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Utilization of Hydroxyapatite-Chitosan-Bay Leaf Oil (*Syzygium Polyanthum* W.*)* as Antibacterial for Streptococcus Mutants

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Abstract: Dental caries is a disease that can damage hard tooth tissue caused by the activity of Streptococcus mutans bacteria. Streptococcus mutans bacteria are the leading cause of dental caries disease. Efforts to prevent dental caries can be made using toothpaste, but toothpaste cannot reach areas that are difficult to reach with a toothbrush. Another method that can be used is mouthwash. This study was developed with the active ingredient compound HAp-chitosan-bay leaf oil in mouthwash to have an antimicrobial effect to prevent dental caries. This study aimed to determine the chemical physics characteristics and antibacterial activity of mouthwash preparations based on hydroxyapatite-chitosan-bay leaf oil. In this study, the active ingredients hydroxyapatite-chitosan-bay leaf oil was used with variations in the concentration of bay leaf oil, namely 0.5, 1.5, and 2.5 %. FT-IR characterization showed the presence of OH, N-H, CH, C=O, C=C, C-O, and PO_4^{3-} functional groups. The pH test on the mouthwash showed compliance with the quality standard for mouthwash pH, which has a pH range of 5-7. The homogeneity test results in the mouthwash preparation showed no sediment or separation. Antibacterial activity testing showed that the HAp-chitosan-bay leaf oil mouthwash preparation inhibited the growth of Streptococcus mutans bacteria. The inhibition zone in the strong category with an average of 12.75 mm was shown in the HAp-chitosan-bay leaf oil mouthwash preparation formulation with a concentration of 2.5% bay leaf oil with the addition of sodium lauryl sulfate (SLS).

Keywords: Antibacterial; Bay Leaf Oil; Chitosan; Hydroxyapatite; Mouthwash.

Introduction

Bacterial colonies and food debris that stick to the surface of the teeth will cause dental caries by damaging the tooth tissue [1]. Cariogenic bacteria such as Streptococcus mutans are considered the main bacteria that cause dental caries in humans. Streptococcus mutans live by colonizing and usually adhere to the surface of teeth [2,3]. Therefore, maintaining oral health requires good care to prevent diseases of the teeth and surrounding areas.

Cleaning teeth with toothpaste alone is not enough to maintain dental health. Mouthwash is highly recommended to inhibit bacteria from growing in places that a toothbrush cannot reach; the way to prevent it is by using mouthwash [4]. Standard mouthwash components include active ingredients, humectants, surfactants, flavoring agents, and solvents [5].

Hydroxyapatite (HAp) is an essential source of calcium and phosphate; the reaction between calcium oxide and water can produce precursors such as $Ca(OH)_2$ [6]. HAp is biocompatible and very well tolerated by human oral tissues [7] and also has bioactivity, osteoconductivity, non-toxic, and non-immunogenic properties [8]. HAp is used for various applications such as tooth remineralization, oral biofilm control, and tooth sensitivity reduction [9].

The addition of other materials, such as *chitosan*, can be used as an alternative antibacterial material for caries prevention. This is because chitosan is a polycationic natural polymer that is highly reactive and antibacterial [10]. Chitosan contains amine groups ($-NH_2$) as an antimicrobial agent, and hydroxyl reacts with acid ions, increasing the pH of the environment and inhibiting the demineralization process [11]. Wahjuningrum reported that their research produced a significant value of $p < 0.05$, indicating that chitosan exerts antibacterial activity against Streptococcus mutans bacteria [12]. Chitosan is safe to use as an antimicrobial agent in mouthwash and can replace commercial mouthwash, which is more effective, secure, and natural [13].

The properties of hydroxyapatite composites with chitosan material can show a dual effect by acting as a tooth remineralization and antibacterial at the same time. This is evidenced in studies showing that chitosan can reduce plaque formation in the teeth and reduce the bacteria S.mutans that cause caries [14]. HAp-chitosan composites have antibacterial activity, both on gram-negative bacteria. Positive and negative bacteria due to the active ingredient chitosan and being able to increase the effectiveness of HAp in binding bioactivity [15].

Other active ingredients commonly added in health science besides HAp and chitosan are herbal. Natural ingredients derived from plants contain bioactive compounds that can inhibit the growth of bacteria in the oral cavity. One of the herbal ingredients used is the salam plant (Syzygium polyantha Wight). Secondary metabolites in Salam (Syzygium polyanthum) leaves include flavonoids, tannins, triterpenoids, and essential oils [16]. The dominant compounds contained in bay leaf essential oil

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are terpenoids. The primary ingredient in bay leaf essential oil is α-pinene (30.88%) of the oil's composition [17]. This bioactive substance dramatically slows the growth of gram-positive bacteria, such as the dental caries-causing Streptococcus mutans [18].

Based on this description, this study aims to determine the benefits, physical-chemical characteristics, and antibacterial properties of mouthwash containing HAp based on cow bone (Bos taurus), chitosan, and bay leaf oil with various concentrations of bay leaf oil. The mouthwash was then subjected to several tests, including homogeneity, pH, and identification of functional groups using a Fourier Transform Infrared (FTIR) spectrophotometer and S. mutans antibacterial activity test.

Research Methods

This research was conducted in several stages. First is the synthesis of hydroxyapatite and chitosan. Second, formulate the mouthwash. Next, test the chemical characteristics with FTIR. Then, conduct pH and homogeneity tests. The last is the antibacterial test.

Hydroxyapatite synthesis was made using cow bone waste. Hydroxyapatite 2% was prepared by mixing 0.4 grams of HAp with 20 mL of phosphoric acid [8]. Chitosan synthesis was made using shrimp shell waste—1% chitosan by dissolving 0.2 grams in 20 mL of 2% acetic acid. The solution was stirred with a magnetic stirrer until homogeneous [10].

The mouthwash was made by adding the active ingredients of bay leaf oil with tween 80. Propylene glycol was dissolved with distilled aquades in a separate container and stirred until homogeneous using a magnetic stirrer. Then, the active ingredients of chitosan and HAp were added and mixed until homogeneous using a magnetic stirrer. After that, sodium benzoate-saccharin and sodium lauryl sulfate solutions were added and stirred until homogeneous using a magnetic stirrer. Next, bay leaf oil mixed with tween 80 is added. This is followed by the addition of menthol as a refresher, and distilled aquades are added as a solvent until the volume of the mouthwash preparation becomes 100 mL. The mouthwash formulation made from hydroxyapatite-chitosan-bay leaf oil is presented in Table 1.

Table 1. Hydroxyapatite and Chitosan-Lay leaf oil mouthwash formulation

Material	Formulation (%v/v)						Function
	F1	F2	F3	F4	F5	F6	
Bay leaf oil (ml)	0.5	1.5	2.5	0.5	1.5	2.5	Active ingredients
HAp 2% (mL)	1	1	1	1	1	1	Active ingredients
Chitosan 1% (mL)	1	1	1	1	1	1	Active ingredients
Propylene glycol (mL)	10	10	10	10	10	10	Humectants
Tween 80 (mL)	5	5	5	5	5	5	Surfactants
Sodium benzoate (gr)	0.4	0.4	0.4	0.4	0.4	0.4	Preservatives
Saccharin Sodium (gr)	0.4	0.4	0.4	0.4	0.4	0.4	Sweetener
Mentol	0.3	0.3	0.3	0.3	0.3	0.3	flavoring agents
SLS (gr)	-	-	-	1	1	1	Foaming
Aquades (mL)	ad 100	ad 100	ad 100	ad 100	ad 100	ad 100	Solvent

An FTIR spectrophotometer was used to examine the hydroxyapatite-chitosan-bay leaf oil mouthwash to determine the success of the reaction. FTIR spectra were recorded at room temperature in the 4000-400 cm-1 range.

Using a pH meter that has been calibrated using a standard buffer solution for a few minutes, place the pH meter inside the mouthwash. Measurements were taken at room temperature. In the centrifugation method, the homogeneity test will be carried out. A 5 mL mouthwash preparation was put into a centrifuge tube and then centrifuged at 3000 rpm for 30 minutes. Separation can be observed in the centrifugation results [19].

In this study, an antibacterial test was conducted to measure the antibacterial activity of mouthwash preparations using the disc diffusion method. The antibacterial test was carried out by preparing muller hinton agar (MHA) and nutrient agar (NA) media. After that, prepare a suspension of Streptococcus mutans bacteria from nutrient agar (NA) media by taking one ose of the colony into a test tube containing 0.9% NaCl. The turbidity of the test colony suspension was standardized to 0.5 McFarland standard (approximately 1.5 x 10 CFU/mL). Suspensions of Streptococcus mutans bacteria were inoculated on muller hinton agar (MHA) media. After everything was ready, the disc paper was placed in a Petri dish, and 25 µl of mouthwash preparation was given. Next, it was incubated at 37 °C for 24 hours. After that, the Petri dish was removed from the incubator, and the clear zone was measured around each paper disk using a calliper [20].

Results and Discussion

Fourier Transform Infra-Red (FTIR)

Functional group analysis with Fourier Transformed Infrared (FTIR) was used to determine the presence of functional groups in the hydroxyapatite-chitosan-bay leaf oil composite. This analysis determines the reaction characterized by an increase, decrease, and shift in intensity of the peak obtained in the wave number area 4000-400 cm^{-1}. The results of the FTIR analysis of the Hydroxyapatite-Chitosan-Bay leaf oil composite are shown in Figure 1.

Figure 1. shows The spectra of HAp-chitosan-bay leaf oil, showing the presence of OH, N-H, CH, C=O, C=C, and PO_4^{3-} functional groups. The vast and sharp absorption at wave numbers 3000-3600 cm^{-1} interprets the stretching vibrations of several functional groups, namely, OH- and N-H. The OH^- group comes from HAp and bay leaf oil, while the N-H group is associated with the OH- group from chitosan. This group includes stretching vibrations from the

-OH and -NH groups; there is a spectral shift at wave number 3346.855 cm^{-1} [21]. It can be seen that the C-H functional group in the FTIR spectra of bay leaf oil (d) appears in the FTIR spectra of hydroxyapatite-chitosan bay leaf oil (a). The wave numbers 3100-2800 cm^{-1} show the presence of -CH stretching vibrations of CH, CH_2, and CH_3 groups with peaks at 2280.16 cm^{-1}, 2813.63 cm^{-1} and 2719.13 cm^{-1}. The presence of C carbonyl atoms was shown at wave number 1636.30 $cm^{-1,}$ which interpreted the stretching vibrations of C=O functional groups from bay leaf oil, C=O groups from chitosan, and C=O groups from CO_3^{2-} from HAp [22]. The C=C group was found at a wavelength of 1531.20 cm^{-1}. At a wavelength of 1258.32 cm^{-1}, a C-O group from bay leaf oil was discovered. The -CH bending vibration of bay leaf oil aromatic compounds was also shown at wave numbers 911, 816, and 793 cm^{-1}. The PO_4^3 group was shown to have absorption at wave numbers 1091.51 cm-1 and 965.19 cm^{-1} [23].

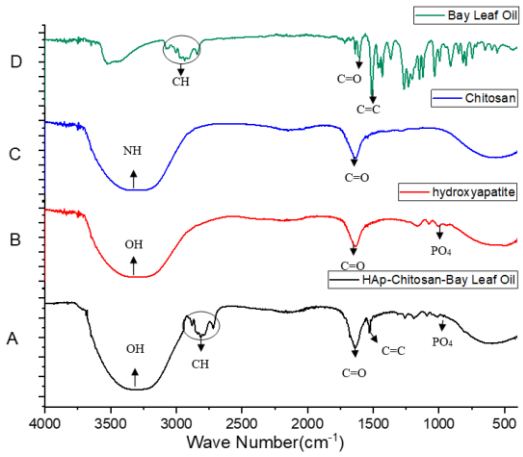

Figure 1. FTIR spectra of (a) Hydroxyapatite-Chitosan-Bay Leaf Oil, (b) Hydroxyapatite, (c) Chitosan, (d) Bay Leaf Oil

pH and Homogeneity test

The mouthwash preparation was tested for pH and homogeneity; the results are shown in Table 2.

Table 2. pH test results and homogeneity

Formula	pH	Homogeneity
F1	5.7	Homogenous
F2	5.6	Homogenous
F3	5.5	Homogenous
F4	5.8	Homogenous
F5	5.7	Homogenous
F6	5.6	Homogenous

Table 2. shows that there is no significant change in the pH of the mouthwash preparation at room temperature. The results of the pH examination of the mouthwash preparation showed that the six samples had the smallest pH of 5.6 and the most significant pH of 5.8. The results are based on the quality standards of mouthwash pH, which has a pH range of 5-7 [24]. The pH observation indicates the acidity of the mouthwash so that the mechanism of action of the mouthwash reacts optimally without affecting the oral mucosa. Suppose the mouthwash preparation has a pH <5. In that case, it will cause more growth of acidogenic bacteria such as Streptococcus mutans, which can cause damage to tooth enamel and increase the risk of cavities (caries) [25]. Meanwhile, if the mouthwash preparation has a pH >7, it will trigger mould growth and cause problems such as plaque deposition and coral formation [26].

Homogeneity testing of mouthwash using a centrifuge is one of the methods used to evaluate the physical and chemical stability of the product. The centrifuge is used to accelerate the particle separation process and test the stability of the mouthwash [19]. The homogeneity test results were carried out with a centrifugation test to see whether there was separation. Table 2 shows that the mouthwash preparation made from HAp-chitosan-bay leaf oil does not occur in separation and can be interpreted as a homogeneous formula. A good preparation will not experience phase separation because this will impact the long-term use of the preparation and reduce its efficacy [27].

Antibacterial test

Antibacterial activity was tested against Streptococcus mutans bacteria in mouthwash preparations made from HAp-chitosan-bay leaf oil using the disc diffusion method. Observations were made 1x24 hours, marked by the appearance of a clear zone. Since a clear zone formed around the disc paper, it is possible to determine the sample's diameter and that it can limit bacterial growth. The antibacterial activity increases with the size of the obtained clean zone.

This test was made in three concentration variations to determine the inhibition with the addition of bay leaf oil, namely 0.5%, 1.5%, and 2.5%, to see antibacterial activity against Streptococcus mutans bacteria. Distilled water was used as a negative control comparator because it has no antibacterial activity and as a solvent in mouthwash preparations. Listerine zero mouthwash is a favourable control comparison circulating in the market because it has an antibacterial effect and can reduce the number of bacteria. The results of the antibacterial activity test of mouthwash preparations made from Hydroxyapatite-chitosan-bay leaf oil can be seen in Table 3.

The precise zone diameter is the inhibition zone formed; the low inhibition area category is in the range of <5 mm, the medium inhibition area category is in the range of 5-10 mm, the potent inhibition area category is in the range of 10-20 mm and the inhibition area >20 mm is categorized as very strong [28]. Table 3 shows that of the six sample variations, it can be seen that the concentration of 2.5% with SLS has the most excellent antibacterial activity in the strong category with the formation of an average inhibition zone of 12.75 mm. Listerine Zero, used as a positive control, has an average inhibition zone diameter of 11.45 mm, meaning that the inhibition response of the positive control is in a strong category. This shows that this study is based on the hypothesis at the beginning of the study, namely that there is inhibition in mouthwash preparations made from active HAp-chitosan-bay leaf oil against S.mutans bacteria that cause dental caries.

In the calculation of the inhibition zone, it was found that the mouthwash made from HAp-chitosan-bay leaf oil containing SLS had a higher average inhibition zone measurement than the mouthwash made from HAp-chitosan-bay leaf oil that did not contain SLS. This shows that the HAp-chitosan-bay leaf oil content with the concentration added with SLS can inhibit bacteria to a greater extent. According to Sälzer, the detergent content

used in mouthwash is SLS, which can produce foam and increase the effect of a fresh taste sensation. SLS can also bind to bacterial proteins, causing inhibition of bacterial attachment to the teeth [29].

Table 3. Antibacterial Test Results of hydroxyapatite-chitosan-bay leaf oil Mouthwash Against S.Mutants Bacteria

Formulas	Zone of Inhibition (mm) Repetition			Rata-rata
	I	II	III	
Hydroxyapatite -Chitosan-bay leaf oil 0.5%	11.61	10.79	9.89	10.76
Hydroxyapatite -Chitosan-bay leaf oil 1.5%	11.92	10.54	9.94	10.80
Hydroxyapatite -Chitosan-bay leaf oil 2.5%	11.52	10.89	11.71	11.37
Hydroxyapatite -Chitosan-bay leaf oil 0.5% with SLS	10.48	11.62	11.55	11.21
Hydroxyapatite -Chitosan-bay leaf oil 1.5% with SLS	12.37	13.46	12.25	12.69
Hydroxyapatite -Chitosan-bay leaf oil 2.5% with SLS	13.75	12.00	12.51	12.75
Positive Control	12.32	10.84	11.20	11.45
Negative Control	0	0	0	0

It can be seen from the inhibition zone formed indicating that the mouthwash preparation formulation made from Hydroxyapatite-chitosan-bay leaf oil is proven to be able to inhibit the activity of Streptococcus mutans bacteria. The active ingredient chitosan can also interact with the phospholipids of S.mutans. Chitosan has cationic properties, which means it has a positive charge due to the presence of amine groups in its structure. When chitosan is near bacteria, it interacts with the negatively charged bacterial cell wall. This interaction causes destruction or damage to the bacterial cell wall. Chitosan can penetrate the bacterial cell membrane and cause changes in permeability. This can cause leakage of essential ions and nutrients from the bacterial cell, leading to the death of the microorganism [29,30]. Mediouni reported that the α-pinene compound is a lipophilic terpene compound, which is fat soluble. So that there can be an interaction of α-pinene with lipid membranes that can pass through and damage cell structures by entering lipopolysaccharides in cell membranes, which will disrupt cell permeability [32]. This can cause leakage and damage to nutrients' entrance and exit, so bacterial growth is inhibited and dies [33]. Based on the antibacterial activity, the HAp-chitosan-bay leaf oil 2.5% with SLS mouthwash formulation inhibited the growth of Streptococcus mutans bacteria with the largest inhibition zone in F9, which was 12.75 mm.

Conclusion

Based on the results of this study, FTIR characterization of hydroxyapatite-chitosan-bay leaf oil showed the presence of OH, N-H, CH, C=O, C=C, C-O, and PO_{43}^{-} functional groups. The characterization of the mouthwash with pH was produced by the mouth's pH, which is in the range of 5-6, and the results of the homogeneity test carried out did not experience separation in the mouthwash preparation. The measurement results of the antibacterial activity test showed that the hydroxyapatite-chitosan-bay leaf oil mouthwash preparation was able to inhibit the growth of Streptococcus mutans with the largest average diameter of inhibition in the addition of 2.5% bay leaf oil with the addition of sodium lauryl sulfate (SLS), which was 12.75 mm.

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