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Development of Mouthwash Formulations based on Natural Ingredients with Antimicrobial Activity

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Abstract

Dental and oral health problems are one of the problems that need attention. The oral cavity is colonized by various microflora and some bacteria, so it is necessary to develop additional oral cleaning methods besides brushing teeth. Mouthwash with natural ingredients, such as a combination of chitosan and Eucalyptus grandis, is the right choice. Chitosan is a type of water-soluble chitosan characterized using FT-IR. Meanwhile, Eucalyptus grandis was isolated using the Stahl method to obtain its essential oil. Mouthwash was evaluated through organoleptic testing, pH, viscosity, and antimicrobial testing. The most optimal formula is formula III, with an inhibition zone diameter of 11.9 ± 1.5 mm for Streptococcus mutans bacteria, 12.8 ± 0.9 mm for Staphylococcus aureus, and 12.2 ± 0.15 mm for Candida albicans.

Keywords: Chitosan, *eucalyptus grandis*, *streptococcus mutans, staphylococcus aureus, candida albicans*

Introduction

Oral hygiene maintenance is essential in preventing plaque buildup, a sticky layer of bacteria and food from accumulating on the teeth. Oral hygiene measures can be carried out using mechanical aids such as toothbrushes, interdental cleaners, and chemotherapeutic agents such as camphor, toothpaste, and chewing gum (Patil et al., 2020). However, mouthwash is more effective because it can reach areas in the oral cavity that cannot be reached by brushing (Iskandar et al., 2022). Mouthwash is a concentrated aqueous antibacterial solution used against oral microbes to fight oral infections, cleanse, remove bad breath refresh, and be antiseptic (Banu & Gayathri, 2016).

WHO has advised researchers to investigate the possible use of natural products in mouthwash (Patil et al., 2020). For several decades, medicinal plants have been reported to be important in curing diseases because they have antimicrobial and antifungal activities against human pathogens. Herbal mouthwashes are in great demand because they can fight oral pathogens, relieve pain instantly, and have fewer side effects (Banu & Gayathri, 2016).

One of the natural ingredients that can be used is chitosan. Chitosan, a derivative of chitin (obtained by deacetylation) and is used as the leading cause of tooth loss (Li et al., 2017) is a high molecular weight polysaccharide having an active amino group that gives it some biological properties (Costa et al., 2014). Vilasan et al. (2020) researched the manufacture of mouthwash with a combination of chitosan and chlorhexidine, which can control plaque. Besides the advantages, this mouthwash has long-term side effects because of its chemical, namely chlorhexidine. So it would be better to combine chitosan with other natural ingredients to manufacture mouthwash such as *Eucalyptus*.

The genus Eucalyptus has a long history of use in traditional medicine. It has attracted significant interest worldwide for its antibacterial, antiviral, antifungal, anti-inflammatory, and insect-repellent properties for cosmetic, pharmaceutical, nutraceutical, and furniture purposes (Souza et al., 2021).

Many species of microbes exist in the oral cavity. However, the most common microbes are *Streptococcus mutans, Staphylococcus aureus,* and the fungus *Candida albicans* which cause many oral problems. *Streptococcus mutans* is a facultative anaerobic gram-positive bacterium and the most crucial cariogenic agent in developing dental caries (Li et al., 2017). *Staphylococcus aureus* is a Gram-positive, aerobic bacterium (Troeman et al., 2019) and the most pathogenic member of the *Staphylococci* genus and the etiologic agent of a wide

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variety of diseases ranging from superficial skin abscesses to food poisoning, which can cause various diseases (Bitrus et al., 2018). *Candida albicans* is the most common microbial agent causing opportunistic fungal infections affecting the oral mucosa, and it also has an important role in caries formation (Souza et al., 2021). So, this study was conducted to analyze the most optimal mouthwash formulation based on physical stability testing and antimicrobial activity.

Methods

Tools and materials

The tools used in this study include glassware, analytical balance, pH meter, magnetic stirrer, Ostwald viscometer, Stahl distillation, oven, sample refrigerator, Agilent Cary 630 FT-IR Spectrometer, Shimadzu QP 2010 GC-MS using RTX-5MS column with uHP grade helium as a carrier gas, antimicrobial test equipment. The materials used in this study include chitosan, *Eucalyptus grandis*, glycerin, Tween-80, sorbitol 70%, sodium benzoate, lactic acid, anhydrous Na_2SO_4, and aquadest.

Sample preparation

(1) Water-soluble chitosan

The type of chitosan used came from shrimp shells obtained from PUI Chitosan, University of North Sumatra. Water-soluble chitosan is obtained by reducing the molecular weight of chitosan through the depolymerization process with H_2O_2. After that, the water-soluble chitosan obtained was characterized using FT-IR.

(2) Eucalyptus essential oils

The leaves of *Eucalyptus grandis* used were from PUI Eucalyptus, University of North Sumatra, which were isolated using the Stahl method for ± 4-5 hours at a temperature of ± 110 °C until the oil evaporated completely. The distillate was decanted with Na_2SO_4 to bind the water mixed with the isolation.

Mouthwash formulation

The design of the mouthwash formulation is shown in **Table 1**.

Table 1. Mouthwash formulation

Composition	Control	Formulas			Function
		I	II	III	
E. grandis extract (mL)	-	0.1	0.2	0.3	Active substance
Water-soluble chitosan (%)	2	2	2	2	Active substance
Tween-80 (mL)	3	3	3	3	Surfactant
Gliceryn (mL)	5	5	5	5	Humectant
Sorbitol (mL)	2	2	2	2	Sweetener
Benzoic acid (g)	0.2	0.2	0.2	0.2	Buffer
Aquadest (mL)	100	100	100	100	Solvent

Preparation of mouthwash

Preparation begins with dissolving *Eucalyptus grandis*, which is insoluble in water emulsified with tween-80, and then adding water-soluble chitosan. Then, glycerin and sorbitol 70% were added to the preparation and stirred until homogeneous. Next, add sodium benzoate, dissolved in the water previously. After all the ingredients are put into the preparation, add aquadest until the preparation becomes 100 mL. It was then homogenized with a magnetic stirrer. Put the preparation into a mouthwash bottle. Do the same for the other variations.

Evaluation of mouthwash preparations

(1) Organoleptic observations

Observable preparations include color, taste, and odor at room temperature, visual characteristics that can be observed directly (Handayani et al., 2017).

(2) pH test

A crucial parameter to determine the feasibility of a mouthwash formula is the pH value. In this study, testing was carried out for 16 weeks and evaluated every 4 weeks. The pH quality standard for mouthwash is between 5-7 (Hidayanto et al., 2017).

(3) Viscosity testing

The preparation is inserted into tube B on the *Ostwald* viscometer. Then the solution is sucked until the liquid passes through part and the viscometer's upper limit. The stopwatch is turned on when the rubber ball is released, and the time is calculated when the preparation flows from the upper limit to the lower limit. Then the stopwatch is turned off, and the time of mouthwash preparation is recorded (Handayani et al., 2017).

Antimicrobial test

Mechanism of antimicrobial activity testing using agar diffusion method. According to Davis & Stout (1971), the criteria for the strength of antimicrobial activity are classified into 4, namely, if the diameter of the inhibition zone > 5 mm is categorized as weak, 5-10 mm is categorized as moderate, 11-29 mm is categorized as vital, and > 20 mm is categorized as very strong. The mouthwash formula has a negative control comparison, namely mouthwash without adding chitosan and *E. grandis* extract. The repetition was carried out three times for the antibacterial activity of *S. mutans* and *S. aureus*, as well as antifungal *C. albicans* (Handayani et al., 2016).

Results and Discussion

Chitosan characterization

Analysis of water-soluble chitosan using FT-IR yielded the spectrum shown in **Figure 1** and **Table 2**.

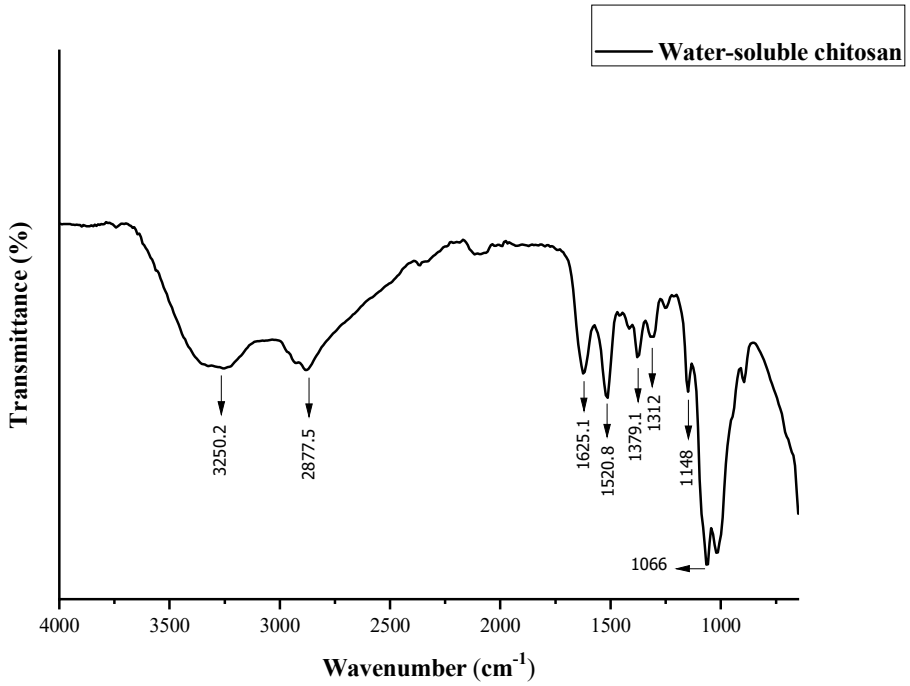

Figure 1. Spectrum of water-soluble chitosan

The absorption area of chitosan is shown in **Table 2**.

Table 2. Chitosan spectrum data

No	Functional groups	Wave number (cm^{-1})
1	(vs) O-H, (vs) N-H	3280.1
2	(vs) C-H aliphatic	2870.1
3	(vs) C=O	1640
4	(vb) N-H	1580.4
5	(vb) C-H	1423.8
6	(vs) C-N	1319.5
7	(vs) C-O-C	1028.7

Description vs: stretching/stretching vibration vb: bending vibration

Based on the tabulation of the FT-IR test in **Table 2**, there is a broad absorption at a wavenumber of 3250.2 cm^{-1}, which indicates the presence of OH group strain and N-H symmetrical vibration. In addition, there is absorption at a wavenumber of 2877.5 cm^{-1}, indicating the presence of an aliphatic CH functional group with stretching vibrations. Three types of chitosan amide groups identified at a wavenumber of 1625.1 cm^{-1} indicate the presence of a stretching vibrational C=O group (amide I), wavenumber 1520.8 cm^{-1} indicates the presence of a bending vibrational N-H functional group (amide II) and a wavenumber of 1312 cm^{-1} C-N stretching vibration (amide III).

Furthermore, the wavenumber 1379.1 cm^{-1} is a bending vibration C-H functional group, and the wavenumber 1148 cm^{-1} indicates the presence of an asymmetric C-O-C functional group with stretching vibrations. In addition, there is a wavenumber of 1066 cm^{-1}, indicating the presence of glycosidic bonds, namely Ω β-1,4-glycosidic. The spectral data of water-soluble chitosan are not much different from that of ordinary chitosan. This is in line with Valgas et al. (2007), who have characterized water-soluble chitosan and water-insoluble chitosan and produced almost the same spectral data.

Evaluation of mouthwash preparations

(1) Organoleptic observations

The mouthwash preparation has a clear color, a distinctive *Eucalyptus* aroma, and a strong mint taste

(2) pH test

There was no significant change in the pH value of mouthwash preparations stored at room temperature for 16 weeks. The greater the composition of the active substance added to the mouthwash, the resulting pH will also be more acidic. Anisa (2020) reported that mouthwash preparations were safe and did not experience drastic changes in pH at room temperature storage. The results of the examination of the pH of the mouthwash preparation show conformity with the pH quality standard for mouthwash, which is between 5-7 (Hidayanto et al., 2017).

Mouthwash pH test data is shown in **Table 3**.

Table 3. pH of mouthwash preparation

Sample	pH value Week of				
	0	4	8	12	16
C	6.7	6.7	6.7	6.6	6.6
I	6.5	6.5	6.4	6.4	6.4
II	6.4	6.4	6.4	6.3	6.2
III	6.2	6.2	6.2	6.2	6.1

(3) Viscosity test

Viscosity testing was carried out using an Ostwald viscometer to determine the ease of application of mouthwash based on the viscosity level. The greater the viscosity of a fluid, the more difficult it is for the liquid to flow (Nurdianti et al.,

2020). The results of the viscosity test of the preparation at room temperature can be seen in **Table 4**.

Table 4. The viscosity of mouthwash preparation

Week of-	Viscosity (Cps)			
	C	I	II	III
0	1.06	1.14	1.16	1.2
4	1.08	1.15	1.17	1.2
8	1.07	1.16	1.18	1.22
12	1.09	1.17	1.19	1.23
16	1.09	1.17	1.19	1.23

Based on the table, the viscosity of the mouthwash at room temperature storage for 16 weeks did not increase significantly. Formulas with a larger composition of active ingredients have a greater viscosity because the substance's composition affects the viscosity level. The viscosity values for all formulas are almost close to the water viscosity values of 0.89 Cps. It means mouthwash preparation complies with the requirements for the viscosity of the mouthwash, which is easy to apply (Permatasari et al., 2022).

Antimicrobial test preparation

The antimicrobial activity test was carried out using the agar diffusion method. This method has the advantage that it is easier to measure the area of the inhibition zone formed because the bacteria are active not only on the top surface of the agar nutrient but also down to the bottom (Nurhayati et al., 2020). This test was conducted to determine whether there was an antimicrobial activity of the bacteria *S. mutans*, *S. aureus,* and the fungus *C. albicans* in the mouthwash preparation.

Streptococcus mutans antimicrobial test results are tabulated in **Table 5**.

Table 5. Inhibition zone diameter of *S. mutans*

Repetition of	Inhibition zone diameter (mm)			
	C	I	II	III
1	0	9.5	10	10.7
2	0	9.5	11.7	11.4
3	0	12	13.6	13.5
Mean ± SD	0	10.3 ± 1.4	11.8 ± 1.8	11.9 ± 1.5

Souza et al. (2021) reported that the average diameter of the *Eucalyptus* inhibition zone in inhibiting the growth of *S. mutans* was 4.5 mm. Meanwhile, Wahjuningrum et al. (2022) who examined the antifungal activity of *S. mutans* from chitosan 2%, obtained an average diameter of 5.5 mm inhibition zones.

If the two active substances are combined, the antifungal activity is higher, as shown in Table 5 and the most optimum mean diameter of the inhibition zone is formula III, which is 11.9 ± 1.5 mm. The control did not have an inhibitory zone against *S. mutans* bacteria because it did not have an active substance that functions as an antimicrobial agent. The antimicrobial strength of the average diameter of the inhibition zone of each formula showed a strong category, while the control did not have an inhibition zone. The zone of bacterial inhibition is shown in **Figure 2**.

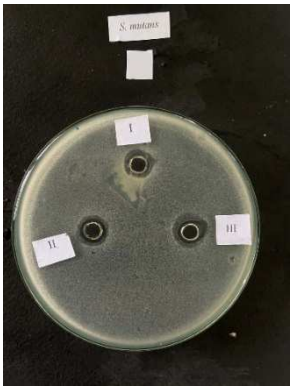

Figure 2. Inhibition zone diameter S. mutans

The results of the *Staphylococcus aureus* antimicrobial test can be seen in **Table 6**.

Table 6. Inhibition zone diameter of *S. aureus*

Repetition of	Inhibition zone diameter (mm)			
	C	I	II	III
I	0	10.8	10.9	12.7
II	0	10.8	12.1	11.9
III	0	12.4	12.1	13.7
Mean ± SD	0	11.3 ± 0.92	11.7 ± 0.69	12.8 ± 0.9

Bachir & Benali (2012) reported that the average diameter of the *Eucalyptus* inhibition zone in inhibiting the growth of *S. aureus* was 8 mm. Meanwhile, Escárcega-Galaz et al. (2017) who examined the antifungal activity of *S. aureus* from chitosan 2%, obtained an average diameter of mm inhibition zones.

If the two active substances are combined, the antifungal activity is higher, as shown in **Table 6** that the most optimum mean diameter of the inhibition zone is formula III with an inhibition zone of 12.8 ± 0.9 mm. At the same time, the control did not have an inhibition zone against *S. aureus* bacteria. The antimicrobial strength of the diameter of the inhibition zone of each formula showed a strong category, while the control did not have an inhibition zone. The bacterial inhibition zone is shown in **Figure 3**. The results of the *Candida albicans* antimicrobial test can be seen in **Table 7**.

Figure 3. Inhibition zone diameter S. aureus

Table 7. Inhibition zone diameter of *C. albicans*

Repetition of	Inhibition zone diameter (mm)			
	C	I	II	III
1	0	11.9	12.2	12.2
2	0	12.2	11.4	12.3
3	0	10.7	12.6	12
Mean ± SD	0	11.6 ± 0.79	12.1 ± 0.61	12.2 ± 0.15

Mohammed (2014) reported that the average diameter of the *Eucalyptus* inhibition zone in inhibiting the growth of *Candida albicans* was 5.1 mm. Meanwhile, Komariah & Latifah (2019) who examined the antifungal activity of *Candida albicans* from chitosan, obtained an average diameter of 7.7 mm inhibition zones.

If the two active substances are combined, the antifungal activity is higher, as shown in **Table 7** that the most optimum diameter of the inhibition zone is formula III with a bland zone of 12.2 ± 0.15 mm. The diameter of the control inhibition zone did not have an inhibition zone against the fungus *C. albicans*. The antimicrobial strength of the diameter of the inhibition zone of each formula showed a potent category. The inhibition zone of the fungus *C. albicans* is shown in **Figure 4**.

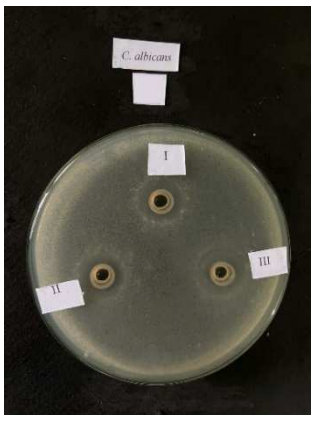

Figure 4. Inhibition zone diameter C. albicans

Zhang et al. (2021) reported that mouthwash containing water-soluble chitosan showed lower toxicity and higher antimicrobial activity compared to commercial mouthwash with or without alcohol.

E. grandis essential oil has antimicrobial activity due to 1,8-cineol compounds. The compound 1,8-cineol is a monoterpene hydrocarbon group. The mechanism of action of this compound binds to proteins through hydrogen bonds, causing the protein structure to be damaged. So most of the cell wall structures and bacterial cytoplasmic membranes contain proteins and fats. Instability in the cell wall and cytoplasmic membrane of bacteria causes the function of selective permeability, active transport function, controlling the protein composition of bacterial cells to be disturbed (Mączka et al., 2021).

Eucalyptus grandis essential oil was found to have compounds with biochemical properties known to apply in the medicinal world. These compounds have individual antifungal and antibacterial properties property ability, but their effectiveness can be increased synergistically (Tum et al., 2016).

Meanwhile, according to previous studies, chitosan has also been shown to have antimicrobial activity. The most plausible mechanism refers to the electrostatic interaction of the amino groups of chitosan with the negatively charged target cell membrane leading to cell wall permeability, leakage, and ultimately, cell death (Fakhri et al., 2020). The other mechanism of action of chitosan as an antimicrobial is that the positive charge of the amino group of chitosan interacts with the negative charge of the microbial cell membrane, causing the loss of proteins and other intracellular constituents of microorganisms (Yan et al., 2021).

Hedonic test of mouthwash based on all test results, it can be seen that formula III is the most optimal because it fulfills all test requirements, so the hedonic test is carried out only on formula III. The hedonic test parameter questionnaire on mouthwash preparations, including color, taste, and aroma, was conducted by 20 untrained respondents. The results of the respondents' assessment are shown in **Figure 5**.

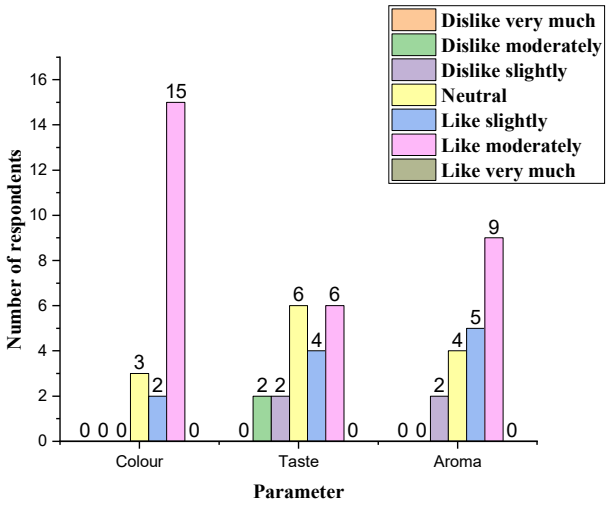

Figure 5. The chart of mouthwash hedonic test

Figure 5 shows that on the color parameter, there are 15 respondents in the like moderate category, 2 respondents choose the like slight category, and 3 respondents choose neutral. From the results of the questionnaire, it is known that the reason why 5 respondents did not choose the like category is that they are more interested if the color of the mouthwash is not clear. Meanwhile, for the taste parameter, 6 respondents chose the like moderate category, 4 respondents chose somewhat like slightly, 6 respondents chose neutral, 2 respondents somewhat disliked slightly, and 2 others chose not to dislike moderately. This is because the mouthwash has a strong mint taste, so some respondents who do not like the spicy taste too much choose the category they do not like or do not like.

As for the aroma parameter, 9 respondents chose the like moderate category, 5 respondents chose like slightly, 4 respondents chose neutral, and 2 others chose dislike slightly. This is because the distinctive aroma of *Eucalyptus* grandis is the same as that of commercial eucalyptus oil, so some respondents who do not like the aroma of *Eucalyptus* oil will feel less like the aroma of the mouthwash.

Conclusions

Mouthwash was evaluated through organoleptic testing, pH, viscosity, and antimicrobial testing. The most optimal formula is formula III, with an inhibition zone diameter of 11.9 ± 1.5 mm for *Streptococcus mutans* bacteria, 12.8 ± 0.9 mm for *Staphylococcus aureus,* and 12.2 ± 0.15 mm for *Candida albicans*.

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