ANTIBACTERIAL EFFECT OF NANNOCHLOROPSIS OCULATA AS ROOT CANAL STERILIZATION MATERIAL ON STREPTOCOCCUS MUTANS BIOFILM

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ABSTRACT

Background: *Streptococcus mutans in the most frequent microbiota that causes pulp necrosis because of caries. The microorganism that is colonized and embedded in the biofilm matrix is resistant to antimicrobials compared to planktonic cells. Root canal sterilization materials must have good biocompatibility with tissues. Nannochloropsis oculata is an algae that contains various compounds such as terpenoids, alkaloids, and flavonoids that have potential as antibacterial and antioxidant and can be used as alternative to root canal sterilization.*
Method: *This research was true experimental laboratory research with post-test only control group design. The antibacterial potential of Nannochloropsis oculata was tested using the biofilm method, divided into 5 groups. The control group was: K- (aquadest), K+ (calcium hydroxide), and the treatment group was given Nannochloropsis oculata: P1 (0.625%), P2 (1.25%), and P3 (2.5 %). Congo Red method test was to determine the formation of biofilm that shows black strains on agar. While biofilm test with Microtiter Plate Assay to measure the value of biofilm that were inhibited in Optical Density (OD) value in the ELISA Reader. The lower the value, the more biofilm inhibited, with OD value, inhibition percentage could counted*
Result: *The result of all treatment groups were increasing in percentage inhibition value shows inhibition in biofilm growth ($p < 0.05$).*
Conclusion: *Nannochloropsis oculata had an antibacterial effect on the biofilm of Streptococcus mutans*

BACKGROUND

Pulp necrosis requires root canal treatment, which is dental treatment by removing the entire pulp tissue, both in the pulp chamber and root canal[1]. Root canal treatment can be divided into three stages, biomechanical preparation of root canals, disinfection and obturation.

The most frequent microbiota that causes pulp necrosis because of caries encountered is *Streptococcus mutans (S. Mutans)*. This microorganism produces organic acids, especially lactic acid by fermenting carbohydrates on the surface of the teeth and make a decrease in salivary pH below 5.5 which will result demineralization of the tooth surface and dental caries will formed[2]. Biofilm are colonies of microorganisms that consist of cells from various kinds of bacteria that attach to one another on the surface of the teeth in irreversible form[3]. The formation of biofilm is a defense mechanism for bacteria to stay alive in environmental conditions in the oral cavity and is not easily attacked by the immune response of the host or drugs[4]. This formation process occurs through several phases, which are the formation of

pellicles on the surface of the tooth, the beginning of attachment of bacteria, bacterial colonization, maturation, and release of biofilm cells[5]. The formation of biofilm can be influenced by several things such as pH levels, nutrients, and host defense[6].

Calcium hydroxide is the first choice intracanal medicament and is commercially available as a paste or pure powder mixed with water or saline to make a paste of the desired consistency. The characteristics of calcium hydroxide are having a high pH (12) by working synergistically with sodium hypochlorite to degrade pulp tissue, broad-spectrum antimicrobial agents, prolonged anti-microbial effect to control the serous exudate. Disadvantages of calcium hydroxide is it can weaken dentin if left in the root canal for a long period of time[7]. The highest antimicrobial effect of calcium hydroxide and the lowest toxicity is at a concentration of 60%[8]. One of the requirements for materials used in dentistry should be non-toxic, not irritating and must have biocompatibility properties or the material produced must not have a detrimental effect on the biological environment both locally and systemically. Ideally, a root canal sterilization material must have good biocompatibility with the tissue. This biocompatibility includes the degree of cytotoxicity, mutagenicity, and carcinogenicity in addition to having antimicrobial properties[9].

Algae is rich in fatty acid-derived compounds, oxylipin, which functions as an antibacterial for pathogenic bacteria[10]. Oxylipin is a secondary metabolite product that has antibacterial properties, besides that, marine algae are also rich in flavonoid compounds which have antimicrobial benefits by damaging bacterial cell walls[11,12]. *Nannochloropsis oculata* microalgae is an algae that contains various compounds such as terpenoids, alkaloids, and flavonoids that have potential as antibacterial and antioxidant[13]. Previous studies conducted by Revianti and Parisihni, showed that the extract of *Nannochloropsis oculata* had a toxicity effect at concentrations above 2.5% and did not show a toxicity effect at concentrations below 2.5% [14].

Based on this data, the researcher wants to develop a research on the antibacterial power of green microalgae *Nannochloropsis oculata* on the biofilm of the bacterium *Streptococcus mutans* which is included in the bacteria that causes dental caries disease which can cause pulp death.

MATERIAL AND METHOD

This research was conducted based on approval from the Research Ethics Commission of the Faculty of Dentistry, Hang Tuah University with number EC/006/KEPK-FKGUHT/VII/2019. This type of research was a true experimental laboratory research. The groups were divided into 5, which were the negative control group (*S. mutans* 0.1mL + 0.1 mL distilled water (K-), the first positive control group (*S. mutans* 0.1mL + 60% calcium hydroxide (K +), the treatment group that was given *Nannochloropsis oculata* with a concentration of 0.625 % 0.1 mL (P1), the treatment group given *Nannochloropsis oculata* with a concentration of 1.25% 0.1 mL (P2), and the treatment group given *Nannochloropsis oculata* with a concentration of 2.5% 0.1 mL (P3).

The materials used in this research were suspension of *Streptococcus mutans*, BHIB, Congo red agar, green microalgae *Nannochloropsis oculata* with concentrations of 0.625%, 1.25%, and 2.5%, calcium hydroxide 60%, crystal violet 0.1%, Tween 80 concentration of 2%, and sterile distilled water.

Streptococcus mutans bacteria were cultured in liquid BHI media which had been

incubated for 2x24 hours in an anaerobic atmosphere. Then making suspension of *Streptococcus mutans* bacteria in the BHIB turbidity was synchronized with the 0.5 *Mc Farland* standard.

The process of making green microalgae *Nannochloropsis oculata* for a concentration of 2.5% was obtained by weighing the microalgae powder of *Nannochloropsis oculata* as much as 0.5 mg and then dissolving it in 20 mL of distilled water. A 1.25% concentration was obtained from 0.25 mg of *Nannochloropsis oculata* powder dissolved in 20 mL of distilled water. The concentration of 0.625% *Nannochloropsis oculata* was obtained from 0.125 mg of *Nannochloropsis oculata* powder dissolved in 20 mL of distilled water.

A qualitative test to detect biofilm formation was carried out using the Congo red agar method. *Streptococcus mutans* bacteria were rubbed on Congo red agar and incubated for 48 hours at 37^{o}C in an anaerobic atmosphere. Check the plate after 48 hours, if a black colony is formed then it showed a strain of bacteria that produces biofilm. However, if the colony was red, the strain does not produce biofilm.

Quantitative test for biofilm formation using the microtiter plate assay method. Suspension of *Streptococcus mutans* which was equalized with 0.5 Mc Farland solution was diluted to 1: 100 0.1 mL of *Streptococcus mutans* culture and then put into 96-well rounded bottom plastic tissue culture plates (microtiter plates) and incubated overnight at 37^{o}C. Green microalgae *Nannochloropsis oculata* with concentrations of 0.625%, 1.25%, and 2.5% were applied to each 96-well round-bottomed plastic tissue culture plate (microtiter plate) then incubated overnight at 37^{o}C. The contents of each 96-well round-bottomed plastic tissue culture plate (microtiter plate) were removed and washed 3 times with 0.2 mL phosphate-buffered saline using a micropipette. Biofilm microorganisms attached to the 96-well round-bottomed plastic tissue culture plate (microtiter plate) were painted with crystal violet and rinsed using distilled water and then dried. Quantitative analysis of biofilm formation was done by adding Tween 80, 2% concentration in every 96-well round-bottomed plastic tissue culture plate (microtiter plate). Optical density (OD) measurements were carried out using ELISA Reader with a wavelength of 570 nm. Low OD values indicate the presence of antibacterial power at each concentration of green microalgae *Nannochloropsis oculata.*

The percentage of inhibition was the inhibition of *Nannochloropsis oculata* to the growth of *Streptococcus mutans* bacteria calculated by the formula:

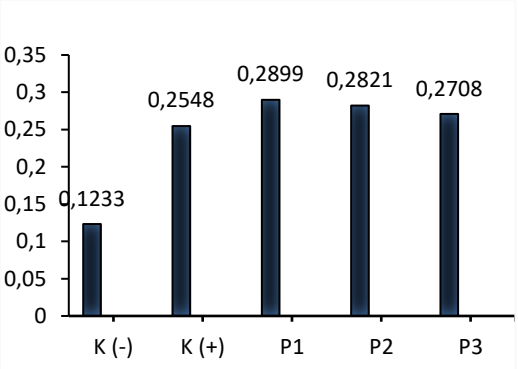
$$\%\text{Inhibition} = 1-(\text{OD K(-)} - \text{OD P}) / \text{OD K (-)} \times 100\%$$

RESULT

The research data were analyzed descriptively to obtain an overview of the distribution and summation of data in order to clarify the presentation of results. Table 1 showed the average percentage value of *Streptococcus mutans* biofilm inhibition.

	Mean	SD
K (-)	.1233	.00519
K (+)	.2548	.01976
P1	.2899	.05793
P2	.2821	.04188
P3	.2708	.02774

Table 1. The mean value of *Streptococcus DE*



Graphic 1. Graphic average percentage of *Streptococcus mutans* biofilm inhibition

Information: K(-) (Aquadest); K(+) (Kalsium Hidroksida 60%); P1 (*Nannochloropsis oculata* 0.625%); P2 (*Nannochloropsis oculata* 1.25%); P3 (*Nannochloropsis oculata* 2.5%).

Based on the average graph of the percentage of inhibition of *Streptococcus mutans* biofilm, the lowest percentage of aquades inhibition, and in the treatment group, the average percentage of inhibition in *Nannochloropsis oculata* was 0.625% (P1) highest compared to the other treatment groups. The presence of antibacterial power against the formation of *Streptococcus mutans* bacterial biofilm was characterized by an increase in the percentage inhibition value.

From the LSD test result, it was known that there were significant differences in the percentage value of biofilm inhibition in the negative control group to the positive control group and the treatment groups P1, P2 and P3. This was evidenced by the significance value of each group, which is p <0.05. There was no significant difference in the percentage value of biofilm in the group K (+) against P1, P2, P3 (p> 0.05).

Group	K (-)	K1 (+)	P1	P2	P3
K(-)		0.000*	0.018 *	0.003 *	0.000 *
K1(+)			0.134	0.239	0.486
P1				0.732	0.405
P2					0.620
P3					

Table 2. LSD Test Result

Information: *p<0,05

DISCUSSION

Streptococcus mutans is a gram-positive cariogenic bacterium, facultative anaerobic[15], non-motile, round in shape, arranged like a chain and does not form spores, grows optimally at temperatures around 18 ℃ - 40 ℃ diameter 0.7 - 09 µm[16]. In this research, bacterial *Streptococcus mutans* biofilm was checked quantitatively method with Congo red agar. The results showed that the black colonies that proved the strains of *Streptococcus mutans* had the ability to form biofilm.

This research used biofilm method because biofilm had a more complex structure than bacterial colonies that do not form biofilm. Biofilm was a collection of bacteria that attaches to a surface and is embedded in the polysaccharide extracellular matrix to protect bacteria from external influences so that they were not easily detached[17]. Extracellular Polymeric Substance (EPS) also caused antimicrobial resistance from biofilm by

inhibiting the transport of antibiotics through biofilm[18]. This formation process occured through several phases, which were the formation of pellicles, the beginning of bacterial attachment, bacterial colonization, maturation, and release of biofilm cells[5].

Nannochloropsis oculata was one of the green microalgae that had potential as an antibacterial and antioxidant[13]. *Nannochloropsis oculata* contains secondary metabolites which were active components that act as antibacterial. The secondary metabolite produced was generally used as an antibacterial[19]. Secondary metabolites in green microalgae *Nannochloropsis oculata* which were contained alkaloids, terpenoids, and flavonoids[20].

This research used *Nannochloropsis oculata* in whole form so that all antibacterial components are still present in the powder used. *Nannochloropsis oculata* powder had been tested quantitatively on alkaloid and flavonoid components performed by the AlCl3 Spectrophotometry method (λ = 415 nm) for determination of flavonoid levels and colorimetric methods, Bromocresol Green reagents (λ = 430 nm) for determination of alkaloid levels. The results obtained were 0.30% w/w flavonoids and 0.05% w/w alkaloids.

There were limitations in this research, *Nannochloropsis oculata* which was used was still in whole form. The antibacterial component in *Nannochloropsis oculata* was still mixed so that it cannot be ascertained that the specific antibacterial component plays a role in inhibiting the formation of biofilm. Although quantitative tests had been carried out, but only on the flavonoid and alkaloid components. There were other antibacterial components in *Nannochloropsis oculata* which played a role in inhibiting the formation of biofilm, but in this research quantitative testing could not be done. This research used *Nannochloropsis oculata* powder whose particles were still coarse, it was estimated that this affected the material in penetrating bacterial cell walls which caused the process of inhibiting the growth of *Streptococcus mutans* bacteria that had not worked optimally. That provided an opportunity for materials to be explored further.

This research was conducted to see the antibacterial power of *Nannochloropsis oculata* solution to the bacterial biofilm of *Streptococcus mutans*. The antibacterial power of *Nannochloropsis oculata* microalgae in inhibiting biofilm was seen from the difference in the average percentage of inhibition. The results showed that the positive control group with calcium hydroxide 60% had antibacterial power that had a significant effect, whereas in the treatment group P1 (concentration 0.625%) had the most influential antibacterial power compared to the treatment group with a concentration of 1.25% or 2.5 %.

In all treatment groups (*Nannochloropsis oculata* solution) 0.625% (P1), 1.25% (P2) and 2.5% (P3) there were increases in the percentage value of inhibition of the concentration of *Nannochloropsis oculata* solution which showed increases in antibacterial power in inhibiting the formation of *Streptococcus mutans* biofilm. *Nannochloropsis oculata* 0.625% (P1) solution group had the highest percentage of inhibition compared to other concentrations which meant that *Nannochloropsis oculata* with a concentration of 0.625% had been effective to inhibit the formation of bacterial biofilm. Based on previous research conducted by Revianti and Parisihni, it was shown that the extract of *Nannochloropsis oculata* was not toxic to fibroblast stem cells with concentrations below 2.5% and showed toxicity effects at concentrations above 2.5% [14].

In the negative control group (distilled water) the lowest percentage of inhibition was obtained compared to the positive control group and the treatment group. This showed that there is no inhibited *Streptococcus mutans* biofilm. Distilled water did not contain antibacterial compounds so it did not affect the growth of *Streptococcus mutans*.

The positive control group with calcium hydroxide had a more influential result in significantly inhibiting the formation of *Streptococcus mutans* biofilm. Calcium hydroxide is a material with a strong base with a pH of 12-13. Calcium hydroxide is the most common medicinal ingredient used today. This material was used as a medicinal material during endodontic therapy visits and has very good antibacterial properties. The antibacterial properties of calcium hydroxide are caused by the decomposition of Ca^{2+} and OH^- ions. The lethal effect of hydroxyl ions was that it can damage bacterial cytoplasmic membranes, denaturated proteins and damage bacterial DNA[21].

Calcium hydroxide and 2.5% *Nannochloropsis oculata* solution had an influential antibacterial power and could equally inhibit the formation of biofilm, but the results of research have *Nannochloropsis oculata* a higher percentage of inhibition. The content of alkaloids and flavonoids in *Nannochloropsis oculata* could inhibit DNA and cell walls in bacteria[22].

The negative control group (Aquadest) showed a significant difference with the positive control of calcium hydroxide and the treatment group of *Nannochloropsis oculata* solution 0.625% (P1), 1.25% (P2), and 2.5% (P3). In the positive control group calcium hydroxide and the treatment of *Nannochloropsis oculata* solution 0.625% (P1) did not show any difference in the value of OD biofilm with the treatment group solution of *Nannochloropsis oculata* 1.25% (P2) and *Nannochloropsis oculata* 2.5% (P3), as evidenced by the value of OD biofilm with the treatment group solution of *Nannochloropsis oculata* 1.25% (P2) and *Nannochloropsis oculata* 2.5% (P3). group significance was p> 0.05.

The difference in the concentration of *Nannochloropsis oculata*'s solution affected the amount of antibacterial content in the solution. In the solution of *Nannochloropsis oculata* the concentration of 0.625% (P1) and 1.25% (P2) might have almost the same antibacterial content so that it did not show significant differences to inhibit the formation of *Streptococcus mutans* biofilm. This showed that both had the same effectiveness in inhibiting the bacterium *Streptococcus mutans* biofilm.

CONCLUSION

Nannochloropsis oculata had an antibacterial power against the biofilm of the bacterium *Streptococcus mutans* with an effective concentration of 0.625% which had almost the same antibacterial power as calcium hydroxide.

CONFLICT OF INTEREST

There is no conflict of interest between authors

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