Dosage Effectiveness of the Combination Calcium Hydroxide-Propolis as an Inhibitor for *Aggregatibacter actinomycetemcomitans* Biofilm

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Abstract

Objective: Calcium hydroxide is widely used as pulp-capping material, but it is resistant to *Aggregatibacter actinomycetemcomitans* bacteria. Because of this deficiency, many researchers are considering using alternative natural ingredients, such as propolis. Propolis has been widely used in dentistry due to its properties and because it has active ingredients that can act as antibacterial agents. It is hoped that when calcium hydroxide and propolis are combined, the combination can overcome the disadvantages. The aim of this study was to determine the dosage effectiveness of the combination calcium hydroxide-propolis as an inhibitor for *A. actinomycetemcomitans* biofilm**. Material and methods:** The sample was divided into five groups. The positive control group is a combination of calcium hydroxide-sterile aquadest, and the negative control group is *A. actinomycetemcomitans* bacteria. Group I is a combination of calcium hydroxide-propolis with a ratio of 1:1, group II has a ratio of 1:1.5, and group III has a ratio of 1:2. Each group consists of five replications. Combination of calcium hydroxide-propolis and *A. actinomycetemcomitans* bacterial suspension was incubated for 2x24 hours then washed three times using PBS. Furthermore, 2% of crystal violet was used for staining and covered with aluminum foil and then incubated for 15 minutes. Isopropanol 200µL was added, followed by a measurement of OD with an ELISA reader, and the OD values were calculated using an inhibition biofilm formula. **Results**: Group II has the largest mean inhibition with 76% compared to groups one and three. **Conclusion:** A combination of calcium hydroxide and propolis with a ratio of 1: 1.5 is more effective than 1:1 or 1:2 to inhibit *A. actinomycetemcomitans* biofilm.

Keywords: *Calcium hydroxide, propolis, herbal medicine, biofilm, Aggregatibacter actinomycetemcomitans*

Introduction

The most common cause of pulp damage is bacterial agents, which enters the pulp chamber through caries. According to Radman *et al.* [1] in their study using the Polymerase Chain Reaction (PCR) method, 80% of *Enterococcus faecalis* bacteria, 32% of *Aggregatibacter actinomycetemcomitans*, and 16% of *Porphyromonas gingivalis* were found. There was a significant decrease in the number of bacteria after the indirect treatment of pulp capping with calcium hydroxide, which decreased *E. faecalis* bacteria by 77.5% and *P. gingivalis* bacteria by 100%, whereas *A. actinomycetemcomitans* bacteria decreased from 32% to 16%. The decrease that occurred in *A. actinomycetemcomitans* bacteria was only 50%, whereby the percentage reduction was smaller when compared to the other two bacteria.

Biofilms in an oral cavity can be produced by some bacteria. One of them is *A. actinomycetemcomitans*, a gram-negative bacterium and facultative anaerob.[2] Biofilms are collections of microorganisms that attach to biotic or abiotic surfaces, and they are encased in a

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hydrated matrix consisting of exopolymeric substances, proteins, polysaccharides, and nucleic acids. Biofilm production in some pathogenic bacteria is regulated by a system of regulating the expression of bacterial genes called quorum sensing in response to the density of the microorganism populations through the production of extracellular signaling molecules or autoinducers. [3] Bacteria that grow in biofilms show higher resistance, both in antimicrobial agents and host defense mechanisms, compared to planktonic cells—cells that form biofilms 10–1000 times more resistant to antimicrobials. [3]

Pulp-capping treatment is required when there is inflammation in the pulp (reversible pulpitis) due to dental caries or iatrogenic factors. Pulp capping is the provision of a layer of protective/treatment material on the pulp. Calcium hydroxide is often used as pulp-capping material because it can stimulate the mineralization of scar tissue formation or reparative dentine, and the high pH of calcium hydroxide can kill microorganisms. [4.5] The inability of calcium hydroxide to eliminate some of the microorganisms found in the dentinal tubules is one of the deficiencies of calcium hydroxide, so many researchers considering using alternative materials derived from nature, such as propolis. [6.7]

Propolis is a natural bee product that has long been used in dentistry. It has anti-inflammatory, anti-microbial, and anti-fungal abilities, and it can heal scars. Existing studies have shown that propolis extract is effective in inhibiting anaerobic periodontal pathogens, namely *A. actinomycetemcomitans*. [8.9] According to Montero and Mori, [7] when calcium hydroxide is combined with propolis, calcium hydroxide can dissociate better into calcium ions and hydroxyl ions so that it can diffuse well into the dentinal tubules. In another study, it was shown that calcium hydroxide combined with propolis did not show any toxic reaction, and it could significantly reduce inflammation and was biocompatible material.[10]

Until now, there has been no research on the effectiveness of the calcium hydroxide-propolis combination in inhibiting the biofilm of *A. actinomycetemcomitans* bacteria; therefore, the aim of this study was to determine the dosage effectiveness of the combination calcium hydroxide-propolis as an inhibitor for *A. actinomycetemcomitans* biofilms.

Material and Methods

This research is an in vitro laboratory experimental study with a post-test only control group study design. The tools used include a Petri dish *(Pyrex, USA),* a brander, a spreader *(Pyrex, USA)*, an anaerobic jar *(Oxoid, UK),* an autoclave *(Hirayama, Japan),* an incubator *(Memmert, Germany),* an analytical balance (*Mettler Toledo, Indonesia*), a micropipette *(Socorex,* Switzerland *),* an osse, an *ELISA reader (Biorad model 550, California)*, a 96-well flat-bottomed plastic tissue culture plate, and a vortex (*Biomol, Germany).* The materials used were calcium hydroxide powder (analysis of Merck, Germany), propolis extract *Apis mellifera* Lawang, bacterial stock *A. actinomycetemcomitans*, trypticase soy broth (TSB) + glucose, aquadest sterilized, 96% ethanol, 1% glucose, phosphate buffered saline (PBS) pH 7.4, 2% crystal violet, aluminum foil, matches, and isopropanol. This research has been conducted ethically feasible test at the Faculty of Dentistry, Airlangga-Surabaya University with numbers 056/HRECC. FODM/V/2018.

Preparation combination of calcium hydroxide and propolis extract

The calcium hydroxide-propolis combination was made by mixing calcium hydroxide powder (analysis of Merck, Germany) and propolis extract (Apis Mellifera, Lawang) into a brownish thick liquid in a test tube at the following ratios: 1:1 (0.125 g calcium hydroxide powder with 0.125 ml propolis extract), 1:1.5 (0.125 g calcium hydroxide powder with 0.1875 ml propolis extract), and ratio 1:2 (0.125 g calcium hydroxide powder with 0.25 ml propolis extract). The combination of calcium hydroxide powder with Apis mellifera propolis extract mixed with sterile aquades to obtain a liquid form so that it can be tested on *A. actinomycetemcomitans* biofilm. To determine the concentration of the amount of propolis extract that is used, the MIC value of the extract against the *A. actinomycetemcomitans* bacteria was inspected. The concentration of the propolis extract was found by diluting 100% propolis extract with a dilution method of 100%, 50%, 25%, 12.5%, and 6.25%, and it was found that the MIC of the propolis extract against the *A. actinomycetemcomitans* bacteria was 12.5%.[11]

Bacteria Preparation

The stock of the *A. actinomycetemcomitans* bacteria was a subculture using the quadrant-streaking method on the *Tryptose Soy Agar* (TSA) medium in an incubator temperature of 37°C for 24 hours. After the pure colony subculture was obtained, one colony was taken into a 10 ml TS-Broth-glucose medium and incubated for 24 hours at 37°C. The subculture in the TS-Broth-glucose medium was measured to find the concentration of bacterial cells (optical density [OD] value) with a wavelength spectrophotometer of 625nm. The spectrophotometer OD results were diluted with 0.9% NaCl to OD 0.1, so we have obtained a 10^8 bacteria / ml suspension of *A. actinomycetemcomitans* bacteria, which is ready to be used for biofilm inhibition testing.

Biofilm formation preparation test

The method that has been selected to examine the formation of the biofilm is a biofilm microtiter plate. After the biofilm is formed, it was injected into the well microtiter plate culture to as much as 100µL / well, after which a combination of calcium hydroxide-propolis was added at 1:1, 1:1.5, and 1:2 ratios to each microplate well (treatment group) to as much as 100µL / well so that the final volume was 200µL / well. The well-positive control groups were given sterile calcium hydroxide and aquadest, and for the negative controls, only *Aggregatibacter actinomycetemcomitans* bacteria, without a combination of calcium hydroxide-propolis, was given. It was incubated again for 2x24 hours at 37°C. Then, the contents of each microtiter plate were aspirated and washed three times with 200µL PBS (pH 7.4) using a micropipette. This function removes planktonic bacteria. The microorganisms attached to the well were stained using 200µL / well crystal violet 2% and then covered with aluminum foil so that it was not exposed to direct light, after which it was incubated at room temperature for 15 minutes and cured overnight. Next, 200 µL / well isopropanol was added, which served to shed the color of the crystal violet on the wall of the well. The microplate was shaken for one minute and placed into an ELISA reader for measuring the OD of each sample with a wavelength of 570 nm. Each treatment group in this procedure was replicated five times.

Observing and measuring biofilm inhibition

The reading result is the absorbance value or OD, which describes the quantity of biofilm formation. To calculate the percentage of biofilm inhbition, it uses a formula with modification as follows. [12]

$$\text{Biofilm Inhibition (\%)} : \frac{\text{OD Control} - \text{OD Experimental Sample}}{\text{OD Control}} \times 100\%$$

Results

Based on the results of the measurements of the OD (Optical Density), the average results of the research are shown in Table 1.

Table 1. Average results of the research

Group	N	Mean (%)	*Std. Deviation*
Positive Control	5	39.0000	±13.03840
Group I	5	42.9540	±27.33372
Group II	5	76.2000	±13.62718
Group III	5	74.8000	±19.96747

Notes: Positive control = calcium hydroxide, group I = combination of calcium hydroxide-propolis 1:1, group II = combination of calcium hydroxide-propolis 1:1.5, group III = combination of calcium hydroxide-propolis 1:2.

Based on the research results of the mean values listed in table 1, it can be seen that the biofilm inhibition of *Aggregatibacter actinomycetemcomitans* bacteria which were treated with a combination of calcium hydroxide-propolis has a greater value than the positive control, so it could be stated that the combination of calcium hydroxide-propolis was effective in inhibiting bacterial biofilms *Aggregatibacter actinomycetemcomitans*, and the ratio of 1: 1.5 is more effective than 1: 1, 1: 2 in inhibiting the *Aggregatibacter actinomycetemcomitans* bacterial biofilm.

To see biofilm formation in the *Aggregatibacter actinomycetemcomitans* bacteria, SEM (Scanning Electron Microscope) was used with the Carl Zeiss (Evo MA 10) brand of England with magnifications used were

500, 1000, 4000, and 10,000x.

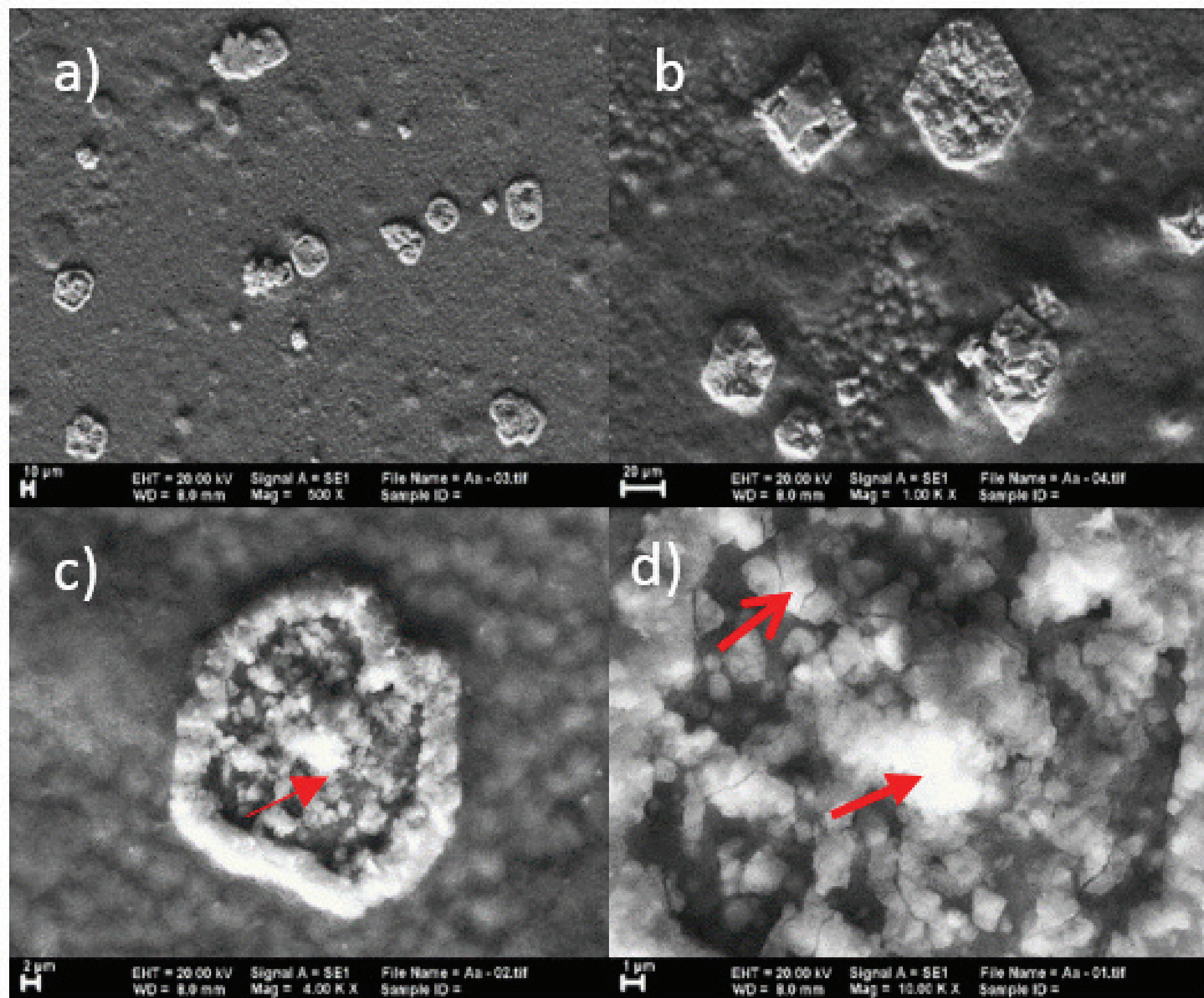

Figure 2. SEM Results ***Aggregatibacter actinomycetemcomitans*** **Bacteria Biofilms (Figure a and b image of colonies forming biofilms at 500 and 1000 magnifications; c and d. Figure biofilm matrix formed at 4000 and 10.00 magnification with arrows indicating bacteria coated with biofilm matrix)**

The normality test used is the One Sample Kolmogorov-Smirnov Test. The test results in all the research groups have a value of $p > 0.05$. This indicates that the data obtained is normally distributed. Homogeneity test with Levene's Test was conducted. The test assay shows a value of $p = 0.358$ ($p > 0.05$), which means that the data is homogeneous. Then, a one-way ANOVA test was conducted. The test results demonstrated a value of $p = 0.011$ ($p < 0.05$), which showed that there were significant differences in the treatment group. Then, the post-hoc LSD analysis was carried out. The test results in each treatment group can be seen below (Table 2). From the table above, it can be seen that there is no significant difference between the positive control group and group I, and there is no significant difference between groups II and III.

Tabel 2. LSD Post-Hoc Analysis Test Results

Group	Positive Control	Group I	Group II	Group III
Positive Control	-	0.736	0.008*	0.010*
Group I		-	0.017*	0.021*
Group II			-	0.911
Group III				-

Notes: * $p < 0.05$ = there was significant difference

Discussion

Pulp-capping treatment with calcium hydroxide is said to be successful if calcium hydroxide can dissociate into ionic forms Ca^{2+} and OH^{-}.[15] This is an ionization process in tissues that explains the antibacterial properties of calcium hydroxide. The dissociation of calcium hydroxide into Ca^{2+} and OH^{-} depends on the carrier that is used. The ion release being influenced by the carrier material is a major factor. The hydroxyl group creates the alkaline condition, which can activate an alkaline phosphatase enzyme that stimulates mineral tissue formation, which helps the repair process. When hydroxyl ions are able to diffuse in the dentine and remain in the pulp at adequate concentrations, the process becomes more effective. It can release the pH needed to destroy bacteria in the root canals and the dentinal tubules. One of the carrier materials that shows good diffusion in dentine is propolis.

Propolis is a combination of organic compounds containing weak acids. Its pH tends to be below 7, while calcium hydroxide is a strong base that has a pH above 8. If propolis and calcium hydroxide are combined, it is possible to observe salting reactions between caffeic acid and calcium hydroxide. If the amount of propolis extract mixed is small, it is possible to neutralize the process of propolis by $Ca(OH)_2$. When calcium hydroxide neutralized the acidic functional group of propolis with a low propolis amount, the inhibitory activity was dominated by calcium hydroxide. Because the active ingredient in propolis is not only acidic but also neutral, there will be synergy where the other active ingredients can suppress the formation of *A. actinomycetemcomitans* bacteria biofilm. Additionally, if the ratio of propolis increases, the inhibition of the biofilm produced will also increase. This happens because calcium hydroxide cannot neutralize all of the acid content in propolis.

The propolis extract is in the form of a brownish thick liquid. Meanwhile, calcium hydroxide is in the form of a powder (fine powder). When calcium hydroxide and propolis extracts are mixed, they create a suspension mixture. Suspensions are liquid preparations containing solid and insoluble particles that are dispersed in the liquid phase.[13] This mixture of suspensions, if allowed to stand, will separate (form deposits) so that the mixture of propolis and calcium hydroxide is not perfect. This imperfect mixture results in the activity of active compounds in propolis decreasing because of uninform impregnation, and the biofilm inhibitory results that are obtained will be different even though the concentration is the same (there are no significant differences).

The method used to observe the microbial biofilm attachments was the Microtitter Plate Biofilm Assay, with a combination of calcium hydroxide-propolis (1:1, 1:1.5, 1:2) put in a 100-µl microtiter plate well after the bacterial preparation on the plate to see the inhibition of the *A. actinomycetemcomitans* bacteria biofilm on the second day.[14] This biofilm inhibition occurs in the initial adhesion phase, which is a nonspecific biofilm formation and bacterial attachment to a reversible surface. If it is permanently attached, the bacteria will

begin to synthesize the insoluble exopolysaccharides (EPS), which attaches the bacteria to a three-dimensional matrix that functions to protect the bacteria from host and antimicrobial immune responses. Therefore, one effective method to inhibit the film formation is to prevent or reduce the initial adhesion of bacteria on the surface.[16]

The inhibition process of *A. actinomycetemcomitans* bacteria biofilm is not only related to the genes that synthesize polysaccharide intercellular adhesion (PIA) but also to the Quorum Sensing (QS) system, which also affects the inhibition of biofilm formation. The bacterium can recognize the presence of the other bacteria in its environment by detecting the autoinducer. Autoinducers, which are mainly in gram-negative bacteria, are AHLs, which have an important role in biofilm formation. [3,17]

The statistical test of this study was to determine the differences between the treatment groups using the LSD post-hoc analysis test. There is no significant difference between the positive control group (calcium hydroxide + sterilized aquadest) and group I (combination of calcium hydroxide-propolis 1:1). There is no significant difference in the value of $p = 0.736$ ($p > 0.05$), which means that the biofilm inhibition in the positive control group is the same as group I. This happened because the active content of the propolis in group I is low, or it can be said that the active ingredient has not been effective in inhibiting the A. actinomycetemcomitans bacterial biofilm, so the inhibitory power obtained is 42%, which is not that much different from 39% for the positive control group (calcium hydroxide + sterilized aquadest).

In addition, in group II and group III there were no significant differences with the value of $p = 0.911$ ($p > 0.05$), which means that the biofilm inhibition that occurs in group II is almost the same as group III. This happened because in group III, the mixture produced was more diluted than in groups I and II. According to Syamsuni (2006)[18], one of the factors that influence the manufacturing process of a suspension preparation is the thickness of a liquid (viscosity), which also influences the velocity of the liquid flow; the thicker the liquid, the lower the flow velocity or the smaller density. The flow velocity of the liquid will also affect the downward movement of the particles contained in it. Thus, by increasing the viscosity solution or the viscosity of the liquid, the movement of the particles they contain will be slowed down. In addition, the number of particles is also a factor regarding why there are no significant differences. If there is a large number of particles in a room, it will be difficult for the particles to move freely because there are frequent collisions between these particles. [18] The movement of ionization between the particles is too wide (free), so the effectiveness of the mixture is also low. Therefore, group III has a smaller inhibition average than group II.

Group II has a greater inhibition because at a ratio of 1:1.5, the suspension formed does not tend to be runny/thick, and the active can be sufficiently ionized so that the effectiveness is higher than group I and group III. In addition, group II is the saturation/critical point of a concentration to be able to inhibit maximum biofilm formation. At certain concentrations, even though concentration is increased, surface tension will be constant by aggregating to form micelles and concentrations, which is called Critical Micelle Concentration (CMC). CMC is related to the minimum value of surface tension. If CMC is reached, the surface tension will decrease. After CMC is reached, the surface tension will be constant.[19,20] The constant surface tension causes no significant differences between group II and group III. This is in accordance with the results obtained where in group II the mean of biofilm inhibition is 76%, while in group III the mean inhibition is 75%. Therefore, it can be concluded that group II (calcium hydroxide-propolis 1:1.5) is the most effective ratio for inhibiting the biofilm of *Aggregatibacter actinomycetemcomitans* bacteria.

The effectiveness of calcium hydroxide in inhibiting the biofilm of *Aggregatibacter actinomycetemcomitans* bacteria is influenced by the active content in calcium salt compounds, which is the result of mixing calcium hydroxide with propolis extract. This compound is formed because of an acid-base reaction between caffeic propolis acid and calcium hydroxide. This calcium salt compound consists of hydroxyl groups and the active compounds that is derived from the extracts of propolis, such as flavonoids (apigenin), tannins, and terpenoids (tt-farnesol). These compounds are thought to be able to inhibit biofilms from the *Aggregatibacter actinomycetemcomitans* bacteria.

Inside the cytoplasmic membrane, the mechanism of the hydroxyl group as an antibacterial occurs because it is the location of enzymatic substance, both extracellular and intracellular. In addition, the pH gradient of the cytoplasmic membrane changes to alkaline (pH 12.5) because of the presence of high hydroxyl ions that affect membrane proteins (protein denaturation), and it also changes the integrity of the cytoplasmic membrane while ultimately damaging the bacterial DNA. This happens due to acidic bacterial DNA turning alkaline because the surrounding environment is alkaline.[21]

Flavonoids are polyphenolic compounds that have three mechanisms. The first relates to the inhibition of nucleic acid synthesis. The second involves the destruction of cell membranes through perforation mechanisms and the perforation mechanisms decrease membrane fluidity. The third inhibits the energy metabolism of bacteria. [22.23] The results of this study are supported by tests of propolis originating from China and Brazil, that the test has shown that propolis is effective A. *actinomycetemcomitans* bacteria. [24] Apigenin (4 ‘, 5, 7-trihydroxyflavone) is a compound of flavones derived from flavonoids that can inhibit the growth of biofilms from bacteria. Apigenin with tt-farnesol will cause a decrease in the number of polysaccharides in microorganism biofilms. Apigenin inhibits the activity of glucosyltransferase and affects the accumulation of S. mutans biofilms by inhibiting the formation of insoluble glucan and increasing the soluble glucan content of the polysaccharide matrix. [25.26]

A tannin is a plant-active compound of the phenol group that has the ability to inactivate the bacterial and enzyme adhesive. Tannins and flavonoids can play a role in inhibiting biofilm formation by inhibiting the expression of icaA and icaD genes. The icaA and icaD genes can regulate the formation of Polysaccharide Intercellular Adhesin (PIA) through the activation of the σ^B factor, which can activate the omega protomer. [27] In addition, tannins and flavonoids play a role in inhibiting biofilm formation by reducing the hydrophobic nature of bacteria, which plays an important role in the process of bacterial adhesion to the substrate. [28]

Additional research was carried out by looking at the morphological form of the *Aggregatibacter actinomycetemcomitans* bacteria using a scanning electron microscope with the British brand Carl Zeiss (Evo MA 10) to see the biofilms form of *Aggregatibacter actinomycetemcomitans* bacteria. The magnifications that have been used are 500, 1000, 4000, and 10,000x. The results of the *Aggregatibacter actinomycetemcomitans* biofilm images studied were not optimal when compared with the existing studies. This might happen because the *Aggregatibacter actinomycetemcomitans* bacteria used for the biofilms is pasase 4, which means that the characteristics were different from the original bacterial stock of *Aggregatibacter actinomycetemcomitans*.

Conclusion

There was no difference between the calcium hydroxide-propolis combination with a ratio of 1:1.5 and 1:2, but ratios 1:1.5 and 1:2 were more effective than 1:1 in inhibiting *Aggregatibacter actinomycetemcomitans* bacteria biofilms.

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