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Research Report

Effectiveness of micro and nano sized propolis with calcium hydroxide combination in inhibiting the formation of *Lactobacillus acidophilus* biofilm

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ABSTRACT

Background: *Lactobacillus acidophilus is involved in secondary caries that can accelerate demineralization of the tooth surface. Bacteria forms biofilm as part of a defense mechanism. One of the caries treatments is direct pulp capping with calcium hydroxide ($Ca(OH)_2$) as the gold standard. Due to its high pH, calcium hydroxide can induce necrosis of pulp tissue when it comes into direct contact. Propolis is a natural material produced by honey bees. Propolis has many benefits including antibacterial effects. Nanoparticles are microscopic particles measuring 1-100 nm, reducing particle size can increase the rate of dissolution and absorption.* ***Purpose:*** *This study aims to prove the difference in the biofilm inhibitory ability of a micro and nano-sized combination of propolis and $Ca(OH)_2$ against L. acidophilus biofilms.* ***Methods:*** *This study used true experimental laboratories research with treatment groups that included a control, combination of micro-sized propolis and $Ca(OH)_2$ and combination of nano-sized propolis and $Ca(OH)_2$.* ***Results:*** *The results showed that there was a significant difference between the micro and nano-sized combination of propolis and $Ca(OH)_2$ in inhibiting biofilm growth, with the combination of nano-sized propolis and $Ca(OH)_2$ showing higher effectiveness compared to the micro-sized one. The combination of nano-sized propolis and $Ca(OH)_2$ produced an inhibition value of 84.35%, while the micro-sized one produced an inhibition value of 77.18%.* ***Conclusion:*** *The combination of nano-sized propolis and $Ca(OH)_2$ had a greater biofilm inhibition compared to the combination of micro-sized propolis and $Ca(OH)_2$.*

Keywords: *nano propolis; nano calcium hydroxide; biofilm; particle size; Lactobacillus acidophilus*

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INTRODUCTION

Dental caries is the most common chronic disease experienced by the most people, and the etiology is multifactorial.[1] Based on the Indonesian Basic Health Research (RISKESDAS) in 2018 data, the prevalence of dental caries in Indonesia is 88.8% with the prevalence of root caries at 56.6%. One of the cariogenic bacteria that causes caries is *Lactobacillus acidophilus* (*L. acidophilus*) which is a gram-positive bacteria that produces lactic acid from carbohydrate metabolism.[2] Although Lactobacillus acidophilus and other lactobacilli are generally poor primary colonizers of tooth surfaces and are thought to participate mainly in the progression rather than the initiation of dental caries[3], recent microbiome studies show that Lactobacillus becomes one of the most abundant genera in deep dentinal caries lesions[4,5].

L. acidophilus is an agent of secondary caries that can accelerate demineralization of the tooth surface.[6] Bacteria forms biofilm as part of a defense mechanism. Bacterial biofilms are collections of bacteria that cling to a surface and/or each other, forming a self-produced matrix.[7] The structural characteristics of biofilms make them more resistant to antimicrobial agents and environmental stress than planktonic cells.[8] The biofilm formation process consists of several stages, starting from the attachment process, bacterial adhesion and aggregation, microcolony formation, maturation, and dispersion. Inhibiting biofilm formation can be done at the stage of bacterial adhesion and aggregation, namely on the 2nd day or after 48 hours. While the bacterial maturation stage occurs on the 5th day.[9]

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Maintaining pulp vitality is critical for tooth survival, pulp feeding, innervation, and immune response mechanisms. There are numerous treatment techniques available to treat caries, one of which is minimally invasive conservative vital pulp treatment (VPT), such as direct pulp capping.[10] Calcium hydroxide has long been considered as the gold standard for pulp capping treatment due to its antibacterial properties and capacity to induce the production of reparative dentin. Calcium hydroxide has a basic pH which alters the local environment, so bacteria cannot grow and creating favorable conditions that stimulate hard tissue growth[1]. Calcium hydroxide has a highly basic pH (pH 12.5); therefore, direct contact with pulp tissue can lead to necrosis.[11]

Propolis is a naturally occurring substance synthesized by honey bees. This material has been used traditionally by ancient civilizations as a traditional medicine, and based on extensive research, propolis has many benefits including antioxidant, antibacterial, antifungal, anti-inflammatory, antiviral and antitumor effects.[12] The main chemical compound groups found in propolis, aside from resin include waxes, polyphenols (phenolic acids, flavonoids), and terpenoids.[13] Propolis has demonstrated the ability to suppress the growth of oral microbes and inhibit the activity of glucosyltransferase (GTF).[14] Propolis is a good calcium hydroxide carrier that allows dissociation to occur and shows greater inhibitory power. A combination of calcium hydroxide ($Ca(OH)_2$) and propolis results in the formation of a calcium salt that incorporates the active antibacterial components present in propolis.[1]

Nanoparticles are tiny particles ranging in size from 1 to 100 nanometers, characterized by a high surface area and distinct chemical or biological reactivity. Their ultra-small dimensions enable them to penetrate and access the intricate structures within the root canal system.[15] While microparticles are particles that have a size ranging from 1–1000 μm and a matrix or reservoir structure that is known to have various different structures.[16] Reducing particle size can increase the rate of dissolution and absorption, thereby increasing the bioavailability of compounds that are poorly soluble in water and can make it easier to enter through the outer membrane of bacteria.[17,18]

In the preliminary study, the minimum inhibitory concentration (MIC) was determined using the Kirby-Bauer diffusion technique to identify the lowest concentration that produced the optimal diameter of the bacterial inhibition zone. The diffusion method was used to measure the inhibition zone, with Mueller-Hinton Agar (MHA) used in this study. The results of the combination of nano-sized propolis and $Ca(OH)_2$ at a ratio of 1:0.4 (100 mg of $Ca(OH)_2$ powder and 40 μl of nano propolis) demonstrated an inhibition zone diameter of 23.83 mm and a setting time of 1 minute. However, to date, no studies have specifically investigated the effectiveness of this combination in inhibiting biofilm formation by *Lactobacillus acidophilus*. This research is expected to provide evidence on whether the combination can effectively suppress the development of *L. acidophilus* biofilms.

MATERIALS AND METHODS

This study employed a true experimental laboratory design, specifically using a randomized post-test only control group design. This approach allows for the assessment of treatment effects by comparing outcomes between randomly assigned groups after the intervention, without pre-test measurements. The tools utilized in this research included 96-well flat-bottomed plastic tissue culture plates, ose, shaker water bath, rotary vacuum evaporator, petri dishes, test tubes, micropipettes, ELISA readers with a wavelength of 570 nm, vortex, brander and spreader, incubator (Memmert, Germany), autoclave (Tomy, Japan), anaerobic jar, analytical balance.

The materials used include Brain-heart Infusion Broth (BHIB), *Lactobacillus acidophilus* bacterial culture that can form biofilms, *Apis Mellifera* bee propolis extract, calcium hydroxide (pro analysis, Merck, Germany), nano propolis extract, nano calcium hydroxide, aquadest, aqua deionization solution, 1% glucose, 2% crystal violet, aluminum foil, 90% ethanol, 40% propylene glycol. This study has been tested for ethical clearance at the Faculty of Dental Medicine, Airlangga University, Surabaya with the number 0812/HRECC.FODM/VIII/2024.

Micro-sized propolis and micro-sized $Ca(OH)_2$ combination was prepared. Calcium hydroxide powder was weighed using an analytical balance as much as 100 mg. Propylene glycol 40% was made using 40 mL of propylene glycol added with distilled water to reach 100 mL. A mixture of calcium hydroxide powder, propolis extract and 40% propylene glycol was prepared using a ratio of 1:1:0.4, so it is equivalent to 100 mg Ca(OH)2 : 100 μL of propolis : 40 μL of PG 40%. The mixture of calcium hydroxide powder, propolis extract and 40% propylene glycol was stirred using a cement spatula until homogeneous.

Nano-sized propolis and nano-sized $Ca(OH)_2$ combination was prepared. Nano calcium hydroxide powder was weighed using an analytical balance to obtain a weight of 100 mg. Nano calcium hydroxide with nano propolis was prepared in a ratio of 1:0.4:0.2, with 40 μL of nano propolis and 20 μL of 40% propylene glycol accelerator measured using a micropipette. The mixture of nano calcium hydroxide powder, nano propolis, and propylene glycol 40% was stirred using a cement spatula until a uniform and homogeneous paste was achieved.

The *Lactobacillus acidophilus* bacteria was prepared. The stock of *Lactobacillus acidophilus* bacteria was sub-cultured in a tube medium and then incubated at 37°C for 24 hours. After obtaining a pure colony subculture, 1 colony was transferred into 10 ml of BHIB medium and incubated at 37°C for 48 hours to produce an active bacterial suspension for further experimentation. The absorbance value (Optical Density) of the subculture in BHIB medium was measured using spectrophotometer (wavelength of 625 nm). The results were diluted with 0.9% NaCl until the OD value became 0.1, then a suspension of *Lactobacillus acidophilus* bacteria of $1.5x10^8$ CFU/ml was obtained which was ready to be used for biofilm inhibition test work.

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Biofilm formation inhibition test was done. A total of 100 µl of the bacterial suspension was added to each well of the microtiter plate, followed by the addition of 100 µl of the combination of nano-sized propolis and $Ca(OH)_2$ (1:0.4) and 100 µl of the combination of micro-sized propolis and $Ca(OH)_2$ (1:1) into the respective wells. In the control group, the bacterial culture on BHIB media was given distilled water. The plates were then incubated for 48 hours at 37°C. After incubation, each well of the microtiter plate was aspirated and washed three times with 200 µl of deionized water using a micropipette to remove non-adherent cells. The aim is to remove the remains of planktonic bacteria and then dried. After the drying step, the microorganisms adhering to the wells of the microtiter plate were stained with 200 µl of 2% crystal violet solution and incubated at room temperature for 20 minutes to allow proper binding of the dye to the biofilm matrix. With a micropipette, 200 µl of 2% crystal violet was discarded, then rinsed 3 times with distilled water. Next, the microtiter plate was added with a solution in each well with 100 µl of 90% ethanol. The microtiter plate was gently shaken for 1 minute to ensure even distribution of the stain, then placed into an ELISA reader to measure the optical density (OD) of each sample at a wavelength of 570 nm.

Biofilm inhibition was observed and measured. The results were determined based on the optical density (OD) values measured using the ELISA reader, which provided quantitative data on biofilm formation in each sample. The inhibitory activity is calculated using the formula with modifications.[19]

RESULTS

Based on the calculated OD values, the averages are summarized in Table 1. It can be seen from Table 1 that the combination of nano-sized propolis and $Ca(OH)_2$ with a ratio of 1:0.4 has a greater inhibitory value than the combination of micro-sized propolis and $Ca(OH)_2$ with a ratio of 1:1. Normality of the data distribution was tested using the Shapiro-Wilk method. Based on the normality test, a p value of 0.988 was obtained in treatment group 1 (a combination of micro-sized propolis and $Ca(OH)_2$) and a p-value of 0.420 in treatment group 2 (a combination of nano-sized propolis and $Ca(OH)_2$). With p-values > 0.05 across all treatment groups, the research data are assumed to follow a normal distribution. Furthermore, the homogeneity of variance was tested using the Levene test. A p value of 0.275 was obtained, which means the p value > 0.05, so it can be concluded that the data variance is homogeneous. As the data followed a normal distribution, a parametric analysis using the independent t-test was conducted to assess differences between groups.

Based on Table 2, the p value < 0.05 is obtained as the basis for making decisions for the independent t-test. It can be concluded that H0 is rejected and Ha is accepted, which means that there is a significant or meaningful difference between the combination group of micro-sized propolis and $Ca(OH)_2$ and the combination group of nano-sized propolis and $Ca(OH)_2$.

Table 1. Average results of the research

Group	N	Mean (%) ± SD
Combination of micro- sized propolis and Ca(OH)2 (1:1)	12	77.18 ± 6.806
Combination of nano-sized propolis and Ca(OH)2 (1:0.4)	12	84.35 ± 5.026

Table 2. Independent t-test results

Group	Treatment group 1
Treatment group 2	p = 0.008*

Notes:
Treatment group 1 = combination of micro-sized propolis and Ca(OH)2;
Treatment group 2 = combination of nano-sized propolis and Ca(OH)2

DISCUSSION

Lactobacillus acidophilus (L. acidophilus) is a bacterium commonly found in the saliva of caries patients. Bacteria forms biofilm as part of a defense mechanism. Due to their structural characteristics, biofilms enhance bacterial resistance to antimicrobial agents and environmental stressors when compared to planktonic cells.[8] Calcium hydroxide ($Ca(OH)_2$) is a widely used endodontic material and has long been considered as the gold standard in pulp capping treatment.[20] High levels of $Ca(OH)_2$ are known to cause damage to pulp tissue, so $Ca(OH)_2$ is combined with natural propolis. Propolis is a honey bee product made from resin collected from various plants. The main chemical compounds found in propolis aside from resin are polyphenols (phenolic acids, flavonoids), terpenoids, tannins and Caffeic Acid Phenethyl Ester (CAPE). According to research by Mori *et al*, $Ca(OH)_2$ combined with propolis does not cause toxic reactions.[21]

Nanoparticles measure about 100 nanometers and are characterized by a high surface-to-volume ratio and distinct chemical or biological properties. Their small size facilitates penetration into the complex anatomy of the root canal system. Reducing particle size can increase the rate of dissolution and absorption, thereby increasing the bioavailability of compounds that are poorly soluble in water and can make it easier to enter through the outer membrane of bacteria.[16] The combination of nano-sized propolis and $Ca(OH)_2$ will form a calcium salt compound through Van Der Waals bonds.

The formation of this calcium salt occurs due to the acid and base reaction between calcium hydroxide and CAPE in propolis. The calcium salt content that has an active role is the hydroxyl group, tannin, flavonoids and terpenoids. Tannins and terpenoids can inhibit the formation of *L. acidophilus* biofilms by inhibiting the formation of EPS. Hydroxyl groups and terpenoids inhibit the formation of *L. acidophilus* biofilms by reducing the production of the GTF

enzyme, the decreased GTF production leads to reduced EPS synthesis, which causes the inhibition of *L. acidophilus* biofilms formation. In this study, a Minimum Biofilm Inhibitory Concentration (MBIC) test was conducted to determine the activity of the combination of nano-sized propolis and $Ca(OH)_2$ in inhibiting biofilm formation in *L. acidophilus*. Experimental laboratory research on the inhibitory power of *L. acidophilus* biofilm was carried out in vitro with a 96-well microtiter plate. The results were obtained in the form of the Optical Density (OD) value of the biofilm measured with an ELISA reader with a wavelength of 570 nm which was then measured using the formula. The concentration comparison of the combination of micro-sized propolis and $Ca(OH)_2$ was 1:1 (100 mg $Ca(OH)_2$: 100 µl propolis) and in accordance with preliminary research that has been carried out. The concentration comparison of the combination of nano-sized propolis and $Ca(OH)_2$ used 1:0.4 (100 mg $Ca(OH)_2$: 40 µl propolis). Based on the calculation of OD value using the data calculation formula, the results showed that the combination of nano-sized propolis and $Ca(OH)_2$ had a higher inhibitory value than the combination of micro-sized propolis and $Ca(OH)_2$ on *L. acidophilus*. Based on the calculation of the IC50 value to obtain the minimum limit of biofilm inhibition, the IC50 value was 82%, which means that a material is considered to have inhibitory activity against biofilm if the calculation of the inhibitory value is above 82%. Based on the average results obtained from the study, the combination of nano-sized propolis and $Ca(OH)_2$ had an average biofilm inhibition value above 82%, which was 84.35%, which means that the combination of nano-sized propolis and $Ca(OH)_2$ possesses inhibitory activity against *L. acidophilus* biofilm. The combination of nano-sized propolis and $Ca(OH)_2$ had a higher biofilm inhibition value than the micro-sized one.

In conclusion, the combination of nano-sized propolis and $Ca(OH)_2$ is more effective in inhibiting the formation of *L. acidophilus* biofilm. Smaller particle sizes result in increased dissolution and absorption rates. Therefore, the particles can enter through the outer membrane of bacteria and prevent the formation of bacterial biofilms.

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