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Research Report

Inhibition of *Streptococcus mutans* biofilm formation using combination of nano brown anchovy (*Stolephorus insularis*) and calcium hydroxide

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ABSTRACT

Background: *One of the important virulence factors of S. mutans is the biofilm formation. Some bacteria in biofilms show resistance to antimicrobials. Therefore, the addition of nano brown anchovy (S. insularis) as antibiofilm agents can potentiate the caries and infection prevention efficacy of endodontic treatment.* ***Purpose:*** *To describe the effect of the combination of nano brown anchovy (S. insularis) 12% and $Ca(OH)_2$ with a ratio of 1:1 on inhibition of S. mutans biofilm formation.* ***Methods:*** *Laboratory experimental research on S. mutans bacteria was carried out in vitro using the crystal violet assay method. Brown anchovy was made into nanoparticles, dissolved to a concentration of 12%, and combined with $Ca(OH)_2$. Biofilm samples were given treatment and observed for inhibition of biofilm growth. Biofilm growth inhibition was seen from the Optical Density $(OD)_{540nm}$ absorbance value measured using a spectrophotometer.* ***Results:*** *In the group that was tested with $Ca(OH)_2$, the average percentage in S. mutans biofilm reduction was 55%, while in the group that was tested with a combination of nano brown anchovy (S. insularis) and $Ca(OH)_2$, the average percentage in S. mutans biofilm reduction was 61%.* ***Conclusion:*** *The combination of nano brown anchovy (S. insularis) 12% and $Ca(OH)_2$ with a ratio of 1:1 can inhibit the formation of S. mutans biofilm.*

Keywords: *nano brown anchovy; antibiofilm; streptococcus mutans; calcium hydroxide*

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INTRODUCTION

One of the essential virulence factors of *S. mutans* is the formation of biofilms on the tooth surface because *S. mutans* is the primary producer of exopolysaccharide (EPS).[1,2] Biofilms play a vital role in various types of human infections that are persistent and difficult to treat, making it very difficult to treat infections associated with biofilms.[3,4]

S. mutans was highly prevalent in asymptomatic and symptomatic endodontic infections, including abscesses.[5] Inadequate endodontic treatment can affect the root canal system and spread beyond its apical foramina, causing the periodontal tissue to develop into abscesses, granulomas, and radicular cysts. Teeth with periodic lesions and infections can heal with non-surgical endodontic therapy, namely root canal dressings based on $Ca(OH)_2$. Calcium hydroxide is used as a root canal dressing to increase tissue alkalinity, create favorable environmental conditions for hard tissue formation, interfere with bactericidal activity, increase mineralization, and induce healing.[6]

The main drawback of $Ca(OH)_2$ root canal dressings is that their effect on biofilms is still not widely known.[7] Since *S. mutans* can be hypervirulent, this limitation may facilitate biofilm formation and contribute to secondary caries development. Although $Ca(OH)_2$ root canal dressings can reduce the number of viable bacteria, it is more challenging to remove biofilms. Therefore, adding anti-biofilm agents can potentiate caries and infection prevention efficacy of $Ca(OH)_2$ root canal dressings.[8]

Natural products exhibit biological activities that make them promising as an alternative or adjunctive therapy to obliterate oral biofilms.[9] One of the additional ingredients needed for root canal dressings is material with anti-biofilm properties. Brown anchovies or Hardenberg's anchovies (*Stolephorus insularis*) are part of the Clupeoidei Sub-Order, the Engraulididae Family, and the Engraulidinae Sub-Family. There are nine species of anchovy in Indonesian waters, one of which is the brown anchovy, found in abundance in the southern to western regions of Indonesia.[10,11] Brown anchovy contains high calories, fat, protein, carbohydrates, vitamins, minerals, fluorine, and CaF_2, most of which are found in the bones. Previous in vitro studies have proven that using brown anchovy solution for topical fluoridation can increase enamel hardness and tooth acid resistance.[12,13]

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Nanoparticles (Np) are fine particles of insoluble constituents with a diameter smaller than 100 nm.[14] In recent decades, with the development of nanotechnology, nanoparticles have shown significant potential for treating biofilm-related infection.[15,16] The small size of the nanoparticles is expected to increase the material's flowability and increase penetration into the root canal.[17] So in this study, the material studied was CaF_2 nanoparticles (CaF_2-NPs). CaF_2-NPs obtained from anchovy showed the ability to reduce *S. mutans* biofilm formation activity. Administration of CaF_2 nanoparticles resulted in almost a 90% reduction in biofilm formation and EPS production. The antibacterial action of CaF_2-NP results from the release of F^- ions.[18] At low pH, fluoride and hydrogen ions bind to each other to create hydrofluoric acid (HF). HF can penetrate the bacterial membrane and dissociate inside the bacteria.[18–20]

Based on the research results of Fa'Izah et al. in 2016, anchovy extract has effective antimicrobial properties against *S. mutans* bacteria at a concentration of 12%.[21] However, until now, no one has specifically examined the effect of nano brown anchovy (*S. insularis*) on the formation of *S. mutans* biofilms. Therefore, this study aims to describe the anti-biofilm ability of a combination of nano brown anchovies (*S. insularis*) and $Ca(OH)_2$ to inhibit the formation of *S. mutans* biofilms. *S. mutans* biofilm formation will be assessed from the results of the optical density readings of the biofilms.

MATERIALS AND METHODS

This research is an in vitro laboratory experimental study using a posttest-only control group design. There were 3 treatment groups namely positive control (K+), negative control (K-), and treatment group 1 (P1) in which each treatment was repeated 10 times according to the calculation of the number of samples.

One kg of brown anchovy was chopped and boiled for 1 hour. Brown anchovies that have been boiled were dried in the sun. A total of 100 grams of dried brown anchovies was macerated with 1 M HCL for 2 hours, then cleaned to neutralize the pH. The anchovies were dried in an oven at 105°C for 2 hours. The brown anchovies were ground using a mortar, filtered through a #60 mesh filter, and ground using High-Efficiency Milling for 1 hour at a speed of 3000 rpm and a milling ball diameter of 0.5 mm. The amount of powder compared to the quantity of milling balls was 1:5. 20gr nano anchovies diluted with saline to a concentration of 12%.

Bacterial culture was taken using sterile osse from *S. mutans* stock cultured in MH Agar. Bacterial concentration adjusted to the standard 0.5 Mc Farland (1.5×10^8 CFU/mL). Dilution was carried out using the serial dilution method until it reached a concentration of 1×10^6 CFU/mL. Bacteria were grown in tubes containing BHIB and 5% sucrose then incubated for 24 hours at 37°C.

The positive control group was prepared by mixing $Ca(OH)_2$ powder with sterile distilled water at a ratio of 1:1 (0.9 g $Ca(OH)_2$ powder and 0.9 mL sterile distilled water). Pure $Ca(OH)_2$ powder and sterile distilled water are mixed and placed in a measuring cup.

Treatment group 1 was prepared by mixing $Ca(OH)_2$ powder and 12% nano brown anchovy extract solution with a ratio of 1:1 (0.6 g $Ca(OH)_2$ powder and 0.6 mL of nano brown anchovy extract).

After incubation, the medium was decanted, and any remaining planktonic cells were removed with sterile water rinses. Adhered biofilm was stained with 200 µl of 0.1% CV dye for 15 min at room temperature. Biofilms were gently washed with distilled water, and excess dyes were removed from cells with 100 µl 98% ethanol. For the full release of the dye, the tubes were kept in the shaker for 5 minutes. The tube is taken from the shaker and inserted into the spectrophotometer, and the wave is set to 540nm. Light is passed through a tube containing a suspension of microorganisms which is then captured by a detector. The number of light absorption intensities (optical density) captured by the detector is read as a measurement result. A greater light absorbance indicates that there are more bacteria present in the suspension.

The data obtained from the Optical Density measurement was analysed statistically using SPSS v.26. The statistical test begins with a normality test for each group using the Shapiro-Wilk Test (n <50) to see if the data is normally distributed. A homogeneity test was carried out for each group using the Levene Test to see if the data was homogeneous. Nonparametric comparative statistical analysis was carried out using One Way ANOVA to see if there was a difference between the negative control, positive control, and treatment group 1 after being given treatment. A follow-up test (Post Hoc) was carried out using Tukey HSD to see which groups were significantly different.

RESULTS

The anti-biofilm activity of a combination of nano brown anchovy (*S. insularis*) 12% and $Ca(OH)_2$ against *S. mutans* bacteria was tested and read using a spectrophotometer with a wavelength of 540 nm expressed in OD units. The results of the research OD reading can be seen in Table 1. From the table of biofilm OD readings, the average *S. mutans* biofilm OD or growth value for each group can be calculated

Table 1. Optical Density (OD) reading results

Sample	K-	K+	P1
1	0.966	0.426	0.365
2	0.978	0.431	0.376
3	0.924	0.432	0.384
4	0.962	0.423	0.366
5	0.955	0.436	0.359
6	0.967	0.451	0.376
7	0.951	0.418	0.371
8	0.942	0.421	0.381
9	0.944	0.433	0.354
10	0.932	0.429	0.336

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(Table 2). Based on the results of reading the OD of *S. mutans* biofilm using a spectrophotometer, it was found that treatment group 1 (a combination of nano *S. insularis* 12% and $CaOH_2$) and positive control ($Ca(OH)_2$) had a lower biofilm OD value than the negative control (Figure 1).

Table 2. Average Optical Density (OD) results

Treatment Groups	N	Mean OD	SD
K-	10	0.9521	0.016
K+	10	0.43	0.009
P1	10	0.3668	0.014

Table 3. Percentage of reduction in *S. mutans* biofilm formation

Sample	K-	K+	P1
1	0%	56%	62%
2	0%	56%	62%
3	0%	53%	58%
4	0%	56%	62%
5	0%	54%	62%
6	0%	53%	61%
7	0%	56%	61%
8	0%	55%	60%
9	0%	54%	63%
10	0%	54%	64%

As shown in Table 3, in the negative control group, there was 0% reduction in biofilm formation because the negative control was only consisted of *S. mutans* grown on media and was not given any treatment. In the positive control group, the percentage in *S. mutans* biofilm formation ranged from 53%-56%. In treatment group 1, the percentage reduction in *S. mutans* biofilm formation ranged from 58%-64%. From the data in Table 3, the average percentage of reduction in *S. mutans* biofilm formation for each group can also be calculated (Figure 2).

The results of the Shapiro-Wilk Test showed that the research data were normally distributed. The results of the Levene Test showed that the research data was homogeneous. The results of One Way ANOVA showed that there was at least one group that had a difference after being given treatment. From the Post Hoc Test results table, it was found that all groups showed significant differences in inhibiting biofilm formation when compared to other groups.

DISCUSSION

The presence of anti-biofilm properties in the anchovy nano is in the form of CaF_2, which is capable of inhibiting bacterial growth. The results of the analysis carried out at BPPKI Ketintang showed that the amount of CaF_2 in nano

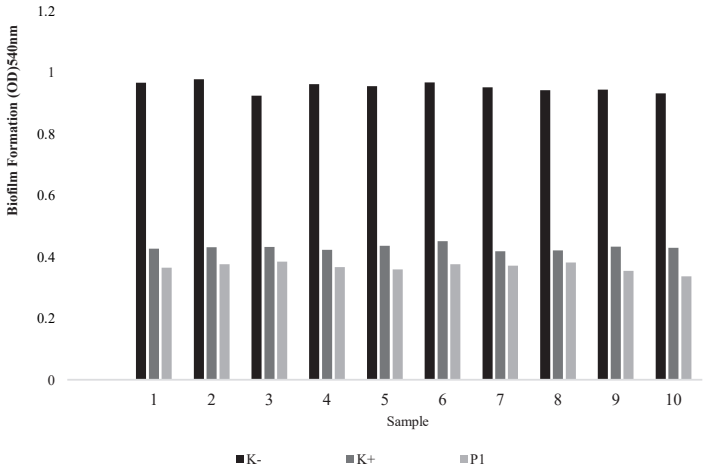

Figure 1. Graph of reduction in *S. mutans* biofilm formation

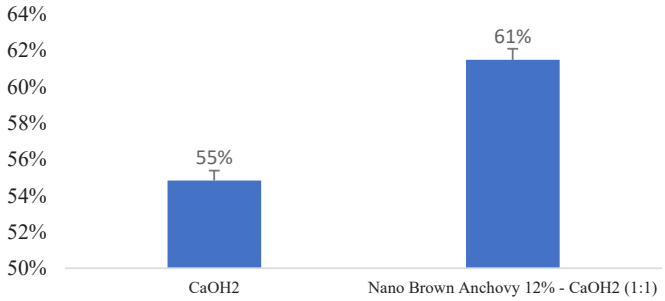

Figure 2. Graph of the average percentage of reduction in *S. mutans* biofilm formation.

brown anchovy was 21.05 mg/100g. There is also fluor, as much as 4.03 mg/100g.

The most effective nano-anti-biofilm power comes from the CaF_2-NP compound, which releases F^- ions. These ions work by reducing the ability of *S. mutans* bacteria to produce acid and can interfere with glycolysis by inhibiting enolase activity. Furthermore, F ions act on the proton pump (H-ATPase) by inhibiting and reducing cellular ATP levels.[18]

From the results of the average decrease in biofilm growth, it can also be concluded that the anti-biofilm capacity of the combination group of nano brown anchovy (*S. insularis*) and $Ca(OH)_2$ against *S. mutans* biofilms is more potent than the group that was only tested with $Ca(OH)_2$. This is because even without the addition of nano brown anchovies, there is already a bactericidal effect from $Ca(OH)_2$ itself. In an aqueous environment, $Ca(OH)_2$ releases hydroxyl ions. The lethal effect of hydroxyl ions on bacterial cells may result in damage to the DNA and bacterial cytoplasmic membrane, or protein denaturation. Therefore, it is speculated that calcium hydroxide has the ability to damage the EPS biofilm matrix and inhibit biofilm growth.[7,22]

The effect of $Ca(OH)_2$ on the morphology, structure, and physicochemical properties of biofilms must also be considered. According to research conducted by Momenijavid et al. (2022), the alkaline pH of the environment increases Ca^{2+} uptake when biofilms are treated with $Ca(OH)_2$. The presence of Ca^{2+} ions caused biofilms that was grown in vitro to become denser with more cavities present, and there are indications of an increase in EPS. The presence of these ions also creates a granular surface on the biofilm. The increase in the thickness, colony size, and volume of the biofilm, as well as the decrease in the surface-to-biofilm ratio, are the result of Ca^{2+} ions in the biofilm.[23] Because Ca^{2+} ions in $Ca(OH)_2$ are thought to be able to increase biofilm EPS, that is why the group treated with $Ca(OH)_2$ solution alone cannot inhibit *S. mutans* biofilm growth more effectively.

With the addition of CaF_2-NPs, there was a decrease in adhesion or interference with the attachment of bacteria to the tooth surface. The addition of CaF_2-NPs with $Ca(OH)_2$ is expected to result in a concentration decrease of polysaccharides which causes an increase in the permeability of EPS biofilms. Then, there was a disturbance in the EPS biofilm balance which resulted in the dissolving of EPS.[24] Due to barriers to the formation and reduction of *S. mutans* EPS production, there was a decrease in biofilm EPS volume. The decreased EPS volume will inhibit the formation of *S. mutans* biofilms. In a study by Zhu et al. (2018), drug-free nanoparticles (NPs) were made as surface charge regulators. Then systematically investigated the interaction between nanoparticles and bacteria and their natural bioactivity on mature *S. mutans* and *S. mutans* biofilms in a planktonic form. Possible mechanisms behind the disruption of mature biofilms by nanoparticles can be related to the direct inactivation of bacterial cells, inhibition of new biofilm formation, interruption of bacterial cell-to-cell and cell-to-EPS interactions, and biofilm disintegration.[15,16]

The findings of a study conducted by Kulshtrestha et al. (2016) showed that the anti-biofilm mechanism of CaF_2-NPs on *S. mutans* was a combination of enzymatic activity suppression and gene suppression. These mechanisms can cause disturbances in all metabolic tissues, which in turn inhibits bacterial pathogenesis. Considerable reductions in biofilm-forming ability and EPS production in *S. mutans* were observed in the presence of CaF_2-NPs, and the reduction was concentration-dependent. When the concentration of nanoparticles increases, the ability to form biofilms and EPS production decreases. Different CaF_2-NP concentrations were also used to evaluate the effect of the treatment on preformed *S. mutans* biofilms. Around a 5-11% reduction in the biofilm formation at a CaF_2-NPs concentration of 1-4 mg/mL.[18] Based on the results of this study, it can be concluded that the combination of 12% nano brown anchovy (*S. insularis*) and $Ca(OH)_2$ in a ratio of 1:1 can inhibit the formation of *S. mutans* biofilms.

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