate significant differences between groups (p < 0.05) based on Tukey HSD post-hoc test

Visually, the differences in the inhibition zone diameters of the formulas are shown in **Figure 3**. The clear zone around the test hole indicates the level of effectiveness of bacterial inhibition. The holes in Formula 1, Formula 2, and Formula 3 showed an increase in the size of the inhibition zone as the concentration of essential oil increased. Meanwhile, the holes in the positive control showed the widest inhibition zone, and those in the vehicle control appeared to have the smallest.

The One-Way ANOVA test revealed a statistically significant difference in inhibition zones among the treatment groups ($p < 0.05$). Post hoc analysis using the Tukey HSD test showed that increasing the concentration of the essential oil significantly enhanced antibacterial efficacy. Formula 3 (4%) exhibited the strongest inhibition among the herbal formulations and was significantly different from the Vehicle Control ($p < 0.05$), indicated by the distinct superscript letter (c vs a). However, the Positive Control (Povidone Iodine) still displayed the highest efficacy (18.03 ± 1.16 mm), which was significantly superior to all herbal formulations.

Justification of the control in this study: Povidone-Iodine (10%) was employed as the positive control. Although Chlorhexidine 0.12% is widely regarded as the gold standard for anti-plaque efficacy (Mathew et al., 2022), Povidone Iodine was selected due to its broad-spectrum antiseptic properties and its wide availability as a standard oral care intervention in primary healthcare settings in Indonesia (Amtha & Kanagalingam, 2020).
Furthermore, the study used a vehicle control (Tween 80 and other excipients without *Piper betle* oil) rather than a simple vehicle control. The results showed that, while the vehicle exhibited a baseline inhibition zone (likely due to the preservative sodium benzoate or the peppermint additives), the addition of Piper betle essential oil in Formulas 1, 2, and 3 resulted in larger inhibition zones. This confirms that the Piper betle essential oil provides significant active antibacterial reinforcement beyond the capacity of the basic emulsion system/vehicle.

This finding is in line with Rizkita et al. (2017), who reported that the essential oil from green betel leaves showed antibacterial activity against *Streptococcus mutans*, with an inhibition zone of 10.20 mm at 20% concentration. In the study, green betel leaf oil was specifically separated from the red betel type and still showed strong antibacterial activity, although slightly lower than that of red betel oil. This indicates that green betel alone contains bioactive components effective against caries-causing bacteria.

Although GC-MS analysis was not performed in this study, recent profiling of green betel leaf (*Piper betle* L.) essential oil by Septiani et al. (2024) revealed that the oil is rich in bioactive terpenes and phenolic compounds, identifying sabinene (18.3%), phenol (11.66%), and chavicyl acetate (10.70%) as major constituents. This quantitative data complements the findings of Rizkita et al. (2017), who reported that *Piper betle* essential oil exhibits specific antibacterial potency against *Streptococcus mutans*, primarily driven by its phenolic content. The presence of these lipophilic compounds allows the oil to penetrate the bacterial cell membrane, leading to leakage of intracellular materials and cell death, which explains the inhibition zones observed in this study (Nadia Zahra & Maryati, 2024; Rizkita et al., 2017; Septiani et al., 2024).

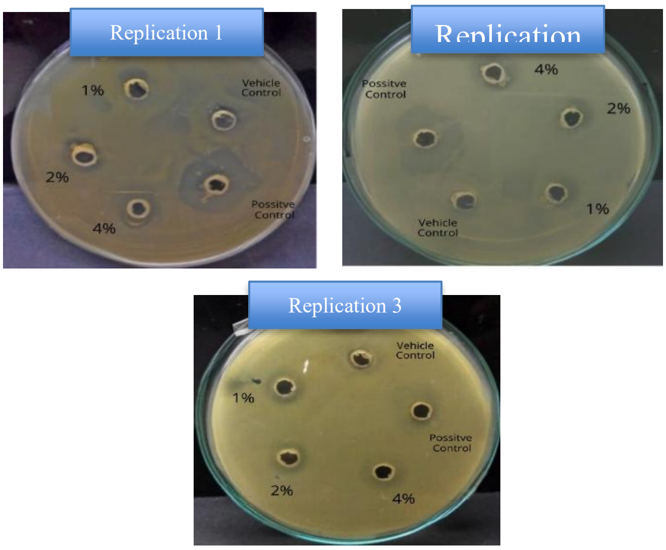

Figure 3. Antibacterial activity of mouthwash formulations against *Streptococcus mutans*. Clear zones around the wells indicate bacterial inhibition. Vehicle control showing no inhibition, Povidone iodine 10%, *Piper betle* essential oil formulations showing concentration-dependent inhibition zones

Relationship between Physical Parameters and Activity

Based on the evaluation results, no significant relationship was found between physical parameters, such as pH and specific gravity, and the antibacterial activity of betel leaf essential oil mouthwash preparations against *Streptococcus mutans*. Although physical parameters play an important role in ensuring the physical stability and quality of the preparation, they do not directly affect the antibacterial effectiveness.

The pH values of the three formulas were in the neutral range (6.46-6.82), which corresponds to the safe range for oral product use, but not extreme enough to produce a bactericidal effect against microorganisms. This suggests that the antibacterial activity is more determined by the concentration and potency of the active compounds than by pH alone. Similarly, the relatively uniform specific gravity of the preparation is more reflective of the system's physical stability, such as homogeneity and viscosity, without directly contributing to the mechanism of inhibiting bacterial growth.

These results are in line with the findings of Karim et al. (2023) in a study of red shoots leaf extract mouthwash *(Syzygium myrtifolium)*, which showed no correlation between physical parameters (pH and density) and the diameter of the inhibition zone against *Streptococcus mutans*. A similar study by Sarifuddin (2022) on the preparation of beluntas (*Pluchea indica*) leaf mouthwash also revealed that although the formula was within the ideal pH range and showed good physical stability, there was no significant increase in antibacterial activity.

Limitations of the Study

Several limitations of this study should be noted. First, the chemical profile of the essential oil was not analyzed using GC–MS, so the presence of phenolic constituents is inferred from prior studies on Indonesian Piper betle L. and supported by its organoleptic characteristics. Second, physical stability testing was limited to pH, specific gravity, and visual observation without rheological or droplet-size analysis, although no phase separation occurred during storage. Third, antibacterial activity was assessed using the well-diffusion method, which serves as a preliminary screen and does not provide quantitative MIC values. Finally, Povidone Iodine was used as the positive control, but future studies should include 0.12% Chlorhexidine as the gold-standard comparator for clinical antiplaque efficacy

CONCLUSION

Green betel leaf essential oil showed significant antibacterial activity against *Streptococcus mutans*, with greater effectiveness at higher concentrations in mouthwash preparations. Formula number 3 (4%) exhibited the highest antibacterial inhibition. All formulations maintained stable physical characteristics during 7 days of storage at room temperature (27 ± 2°C) and were organoleptically acceptable. However, physical parameters such as pH and specific gravity are not directly associated with antibacterial activity. Therefore, betel leaf essential oil-based mouthwash preparations have the potential to be developed as effective, stable, and safe herbal products to support the prevention of dental caries.

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AUTHOR CONTRIBUTION

PA : Conceptualization, methodology, formal analysis, investigation, resources, writing original draft; SM: Methodology, formal analysis, resources; AF: Investigation, writing original draft; NDAC : Writing original draft; NS: Reviewing draft, supervision

CONFLICT OF INTEREST (If any)

None to declare

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