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Chitosan–Lactic Acid Mouthwash Enriched with Eucalyptus Grandis Oil: Formulation and Antimicrobial Properties

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Abstract. Oral health needed improvement through additional mechanical methods, particularly the use of mouthwash formulated with natural ingredients such as chitosan and Eucalyptus grandis. This study aimed to evaluate the physical stability and antimicrobial activity of mouthwash formulations and to identify the most optimal formula among the combinations. Chitosan was characterized using Fourier Transform Infrared Spectroscopy (FT-IR), while Eucalyptus grandis was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). All mouthwash formulations met standard requirements in terms of organoleptic properties, pH levels, and viscosity, indicating good physical stability. The antimicrobial activity was tested against Streptococcus mutans, Staphylococcus aureus, and Candida albicans. The results demonstrated that all formulas exhibited moderate to strong antimicrobial activity. Among the five tested formulas, formula V was found to be the most effective. It showed an average inhibition zone diameter of 11.5 ± 1.94 mm for Streptococcus mutans, 13 ± 1.9 mm for Staphylococcus aureus, and 13.7 ± 0.72 mm for Candida albicans. These findings indicated that formula V had the best combination of physical stability and antimicrobial performance, making it the most optimal formulation.

Keywords : Mouthwash; Chitosan; Eucalyptus grandis

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Introduction

Oral health issues have become a significant public health challenge globally [1]. The main cause of its increasing prevalence is the growing population size [2]. Solutions to reduce dental and oral health problems in society must be implemented as effectively as possible. One way to maintain oral [1] and dental health is by brushing teeth and using supplementary cleaning methods such as mouthwash [3]. Evidence from the literature shows that using mouthwash as an adjunct to mechanical methods in oral cleaning provides better results [4].

Mouthwash is an antiseptic liquid used to clean the oral cavity and eliminate oral pathogens [5]. The mechanism of its use involves holding the preparation in the mouth with the help of contractions from the perioral muscles [6]. Mouthwash offers many benefits, including providing oral and breath freshness, eliminating bad breath, inhibiting and reducing plaque and cavity formation, as well as preventing gingivitis [7]

The active ingredients in mouthwash can come from chemical or natural sources. However, commercial mouthwashes that tend to contain chemical ingredients can dry out the oral cavity, thus promoting microbial growth. Therefore, the active ingredients used in mouthwash should preferably come from natural sources [8]. Chitosan can be used as a natural active ingredient due to its potential to inhibit the growth of bacteria and fungi. This is because chitosan contains amino polysaccharides [9]. This has been proven by the study of Indu et al. [10], which showed that chitosan is effective in inhibiting the growth of Streptococcus mutans and in controlling plaque formation. Therefore, chitosan can be used in new formulations for oral applications, not only as an antimicrobial agent but also for plaque control.

Eucalyptus has also been widely used in essential oil production because its leaves possess strong biological activity. One example is Eucalyptus grandis essential oil, which has been proven to have strong antibacterial activity and is widely used in medicine, food, and the chemical industry [11]. The chemical compounds identified in Eucalyptus grandis that contribute to its antimicrobial activity are 1,8-cineole and α-pinene. Previous studies reported that Gram-positive bacteria are generally more susceptible to its effects than Gram-negative bacteria [12]. Based on the above explanation, the researchers combined chitosan with Eucalyptus grandis essential oil in the formulation of a mouthwash preparation.

Experimental

This study was conducted at the Analytical Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara (FMIPA USU). The equipment used in this research included Pyrex glassware, an Ohaus analytical balance, a Lab 845 pH meter, a Cimarec magnetic stirrer, an Ostwald viscometer, Stahl distillation apparatus, an oven, a sample refrigerator, an Agilent/ Cary 630 FT-IR set, a Shimadzu QP 2010 GC-MS set, and antimicrobial testing equipment. The materials used in this study included chitosan, Eucalyptus grandis, Merck glycerin, Merck Tween-80, 70% Merck sorbitol, Merck sodium benzoate, Merck lactic acid, Merck anhydrous sodium sulfate, and distilled water.

Preparation of chitosan 2%. A total of 2 grams of chitosan was placed into a beaker glass and dissolved in 100 mL of 2% lactic acid while being homogenized using a magnetic stirrer for 24 hours.

Isolation of Eucalyptus grandis Oil. 150 grams of Eucalyptus leaves were prepared and added to a 1000 mL round-bottom flask and sufficient distilled water was added to it. The apparatus was then connected to a Stahl distillation set and heated for approximately 4–5 hours at 110°C until the oil was gone. The resulting distillate was the oil and water solution. Anhydrous Na_2SO4 was utilized as the drying agent to extract the water from the essential oil. The oil fraction was decantated and exported to a securely closed vial and preserved in the fridge.

Characterization. Analysis and characterization of chitosan were characterization using Fourier Transform Infrared (FT-IR) spectroscopy to identify the presence of characteristic functional groups indicating the transformation of chitin into chitosan. Gas Chromatography and Mass Spectroscopy (GC-MS) analysis was conducted to determine the compound content present in the essential oil of Eucalyptus grandis.

Mouthwash formulation design. The mouthwash formulation design is shown in Table 1.

Table 1. Mouthwash Formulation

Composition	Control	Formula					Uses
		I	II	III	IV	V	
E. grandis essential oil (mL)	-	0,1	0,2	0,3	0,4	0,5	Active ingredient
Chitosan (%)	2	2	2	2	2	2	Active ingredient
Tween-80 (mL)	3	3	3	3	3	3	Surfactant
Glycerin (mL)	5	5	5	5	5	5	Humectant
Sorbitol (mL)	2	2	2	2	2	2	Sweetener
Sodium benzoate (g)	0.2	0.2	0.2	0.2	0.2	0.2	Buffer
Distilled water ad. (mL)	100	100	100	100	100	100	Solvent

Preparation of mouthwash formulation. The preparation of the mouthwash formulation begins by emulsifying Eucalyptus grandis, which is insoluble in water, using Tween-80. Next, chitosan dissolved in lactic acid is added. Glycerin and 70% sorbitol are then gradually incorporated into the formulation while stirring until a homogeneous mixture is achieved. Subsequently, sodium benzoate, which has been pre-dissolved in water, is added. After all ingredients have been incorporated, distilled water is added to bring the total volume to 100 mL. The mixture is then homogenized using a magnetic stirrer. The final preparation is transferred into a mouthwash bottle. The same procedure is followed for the other formulation variations.

Evaluation of Mouthwash Formulation

Organoleptic Test. The formulation was observed for its color, taste, and odor at room temperature, as these are organoleptic characteristics that can be directly assessed.

pH Test. One of the key parameters in determining the feasibility of a mouthwash formulation is its pH value. In this study, pH testing was conducted over a period of 16 weeks and evaluated every 4 weeks. The quality standard for mouthwash pH ranges between 5 and 7 [13].

Viscosity Test. The formulation was introduced into tube B of an Ostwald viscometer. The solution was drawn until it passed section A and reached above the upper calibration mark. The stopwatch was started upon releasing the rubber bulb, and the flow time was recorded as the formulation moved from the upper to the lower calibration mark. The stopwatch was stopped once the liquid passed the lower mark, and the flow time of the mouthwash formulation was documented [14] (Handayani et al., 2017). Viscosity plays a crucial role in determining the consistency of a mouthwash during use. The closer the viscosity of the formulation is to that of water, the easier and more comfortable it is for the user to rinse effectively [15].

Cycling Test. Stability testing of the formulation was conducted using a cycling test, in which the samples were stored at 4 ± 2°C for 24 hours, followed by storage at 40 ± 2°C for another 24 hours. This process constituted one cycle. The test was repeated for a total of eight cycles [16].

Antimicrobial Activity Test of the Mouthwash Formulation. According to [17], the strength of antimicrobial activity is classified into four categories: a zone of inhibition >5 mm is considered weak, 5–10 mm is moderate, 11–20 mm is strong, and >20 mm is classified as very strong. The mouthwash formulation was compared to a negative control consisting of a preparation without the addition of chitosan and Eucalyptus grandis extract. Antimicrobial activity tests were performed in triplicate against Streptococcus mutans, Staphylococcus aureus, and the antifungal activity against Candida albicans [18].

Statistical Analysis. The inhibition zone diameter data obtained from the mouthwash formulation were analyzed using One-Way ANOVA with IBM SPSS software version 25.0 at a 95% confidence level. The One-Way ANOVA was employed to determine whether the differences among the formulation variants were statistically significant

Result and Discussion

GC-MS Eucalyptus grandis Analysis. The content and chemical components of the essential oil of Eucalyptus grandis were characterized using Gas Chromatography–Mass Spectrometry (GC-MS) with a Shimadzu QP 2010 instrument, equipped with an RTX-5MS column and ultra-high purity (uHP) helium as the carrier gas. The results of the chemical composition and concentration analysis of the Eucalyptus grandis essential oil are presented in Figure 1.

Figure 1 shows five compounds identified in the Eucalyptus grandis essential oil sample, each with distinct retention times as listed in Table 2. The chemical analysis of Eucalyptus grandis essential oil revealed that the most dominant compound was 1,8-cineole, comprising 79.63% of the total composition. This compound plays a significant role in the antibacterial properties of the oil. These findings are consistent with a study reported by Esposito et al. (2021), in which Eucalyptus grandis extract also contained 1,8-cineole as the major component at 55.2%.

FT-IR Analysis of Chitosan. The characterization of chitosan using FT-IR was conducted to identify specificfunctional groups that indicate the successful transformation of chitin into chitosan. The FT-IR spectrum of the chitosan sample is presented in Figure 2.

The characteristic absorption bands of chitosan are presented in Table 3. The FT-IR spectrum shown in Figure 2 exhibits a broad absorption band at 3280.1 cm^{-1}, indicating the presence of O–H stretching and symmetric N–H vibrations. An absorption band at 2870.1 cm^{-1} corresponds to the aliphatic C–H stretching vibration. Three amide groups characteristic of chitosan were identified: the band at 1640 cm^{-1} corresponds to C=O stretching (amide I), the band at 1580.4 cm^{-1} corresponds to N–H bending vibrations (amide II), and the band at 1319.5 cm^{-1} is associated with C–N stretching (amide III). Additionally, the band at 1423.8 cm^{-1} represents C–H bending vibrations, while the absorption at 1028.7 cm^{-1} is attributed to C–O–C stretching vibrations. The obtained FT-IR data are consistent with previous studies by [19] and [20], which reported that the formation of chitosan is indicated by the reduction of acetyl groups and the decreased intensity of absorption peaks corresponding to C=O and N–H stretching of acetamide groups, which remain prominent in the chitin spectrum.

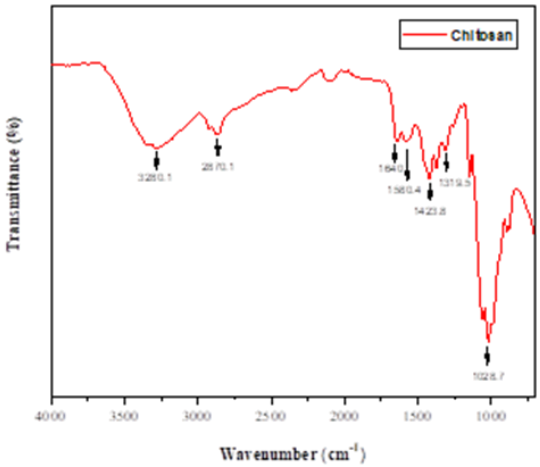

Figure 2. FT-IR Spectrum of Chitosan

Evaluation of the Mouthwash Formulation

Organoleptic Test. The mouthwash formulation appeared clear, with a characteristic Eucalyp-

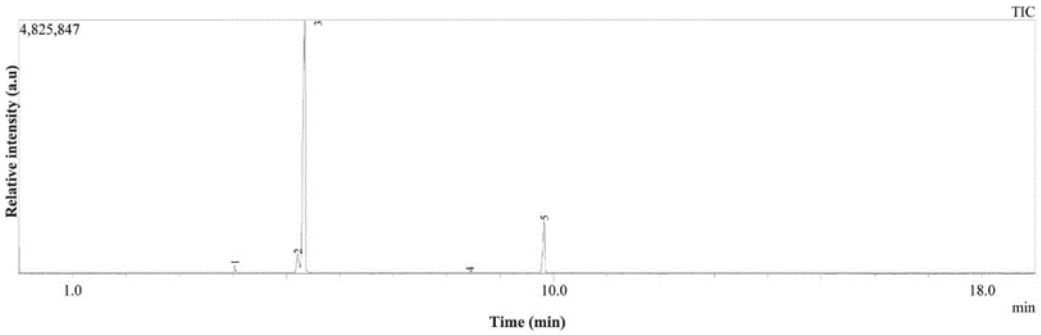

Figure 1. Chromatogram of Eucalyptus grandis essential oil

Table 2. Chemical compounds of Eucalyptus grandis essential oil

Peak	Retention time (min)	Molecular formula	Relative molecular mass	Peak area	Compound
1	4.037	$C_{10}H_{16}$	136	1,21	α-Pinene
2	5.211	$C_{10}H_{14}$	134	5,77	ρ-Cymene
3	5.344	$C_{10}H_{18}O$	154	79,63	1,8-Cineole; eucalyptol
4	8.427	$C_{10}H_{14}O$	150	0,15	2-Cyclohexen-1-one
5	9.820	$C_9H_{14}O_6$	218	13,25	1,2,3-propanetriol triacetate

tus aroma and a strong mint flavor. Based on organoleptic observations—including color, odor, and taste—no significant changes were observed from week 0 to week 16. These results indicate that the formulation remains stable and safe for storage at room temperature. The visual appearance of the mouthwash is presented in Figure 3.

pH Test. The pH values of the mouthwash formulation stored at room temperature over a 16-week period showed no significant changes, as presented in Table 4. Table 4 shows that increasing the concentration of active ingredients led to a more acidic pH, in line with the addition of Eucalyptus grandis essential oil, which has a measured acidity level of 4.12. [21] reported that mouthwash formulations did not undergo significant pH changes during 7 weeks of storage at room temperature. In the present study, the observed pH decrease is attributed to the increasing concentration of E. grandis, which exhibits acidic properties. Nonetheless, the pH values of the mouthwash formulations remained within the acceptable quality standard range of 5 –7 for mouthwash products [13]

Table 3. Spectral data of chitosan

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

Nb : vs : stretching vibration vb : bending vibration

Figure 3. Mouthwash visualization

Table 4. pH of the mouthwash formulation

Sample	pH value week of-				
	0	4	8	12	16
K	6,7	6,7	6,7	6,6	6,5
I	5,9	5,9	5,8	5,8	5,7
II	5,9	5,9	5,8	5,7	5,7
III	5,8	5,8	5,7	5,7	5,6
IV	5,8	5,8	5,7	5,6	5,5
V	5,6	5,6	5,6	5,5	5,5

Viscosity Test. Viscosity testing was performed using an Ostwald viscometer. This measurement aimed to determine whether the formulation could flow easily out of its container, ensuring ease of application. The pourability of a liquid formulation is influenced by its viscosity; the higher the viscosity, the more difficult it is for the liquid to flow [22]. The results of the viscosity test conducted at room temperature are presented in Table 5.

Each formulation did not exhibit significant changes in viscosity over time. However, differences in viscosity were observed among the formulations. Formulations containing higher concentrations of active ingredients demonstrated higher viscosity, as the composition of the formulation directly

Table 5. The viscosity of the mouthwash formulation

Week of-	Viscosity (Cps)					
	K	I	II	III	IV	V
0	1,08	1,19	1,22	1,23	1,26	1,33
4	1,08	1,2	1,23	1,25	1,28	1,35
8	1,09	1,21	1,25	1,26	1,29	1,36
12	1,1	1,21	1,24	1,28	1,32	1,39
16	1,1	1,22	1,26	1,29	1,35	1,4

Tabel 6. pH value in the cycling test

Sample	pH value at cycle				
	0	2	4	6	8
K	6,7	6,4	6,5	6,2	6,1
I	5,9	5,6	5,5	5,6	5,5
II	5,9	5,7	5,6	5,5	5,3
III	5,8	5,7	5,5	5,3	5,1
IV	5,8	5,3	5,2	5	5,1
V	5,6	5,5	5,4	5,2	5

Table 7. Viscosity measurement in the cycling test

Sample	Viscosity (Cp) Cycle of-				
	0	2	4	6	8
K	1,08	1,07	1,08	1,09	1,09
I	1,19	1,2	1,18	1,19	1,2
II	1,22	1,21	1,22	1,23	1,22
III	1,23	1,23	1,22	1,24	1,25
IV	1,26	1,28	1,29	1,3	1,3
V	1,33	1,32	1,33	1,34	1,34

influences its thickness.

Cycling Test. The results of the evaluation showed no significant changes in the organoleptic properties of the mouthwash formulation. The formulation remained clear in color and retained its characteristic Eucalyptus aroma. However, the mint flavor slightly diminished in intensity compared to cycle 0. The pH values obtained from the physical stability testing of the formulation are presented in Table 6.

Table 6 shows that the pH values during the cycling test over eight cycles underwent significant changes, which were attributed to the drastic temperature fluctuations. Lidia et al. [23] reported that pH reduction in mouthwash formulations subjected to cycling tests is caused by the varying storage conditions, particularly alternating exposure to high and low temperatures. The results of the viscosity testing under the cycling test conditions are presented in Table 7.

As shown in Table 7, the mouthwash formulation did not exhibit significant changes in viscosity. However, [23] reported that cycling test conditions can lead to an increase in viscosity, attributed to temperature variations under forced conditions, which may cause the formulation to become more viscous.

Antimicrobial Activity Test of the Formulation. The antimicrobial activity test was conducted using the agar diffusion method, which was selected due to its advantages: it is simple, rapid, easy to perform, and allows precise control over the amount of substance used [24][25]. This test aimed to determine the antimicrobial activity of the mouthwash formulation against Streptococcus mutans, Staphylococcus aureus, and the fungus Candida albicans.

A control group was included using a placebo mouthwash (formulation without active ingredients), which served as a reference to compare the inhibition zone diameters produced by the test samples [26]. The inhibition zone observed against Streptococcus mutans is shown in Figure 4.

Figure 4 shows the inhibition zones formed by both the control and the test formulations against Streptococcus mutans, with the corresponding inhibition zone diameters presented in Table 8. Table 8 shows that the highest mean inhibition zone diameter was observed in Formula V, with a value of 11.5 ± 1.94 mm. In contrast, the control exhibited no inhibition zone against Streptococcus mutans, as it lacked active ingredients functioning as antimicrobial agents. The antimicrobial strength of each formulation, based on the mean inhibition zone diameter, falls within the "strong" category, whereas the control showed no antimicrobial activity. The inhibition zones against Staphylococcus aureus are presented in Figure 5.

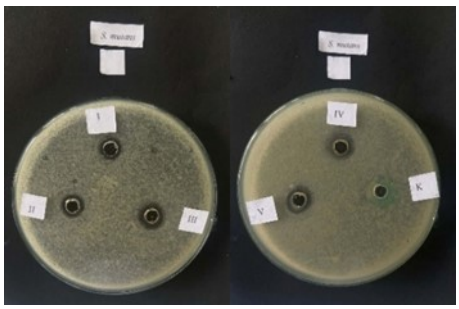

Figure 4. Inhibition zone of Streptococcus mutans

Table 8. Inhibition zone diameters of *S. Mutans*

Replication	Inhibition zone diameter (mm)					
	K	I	II	III	IV	V
I	0	11	11	11,4	11,9	12,8
II	0	9,5	10,1	8,3	10,6	9,3
III	0	9,6	10,1	12,1	10,6	12,5
Average ± SD	0	10 ± 0,84	10,4 ± 0,52	10,6 ± 2,02	11 ± 0,75	11,5 ± 1,94

Table 9. Inhibition zone diameters of *Staphylococcus aureus*

	Inhibition zone diameter (mm)					
	K	I	II	III	IV	V
I	0	10,5	11,1	10	12	11
II	0	10,5	11,3	12,5	12,8	13,1
III	0	11,1	12,9	13,7	13,7	14,8
Average ± SD	0	10,7 ± 0,35	11,8 ± 0,99	12,1 ± 1,89	12,8 ± 0,9	13 ± 1,9

Figure 5 shows the inhibition zones formed by both the control and the test formulations against Staphylococcus aureus, with the corresponding inhibition zone diameters summarized in Table 9.

Table 9 shows that the highest mean inhibition zone diameter was observed in Formula V, with a zone of 13 ± 1.9 mm. The control exhibited no inhibition zone against Staphylococcus aureus. The antibacterial activity against S. aureus produced slightly larger inhibition zones compared to those observed for Streptococcus mutans, although the difference was not substantial. The antimicrobial strength of all formulations falls within the "strong" category, while the control demonstrated no activity. The inhibition zones against Candida albicans are shown in Figure 6.

Figure 6 displays the inhibition zones formed by both the control and the test formulations against Candida albicans, with the corresponding inhibition zone diameters presented in Table 10.

Table 10 shows that the most optimal inhibition zone diameter was observed in Formula V, with a zone measuring 13.7 ± 0.72 mm. The inhibition zones observed against Candida albicans were larger than those against Streptococcus mutans and Staphylococcus aureus. The control showed no inhibition zone against C. albicans. The antimicrobial strength of all tested formulations, based on inhibition zone diameter, falls within the "strong" category.

The antimicrobial activity of Eucalyptus grandis essential oil is attributed to the presence of 1,8-cineole, a compound classified as a monoterpene hydrocarbon. Its mechanism of action involves binding to proteins through hydrogen bonding, leading to structural disruption. Since the bacterial cell wall and cytoplasmic membrane consist largely of proteins and lipids, this disruption compromises the membrane's selective permeability, active transport functions, and protein arrangement [27].

Chitosan has also been proven to possess antimicrobial properties, as demonstrated in previous studies. The mechanism of chitosan's antimicrobial action is based on the positive charge of its amino groups, which interact with the negatively charged microbial cell membranes. This interaction leads to the leakage of intracellular proteins and other constituents, ultimately causing cell death [28].

Statistical Analysis. The inhibition zone diameters of the formulations were analyzed using SPSS software with a One-Way ANOVA test. The results indicated that all formulation variants met the assumptions of normality and homogeneity of variance. The inhibition zone data for Streptococcus mutans, Staphylococcus aureus, and antifungal activity against Candida albicans showed significance

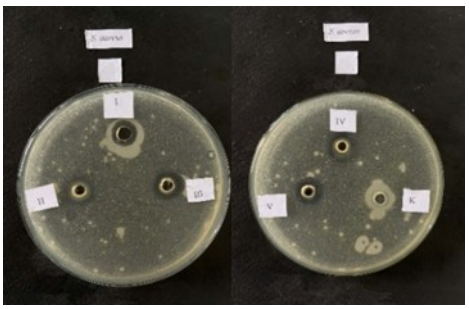

Figure 5. Inhibition zone of *Staphylococcus aureus*

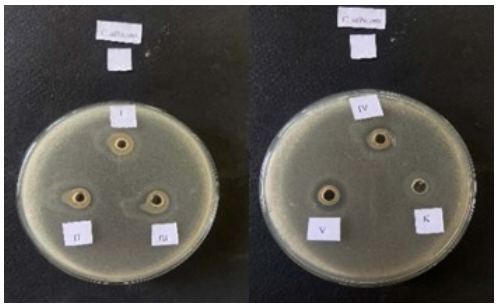

Figure 6. Inhibition zone of *Candida albicans*

Table 10. Inhibition zone diameters of *Candida albicans*

Replication	Inhibition zone diameter (mm)					
	K	I	II	III	IV	V
I	0	11,3	12,1	13,2	11,6	13,2
II	0	9,9	11,1	11,5	12,1	13,3
III	0	10,8	11,4	12,4	13,5	14,5
Average ± SD	0	10,7 ± 0,71	11,5 ± 0,51	12,4 ± 0,85	12,4 ± 0,98	13,7 ± 0,72

values of p = 0.000, which is less than the alpha level (α), indicating significant differences among the formulations. Post hoc analysis using Tukey's test revealed no significant differences between formulations containing active ingredients but showed significant differences compared to the control.

Conclusion

The formulated mouthwash combining chitosan and Eucalyptus grandis demonstrated good physical stability and antimicrobial activity. Among all tested formulations, Formula V showed the most optimal results, with inhibition zone diameters of 11.5 ± 1.94 mm for Streptococcus mutans, 13 ± 1.9 mm for Staphylococcus aureus, and 13.7 ± 0.72 mm for Candida albicans. The antimicrobial activity of the mouthwash formulations falls within the "strong" category, in accordance with the classification by.

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