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The Effectiveness of Propolis Extract against Extracellular Polymeric Substance (EPS) Biofilm Enterococcus Faecalis Bacteria

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Abstract

The resistance of Enterococcus faecalis bacteria in root canals is because it can form a biofilm. Biofilms are communities of microorganisms in a complex and dynamic structure attached to a surface, embedded in Extracellular Polymeric Substance (EPS). Propolis extract contains of flavonoids that can inhibit biofilm growth. To analyze the effectiveness of propolis extracts against Enterococcus faecalis biofilm EPS in vitro.

Enterococcus faecalis biofilm grown on media Tryptic Soy Broth (TSB), dextran conjugate alexa fluor 647 reagent was added, then incubated 24 hours. Enterococcus faecalis biofilm were divided into 4 groups: 3 groups treated by soaking propolis extracts 24 hours with each concentration of 02, 0.8% and 1.2%; 1 control group without extract of propolis. Biofilm samples examined using Confocal Laser Scanning Microscope (CLSM). Yield data was then analyzed using One Way ANOVA and Tukey HSD Test ($p < 0.05$).

The treatment group showed a decrease in the average volume of biofilm EPS than the control group.

Propolis extracts with concentration of 0.2% 0.8% and 1.2%, effectively reducing the Enterococcus faecalis biofilm EPS.

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Introduction

Endodontic treatment is root canal treatment to keep the teeth functioning in the dental arch[1]. Treatment is performed on teeth with irreversible pulpitis, and necrosis accompanied by periapical abscesses due to invading bacteria to the root canal and extending to the periapical tissue. In necrotized root canals there are many bacteria that have potential to spread infection to the surrounding tissue[2].

The objective of endodontic treatment is to remove microorganisms within root canals[3]. The root canal walls which are not completely clean during biomechanical preparation could be a place of bacteria, increase the apical gap, and reduce the adhesion of root canal filling materials. Debris left in the root canal can reduce the adaptation of the fill material to the root canal wall. Poor adaptation of fillers can increase the opportunity of maintenance failure[4].

The most common bacteria found in root canal necrosis are facultative anaerobes and obligate anaerobes[5]. Specimen isolation of bacteria taken from tooth necrosis with periapical pathosis showed resistance to facultative anaerobic gram-positive bacteria *Enterococcus faecalis* in root canals with infection prevalence around 24% -77%[6]. This is due to various defense and virulence factors of *E. faecalis*, including its ability to compete with other microorganisms in their invasion of dentinal

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tubules and to survive in a low nutrient state by utilizing nitrate (NO) as an alternative electron acceptor media. In infected root canals, NO is a virulent factor resulting from polymicrobial metabolism that infects the root canal[7].

Necrotic pulp tissue becomes a favorable environment for multiplication of microorganism because of the presence of organic residues that serve as a nutrient substrate for bacteria[8]. *E. faecalis* bacteria can produce polysaccharides that act as a barrier between the cell wall and the environment, intermediary of host-pathogen interactions, and forming biofilm structures[9]. Biofilm formation is one of the advantages of *E. faecalis* bacteria to increase virulence against the host defense system[2].

Biofilm is a complex and dynamic structured microorganism community attached to a solid surface, embedded in Extracellular Polymeric Substance (EPS) whose main component contains polysaccharide compounds[7]. Polysaccharides not only function to adhere to a surface, but also bind nutritious substances that surround biofilm surface. EPS is the outermost barrier that can increase virulence and protect biofilms to be resistant to antibiofilms. The matrix formed from EPS will protect bacterial cells and facilitate communication between cells through the exchange of biochemical compounds. Virulence of the *Enterococcus faecalis* biofilm is disrupted if it damages the EPS matrix, so that the biofilm resistance is reduced[10].

The main principle of root canal cleaning is that the tool must reach the entire root canal wall and remove debris released by the irrigation solution which functions as a disinfectant and pulp tissue solvent[11]. The root canal irrigation agent should have antibacterial properties to damage, inhibit the reproduction or metabolism of microorganisms, and make the root canal sterile[12]. (Gomes, 2007). Some natural ingredients are known to have antibacterial power so that natural irrigation materials can be used as an alternative to avoid the cytotoxic effects of chemical irrigation materials. One of the ingredients that can be used as an alternative to natural irrigate on materials is propolis[13]. Propolis was chosen because research in the health sector both in vitro and in vivo shows that propolis has anti-oxidant, anti-bacterial, anti-fungal, and anti-inflammatory properties[14] as well as good biocompatibility to Human Periodontal Ligament Fibroblast Cell[13].

Propolis is a product of honey-bees, contains resin and beeswax, is sticky and is collected from plant sources, especially from flowers and leaves tip[15]. The honey-bee species that actively produce propolis are *Apis mellifera* and *Trigona sp*. The compound, color, and aroma of propolis vary greatly depending on the environment, soil conditions, and the season of propolis plants[16]. In this study, the propolis used came from *Apis melifera* bees located in Lawang, East Java.

The anti-bacterial activity possessed by Indonesian propolis extract is influenced by the presence of active compounds in the extract, namely flavonoids (tt-farnesol and apigenin), polyphenols, galangin, quercetin, myrecetine, robinetin, licochalcones AB, caffeic acid, tannins, and essential oils[17]. Each active ingredient has its own mechanism of activity as an antibacterial. Propolis has tt-farnesol and apigenin which can inhibit biofilm growth, because it can reduce the number of polysaccharides in a biofilm and adhesion of bacteria[10]. Based on this description, it is necessary to conduct research to determine the effectiveness of propolis extract against the EPS biofilm of the bacteria *Enterococcus faecalis*.

Materials and methods

This study was divided into 4 groups, which are 1 control group and 3 treatment groups. *Enterococcus faecalis* bacteria were cultivated in TSB media for 24 hours, and then diluted to 1: 100. Bacteria were put on a disc plate with 100 ml Trypticase Soy Broth (TSB) medium supplemented with 1% glucose and stain dextran alexa fluor 647 at 37 ° C in 10% CO_2 to grow *E. faecalis* biofilm incubated overnight. Then 0.1 ml of the biofilm was put into a sterile 24-well flat-bottomed plastic tissue culture plate as a positive control. The 100 µl propolis extract was dissolved in a 24-well microtiter flat-bottomed plastic with a concentration of 0.2% 0.8% and 1.2%, which was put into each microplate that had been labeled with a name. Then incubation at 37° C for 24 hours.

Rinsing the microtiter plate with Phosphate Buffer Saline (PBS) four times, and then drying it. Biofilm samples on the cover slip were fixed using aquadest. Measurement of EPS biofilm with a 40x magnification Confocal Laser Scanning Microscope (CLSM). After the results of the research data were obtained, the

Kolmogorov-Smirnov normality test was then carried out to determine the distribution of the population data for each group. After knowing that the data is normally distributed, the Levene Test homogeneity test is then carried out to determine the similarity of the sample group variations. Furthermore, to determine the differences between groups using Tukey HSD test.

Results

Based on the results of this study on the effectiveness of propolis extract against the EPS biofilm *Enterococcus faecalis*. This study used 3 treatment groups with a concentration of 0.2% 0.8% 1.2% and 1 control group, each with 8 samples. Then the mean EPS of each group is obtained as listed in table 1.

Treatments	X ± SD
Control	942.8465[a] ± 397.53361
Concentration 0,2%	446.4727[b] ± 240.38528
Concentration 0,8%	504.0349[b] ± 289.68023
Concentration 1,2%	678.8631[b] ± 235.72577

Table 1. The average of EPS biofilm *Enterococcus faecalis*.
The different superscript on the same column indicates significantly different.

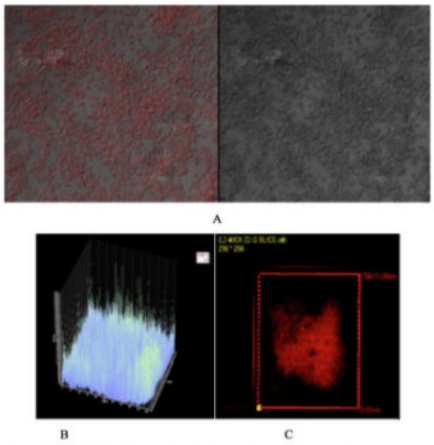

Figure 1. (A) the luminescents colored red is EPS on biofilm (B) average of EPS biofilm of *E. faecalis* (C) surface area of EPS biofilm of *E. faecalis*.

The interpretation of CLSM results in the control and treatment groups can be seen with a red glow (Figure A) which shows EPS formed on the biofilm of *Enterococcus faecalis*. Figure B shows the mean volume of EPS, while image C shows the surface area of the biofilm on a microtiter plate.

In this study, the distribution of data was carried out using the Kolmogorov-Smirnov test. Statistical calculations indicates control group p value = 0.730 (p> 0.05) meaning normal distribution, and from statistical calculations the results in the treatment group concentration of 0.2% p value = 0.750 (p>0.05) meaning normal distribution, the concentration of 0.8% p value = 0.556 (p> 0.05) was normally distributed. Likewise, in the treatment group the concentration of 1.2% that obtains p value = 0.721 (p> 0.05) which means that the distribution is normal.

The homogeneity test was carried out using the Levene test, the results obtained were p = 0.501 (p> 0.05) indicating that the sample used was homogeneous. To determine the significant difference between the control group and the propolis extract with a concentration of 0.2% 0.8% and 1.2%, the One Way Anova test was carried out. It was found that there was a significant difference between the control group and the three treatment groups p = 0.004 (p <0, 05), while there was no significant difference between treatment groups p = 0.317 (p> 0.05). This indicates that the propolis extract is effective in reducing EPS biofilm, but the difference in concentration is not remarkably significant. To determine the significance of the differences between groups, the Tukey HSD test was carried out (p = 0.05)

Based on the results of the Tukey HSD test, it was found that there was a significant difference between the control group and the treatment group, the concentration of 0.2% p = 0.004 (p <0.05), where the value of the control group was greater than the 0.2% group, which means that the extract propolis 0.2% was effective in reducing the mean volume of EPS biofilm E faecalis.

There was a significant difference between the control group and the treatment group with a concentration of 0.8% p = 0.011 (p <0.05), where the value of the control group was greater than the 0.8% group, which means that 0.8% propolis extract was effective in reducing

the average volume of EPS biofilm *E. faecalis*.

There was a significant difference between the control group and the treatment group, the concentration of 1.2% p = 0.043 (p <0.05), where the value of the control group was greater than the 1.2% group, which means that 1.2% propolis extract was effective in reducing the mean volume of EPS biofilm *E. faecalis*.

In all three treatment groups, there was no significant difference in the Tukey HSD statistical test, because the p value was > 0.05. The treatment group of 0.2% compared to 0.8% had a value of p = 0.973, while the group of 1.2% compared to the 0.8% group had a value of p = 0.562, and the 0.2% compared to 1.2% had a value of p = 0.317. This shows that the three concentrations are equally effective in inhibiting the formation of *E. faecalis* biofilm. However, in the terms of CLSM interpretation in the three treatment groups, the EPS biofilm content was different. The propolis extract treatment group had 0.2% lower mean volume of EPS biofilm than the concentrations of 0.8% and 1.2%.

Discussions

This study was conducted to determine the effectiveness of propolis extract against the Extracelullar Polymeric Substance (EPS) biofilm *Enterococcus faecalis*. Biofilms are formed because microorganisms cannot survive in a low nutrient environment, so bacteria become more resistant to unfavorable environments than live planktonically. Biofilms are nutrient traps for the growth of microorganism populations and help the attachment of bacteria to the surface by producing Extracellular Polymeric Substance (EPS) molecular chains, whose main component contains 85% polysaccharide compounds. Polysaccharides are not only useful for attaching to a surface, but also binding and concentrating food substances contained in the water surrounding the surface of the biofilm. Polysaccharides also protect bacterial cells from toxins that can damage biofilms[18].

This study used gram-positive anaerobic facultative bacteria *Enterococcus faecalis* because of its role in causing persistent periradicular lesions after root canal treatment[19]. *E. faecalis* bacteria can survive in unfavorable conditions, such as instrumented and obturated root canals with little available nutrition, because the growth of these bacteria is through the formation of biofilms with one or more communities of microorganisms. The formation of biofilms in this study was carried out in vitro with a single species, so that the addition of 1% glucose is required which functions as additional adheses, and the biofilms can grow rapidly[20].

Based on the results of previous research, the propolis extract contained antibacterial compounds, including flavonoids or tt-farnesol, tannins, and apigenin[15]. In a study by Koo et al., 2005, found that the content of propolis, named apigenin (4,5,7-trihydroxyl flavone) and tt-farnesol (3,7,11-trimethyl-2,6,10- dodecatrien-1-ol), can make a decrease in the number of polysaccharides in microorganisms.

Biofilm studies often encounter difficulties when carrying out microscopic analysis, due to the interference of the fluorescence signal from the staining used and the density of the biofilm layer. So the Confocal Laser Scanning Microscope (CLSM) was chosen for reading *E.faecalis* biofilms, because of its high accuracy for seeing the number of bacteria, components, surface area, and thickness of the biofilm[21]. The staining used alexa fluor 647 dextran conjugate to observe polysaccharides on the EPS biofilm. The luminescence produced by red polysaccharides indicates that the biofilm has a high concentration of polysaccharides. Dextran alexa fluor 647 was chosen for this study because this staining can bind to EPS biofilms that emit red fluorescence under excitation and wavelengths emission for 460 nm and 650 nm[22].

This study used propolis extract with three different concentrations, 0.2% 0.8% and 1.2%. This concentration was obtained from the serial dilution thinning method to determine the minimum inhibitory concentration of propolis extract against *E. faecalis* biofilm. Initial serial dilution obtained a concentration of 0.2% 0.4% 0.8% 1% and 1.2%. Then the three best concentrations were taken through preliminary research to examine the minimum inhibitory concentration of propolis extract against *E. faecalis* biofilm.

From the results of this research, the EPS biofilm in the control group obtained 942.8465 arb. unit height if compared to the treatment group. In the control group, biofilms were grown only without additional propolis extract. This indicates that the polysaccharide content in the EPS biofilm *Enterococcus faecalis* is high. The high polysaccharides contained in the EPS

biofilm causes the virulence of the *Enterococcus faecalis* bacteria to increase on a surface[7], while the results of CLSM interpretation in each treatment group contained different EPS.

In this study, there was a significant difference in the average of EPS between the control group and the treatment group. This is in accordance with the theory which states that a material that has antibiofilm activity can damage in various ways, including damaging the biofilm transmembrane protein so that the bacterial cellular release occurs in the *Enterococcus faecalis* biofilm. Cell release occasion is due to the increase of fluid pressure or degeneration of endogenous enzymes[18].

The main composition of propolis, tt-farnesol and apigenin can inhibit the formation of *Enterococcus faecalis* biofilms by inhibiting the synthesis of the enzyme glucosyltransferases (GTFs) which will inhibit the synthesis of alkali-soluble glucans on biofilms, so that the concentration of polysaccharides decreases. This will disturb the permeability and balance of EPS. The damaged EPS causes decreasing virulence and reduced source of biofilm nutrition for growth. In this study, the damage to EPS was indicated by a decrease in the average EPS biofilm *Enterococcus faecalis* after administration of propolis extract.

In the treatment group with a concentration of 0.2% 0.8% and 1.2%, there was no significant difference in the average of EPS biofilm *Enterococcus faecalis*. Possible this is due to the presence of CMC (critical micelle concentration). CMC is an extract concentration that has reached a critical or saturated point so that the effectiveness of an extract or material used cannot be achieved because of the liquid surface tension that is too high. However, it can be seen that the results of the CLSM readings show that the propolis extract concentration of 0.2% was more effective at inhibiting biofilm formation than the concentrations of 0.8% and 1.2%.

Conclusions

Based on the results of this research, it can be concluded that the propolis extract with a concentration of 0.2% 0.8% and 1.2% is effective in reducing the Extracelullar Polymeric Substance (EPS) biofilm *Enterococcus faecalis.*

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Declaration of Interest

The authors report no conflict of interest.

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