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Antibacterial Power of Nano Brown Anchovy (*Stolephorus insularis*) Against Mixed Bacteria in Deep Dentin Caries

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Abstract

Background: Biodentin is a pulp capping material that has perfected the shortcomings of $Ca(OH)_2$, but has low radiopacity and lower washing out resistance, so it is hoped that there will be natural ingredients that can be used as pulp capping materials. The nano brown anchovy has antibacterial content in the form of fluoride. Its nano size can also facilitate penetration better. **Purpose:** Analyzing the antibacterial potency of nano brown anchovy on mixed bacteria in deep carious dentine. **Methods:** The research was conducted in an experimental laboratory *in vitro* with a post-test only control group design. Brown anchovy was converted into nanoparticles then diluted into several concentrations using the broth dilution method. Direct contact method was used between nano anchovy and various concentrations of bacteria. The values of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bacterial Concentration) were obtained by counting the number of bacterial colonies growing on Mueller Hinton Agar media. Colony growth was calculated manually in Colony Forming Units (CFU). Data were analysed by One Way Anova test followed by a Tukey HSD test. **Results:** The MIC value at a concentration of 0.781% and the MBC value at a concentration of 1.56%. In positive control, there was an average bacterial death of 0%, a concentration of 0.781% had an average bacterial death of 91%, and at a concentration of 1.56% the average bacterial death was 100%. The results showed that the greater the concentration of nano anchovy, the stronger the antibacterial power. **Conclusion:** There is antibacterial potency in nano brown anchovy against Mixed Bacteria in deep dentin caries lesions.

Keywords: Antibacterial, Deep Carious Dentin Bacteria, Nano Brown Anchovy, SDGs, Good Health and Well-Being

1. Introduction

Caries is a chronic process that occurs in the hard tissues of the teeth, namely enamel, dentin, and cementum, due to an ecological imbalance between tooth minerals and biofilm, causing demineralization of the teeth. The process of dental caries involves several factors that bind to each other, namely microorganisms, host, time, and substrate[1].

Dental caries is an infectious disease of the oral cavity, a global public health problem[2]. In Indonesia, the most significant proportion of dental and oral issues is caries (45.3%) and periodontal disease (14%) and children aged five years have a dental caries experience rate (DMFT) 6, including severe early childhood caries[3].

Streptococcus mutans bacteria is the primary bacteria with the highest number in a carious lesion with the prevalence of *S. mutans* bacteria as much as 45.6%, *Lactobacillus spp*. 41.2%, and *S. aureus* 13.2%[4]. Early caries formation is characterized by white spots on the enamel surface. Then, if not treated immediately, caries will progress to the dentin until the pulp forms deep dentin caries. Deep dentin caries can cause pulp inflammation due to bacterial invasion into the pulp cavity, resulting in pulp tissue necrosis, so the treatment is more complex than other caries treatments[5].

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Pulp capping is a treatment carried out on deep dentin caries to maintain pulp vitality by applying biomaterials and accelerating healing after removing all bacteria[6]. Some pulp capping materials include $Ca(OH)_2$, MTA, and Biodentin. $Ca(OH)_2$ is the material most often used as pulp capping because it is considered the best (gold standard). Still, the use of $Ca(OH)_2$ in the long term can cause physical changes in the formation of reparative dentin, which is not dense enough due to discontinuity of the dentinal bridges, leading to areas of necrosis called tunnel defects[7-9].

In 2009, the latest findings in dentistry were found, namely biodentin, which has biocompatibility and a fast setting time[10]. Biodentin as a pulp capping material has the lowest toxic value compared to $Ca(OH)_2$ and MTA. A study showed that within 168 hours, biodentin had a toxicity value of 0.01 and MTA had a toxicity value of 0.05[11]. Besides all its advantages, biodentine still has disadvantages: low radioopacity and low washing-out resistance[10].

Anchovy is a type of fish that can be easily found in Indonesian waters[12]. In 2020, the production volume of anchovy in Indonesia was 175,726 tons with a production value of 2,160 billion rupiahs[13]. Meanwhile, in 2014, anchovy production increased to 199,226 tons[14]. Brown anchovy (*Stolephorus insularis*) is one of the natural ingredients with fluoride content that is resistant and does not dissolve easily in water. Brown anchovy has a high calcium fluoride content, as much as 15.7-38.3 ppm[15]. The use of fluoride has an inhibitory effect on the growth of the cause of dental caries. The mechanism of fluorine in preventing and reducing caries is by inhibiting glycolysis and reducing acid production from caries-causing bacteria, increasing the mineralization process and reducing demineralization[16].

Up to this day, nanotechnology is starting to develop rapidly in dentistry. The limitation of fluoride to penetrate into dental plaque can reduce the inhibitory effect in hard-to-reach areas. Nanomaterials with tiny particle sizes of 0.1-100 nm can facilitate penetration into cell membranes so that the reaction and antibacterial activity become greater[17,18].

Anchovies contain high levels of calcium fluoride (15.7-38.3 ppm) so it is necessary to conduct research to prove the effect of nano antibacterial power of brown anchovy (*Stolephorus insularis*) on mixed bacteria of deep dentin caries by looking at the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

2. Materials and Methods

To manufacture nano brown anchovy, 1 kg of brown anchovy were cut into 2-5 mm in size, boiled for 1 hour, then washed and dried in the sun for approximately one day. 100 g of dried anchovy were taken and then macerated with 1 M HCL for 2 hours and then cleaned to neutralize the pH. Brown anchovy were dried using an oven at a temperature of 105°C for 2 hours. The dried brown anchovies were ground using a mortar and then filtered through a 60 mesh filter. After being filtered, the anchovies were mashed using HEM for 1 hour at a speed of 3000 rpm and a milling ball diameter of 0.5 mm and the amount of powder compared to the milling balls was 1:5[19]. 20 g nano brown anchovy was diluted using a solution of 20 ml of saline with a concentration of 50%; 25%; 12.5%; 6.25%; 3.125%; 1.56%; 0.781%; 0.39%.

In the manufacture of mixed bacteria, for deep dentin caries mixed bacteriafrom Airlangga University Research Center's stock was taken using a sterile osse. To collect the bacteria before, the deep carious dentin lesion without perforation was cleaned of food debris by rinsing it first and the tooth was kept dry from saliva during bacterial collection. The mixed bacteria were taken by thinly scraping the patient's necrotic dentin tissue using a sterile excavator, planted into the BHIB (Brain Hearth Infusion Broth) tube. The tube was then incubated for 24 hours at 37°C. The concentration of bacteria was adjusted to the standard of 0.5 Mc Farland (1.5 x 108 CFU/ml).

In calculating the number of mixed bacteria colonies, nano brown anchovy with several concentrations, namely 50%; 25%; 12.5%; 6.25%; 3.123%; 1.56%; 0.781%; 0.39%. 0.05 ml of mixed bacterial culture suspension was added to the BHIB media. BHIB media was prepared as a positive control.

As a negative control, a test tube containing BHIB media without the addition of test bacteria and nano brown anchovy was prepared to ensure no bacterial growth on the media. All test tubes were incubated for 24 hours at 37°C in an incubator. Then 0.1 ml of BHIB was taken and then planted on Mueller Hinton Agar media using the spreader method and it was incubated at 37°C for 24 hours. Counting bacterial colonies that grew on Muller Hinton media was done manually and expressed

by Colony Forming Unit (CFU). The calculations were carried out by three different observers and repeated three times.

Then the data processing was carried out using the normality test using the Saphiro Wilk test to see if the data obtained were normally distributed. The homogeneity test of variance was tested using the Levene test to determine whether the data variance was homogeneous. One Way ANOVA is used to test the difference in the arithmetic mean of the two sample groups, and the Tukey HSD test is used to compare groups.

3. Result

The test results using the liquid dilution method and spread method on Muller Hinton Agar media showed bacterial growth at a concentration of 0.781%-0.39%. In contrast, at a concentration of 50%-1.56%, there was no bacterial growth on the media (Figure 1). Therefore, the concentrations of 0.781% and 0.39% were used as guidelines for the cultivation of bacteria for colony count purposes.

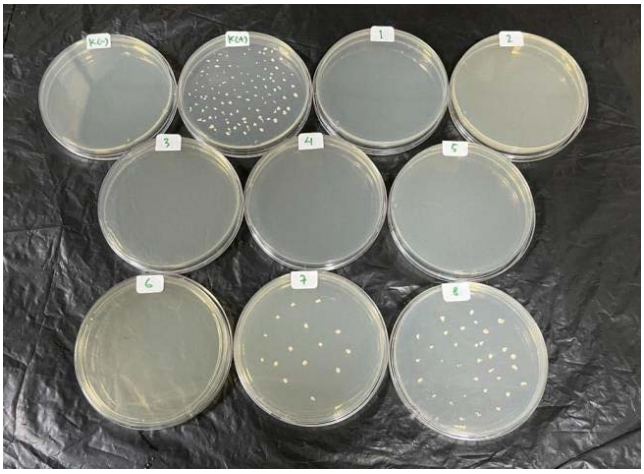

Figure 1. The results of spreading on MH media showed no bacterial growth at a concentration of 50%-1.56%.

Colony count results on Mueller Hinton Agar media showed that at a concentration of 0.781%, the average bacterial death was 91%. At a concentration of 1.56%, average bacterial death was 100%, which indicates the absence of growth of the test bacteria. Therefore, it can be seen that the concentration of 0.781% is the MIC and the concentration of 1.56% is the MBC of nano brown anchovy against mixed bacteria of deep dentin caries. The greater the concentration of nano brown anchovies, the smaller the growth of bacteria (Table 1).

Observations were made on the negative control group. The average percentage of bacterial death was 100%, which means that there was no bacterial colony growth. In the positive control group, the average rate of bacterial death was 0%, which means the percentage of bacterial colony growth was 100%. The average percentage of bacterial colony death at a concentration of 50%-1.56% was 100%, which means that there was no bacterial growth at all. At a concentration of 0.781%, the mean percentage of bacterial colony death was 91%, while at a concentration of 0.39%, the mean rate of bacterial colony death was 79% (Table 2).

The result of gram staining shows that the bacteria are purple, which means that the bacteria are gram-positive with the most forms of *cocci* and *bacilli* (Figure 2). *Lactobacillus* and *Streptococcus* were found among the isolated bacteria.

The first statistical test carried out was the Shapiro Wilk test, and the results showed that the data were normally distributed. The homogeneity test, namely the Levene test, showed that the data variance was homogeneous.

Table 1. Percentage of death of bacterial colonies mixed with deep dentin caries

Sample	Treatment Groups									
	Negative control	Positive control	50%	25%	12.5%	6.25%	3.125%	1.56%	0.781%	0.39%
1	100%	0%	100%	100%	100%	100%	100%	100%	93%	83%
2	100%	0%	100%	100%	100%	100%	100%	100%	91%	79%
3	100%	0%	100%	100%	100%	100%	100%	100%	91%	80%
4	100%	0%	100%	100%	100%	100%	100%	100%	91%	79%
5	100%	0%	100%	100%	100%	100%	100%	100%	92%	81%
6	100%	0%	100%	100%	100%	100%	100%	100%	91%	76%
7	100%	0%	100%	100%	100%	100%	100%	100%	90%	80%

Table 2. The mean value of the death percentage of bacterial colonies mixed with deep dentin caries

Treatment	Number of Sample	Mean Value	Deviation Standard
Negative control	7	100%	0%
Positive control	7	0%	0%
50%	7	100%	0%
25%	7	100%	0%
12.5%	7	100%	0%
6.25%	7	100%	0%
3.125%	7	100%	0%
1.56%	7	100%	0%
0.781%	7	91%	0%
0.39%	7	79%	0%

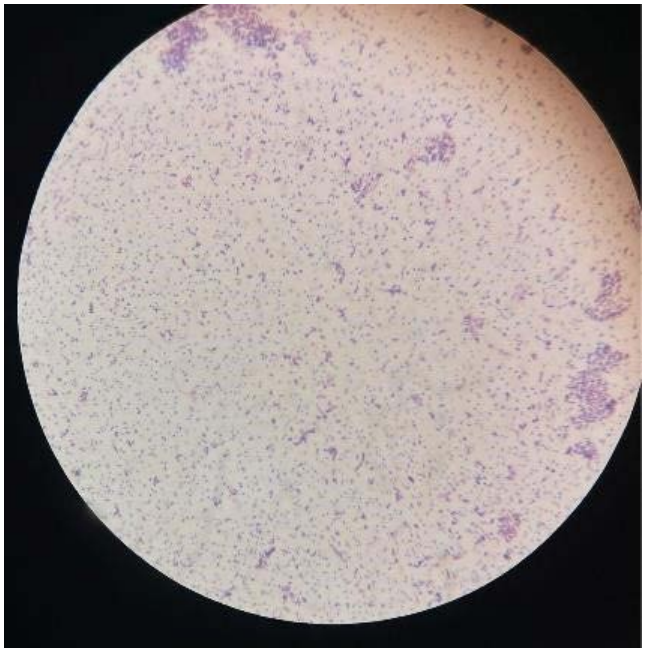

Figure 2. Gram staining of mixed bacteria of deep dentin caries samples.

The One Way Anova test showed significant differences in all treatment groups. The Tukey HSD test showed that all groups had a significant difference between the treatment groups.

4. Discussion

In this study, the results showed the antibacterial power by nano brown anchovy against mixed bacteria of deep dentin caries by determining the MIC and MBC. The MIC from this study was at a concentration of 0.78% because at this concentration, nano brown anchovy could inhibit the growth of mixed bacteria of deep dentin caries by more than 90% compared to the number of bacteria that grew in positive controls. In comparison, the MBC from this study was nano brown anchovy with a concentration of 1.56% because, at this concentration, it could inhibit all bacterial growth.

In positive control, there was an average bacterial death of 0%, a concentration of 0.781% had an average bacterial death of 91%, while at a concentration of 1.56% the average bacterial death was 100%. The results showed that the greater the concentration of nano anchovy, the stronger the antibacterial power. The antibacterial power of nano anchovy against mixed bacteria of deep dentin caries was directly proportional to the concentration of nano anchovy. Based on the Tukey HSD test in this study, all treatment groups have a probability value (p) of 0.000. The probability value (p) was less than 0.05, so it was known that there were significant or significant differences between the treatment groups.

The presence of antibacterial power in nano brown anchovies correlates with its active compound in the form of fluoride, which is able to inhibit bacterial growth. The phytochemical tests carried out at BPPKI Ketintang showed that the fluoride value in nano brown anchovy was 19.81 mg/100 g, or in 100 grams of nano anchovy, there was 19.81 mg of fluoride.

Fluoride's mechanism of bacterial inhibition is by interfering with the action of the bacterial enolase and F-ATPase enzymes. Enolase enzyme acts as a catalyst in Phosphoenolpyruvate (PEP) production during the bacterial glycolysis process. Then, when the enolase enzyme is inhibited by fluoride, PEP production decreases so that bacterial metabolism is disrupted. Disruption of bacterial metabolism results in bacterial death. The F-ATPase enzyme has an essential role in exporting protons and controlling the pH of bacteria to survive in acidic conditions. When the function of the F-ATPase enzyme is inhibited by fluorine, protons (H^+) in the bacterial cytoplasm cannot escape and cause an increased acidic atmosphere in the intracellular bacteria. The increased acidic atmosphere in the intracellular bacteria will be balanced by the decreased acid synthesis in the cells so that bacterial growth is inhibited[20].

This research proves that nano brown anchovy which has a size of 81.80 nm, can inhibit bacteria optimally with a low concentration of 1.56%. With this concentration,

nano anchovy can kill 100% of bacteria. Nano anchovy has better antibacterial power than other pulp capping materials such as biodentin. Biodentin has optimal antibacterial activity against *Enterococcus faecalis* and *Staphylococcus aureus* at a concentration of 25%. In nano form, brown anchovy has a large surface area and has a large number of atoms so that there is maximum contact between the antibacterial and the bacteria and the environment[21].

Several culturable bacteria could be isolated from deep carious lesions. Gram staining was carried out using bacterial samples from the positive control group to see the bacteria that grew. The results of the bacterial colony planting on Muller Hinton Agar media when Gram staining showed purple bacteria, which means bacteria are gram-positive. In this study, the morphology of the bacteria, namely *cocci* and *bacilli*, can be seen. This finding indicates that *Lactobacillus* and *Streptococcus* were found among the isolated bacteria and the proportion is consistent with other studies. In accordance with previous studies that proved that most bacteria contained in mixed bacteria with deep caries were gram-positive bacteria in the form of *cocci* and *bacilli*[22].

5. Conclusion

Based on the research that has been done, it can be concluded that there is antibacterial power of nano brown anchovy against mixed bacteria of deep dentin caries with a Minimum Inhibitory Concentration (MIC) of 0.781% and a Minimum Bactericidal Concentration (MBC) of 1.56%.

6. Acknowledgements

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7. Conflict of Interest

The authors have no conflicts of interest regarding this investigation.

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