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RESEARCH ARTICLE

Antibiofilm activity of *Cyanobacteria spirulina* as an irrigation solution against *Enterococcus faecalis*

Diana Soesilo*✉, Fani Pangabdian*, Eriza Juniar**

*Department of Conservative Dentistry, Universitas Hang Tuah, Surabaya, East Java, Indonesia
**Department of Pediatric Dentistry, Universitas Hang Tuah, Surabaya, East Java, Indonesia
*Jl Arif Rahman Hakim 150, Surabaya, East Java, Indonesia; ✉ correspondence: diana.soesilo@hangtuah.ac.id

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ABSTRACT

Sodium hypochlorite (NaOCl) is currently the golden standard for root canal irrigation. NaOCl at a concentration of 5.25% to 6% can eliminate *E. faecalis*, but this concentration can increase the risk of toxic effects. *Cyanobacteria spirulina* is known to produce several secondary metabolites that have antimicrobial activity against gram-positive and gram-negative bacteria. The aim of this study was to determine the antibiofilm power of *Cyanobacteria spirulina* against the biofilms of *Enterococcus faecalis* at concentrations of 60 mg/ml, 70 mg/ml, 80 mg/ml, and 90 mg/ml. This research was a true experiment with a post-test only group design. The object of the research was divided into 6 groups. Group 1 was a negative control group, group 2 was a positive control group with 5% NaOCl, group 3, 4, 5, 6 were treatment groups given *Cyanobacteria spirulina* solution at a concentration of 60 mg/ml, 70 mg/ml, 80 mg/ml, and 90 mg/ml, respectively. Optical density (OD) of bacteria was bound by staining and analyzed by ELISA auto reader with a wave length of 595 nm (OD 595 nm). The results of the LSD test showed that the significance between K+ and P1, P2, P3 ($p < 0.005$), K+ and P4 ($p=0.129$), P1 and P2 ($p=0.449$), P3 and P4 ($p=0.178$). Significance of $p < 0.005$ showed a significant difference between the groups. The data were analyzed using the one-way ANOVA test followed by a double comparison test with the Least Significance Different (LSD) Post Hoc test method. *Cyanobacteria spirulina* solution at a concentration of 90 mg/ml had the greatest inhibitory effect on the biofilm formation of *Enterococcus faecalis*.

Keywords: biofilm; *Cyanobacteria spirulina*; *Enterococcus faecalis*; irrigation solution; sodium hypochlorite

INTRODUCTION

Pulp and periapical infection are one of the tooth diseases of which the main cause is combination of aerobic and anaerobic bacteria.[1] *Enterococcus faecalis* is a facultative anaerobic gram-positive bacterium that is often found in about 80-90% of teeth with failed root canal treatment.[2,3] *E. faecalis* is resistant to several antibiotics and has the ability to cause a single infection / mono-infection.[4,5]

Biofilm of the biofilm provides an optimal environment for the growth of microorganisms and facilitates the transmission of cellular genetic elements between bacteria. Biofilms play an important role in bacterial virulence because biofilms protect bacteria, especially uro-pathogens, against the antibacterial activity of antibiotics. This bacterial defense mechanism is formed due to changes in the characteristics of bacterial cells in environmental adaptation, such as inhibition of bacterial growth or in dormant metabolic conditions, which can increase resistance and tolerance to antibiotics.[6] Elimination of bacteria in root canals can be done by root canal treatment. Root canal irrigation is one of the stages that affects the success of bacterial elimination in root canal treatment.[7] Irrigation material is good if it is antimicrobial as well as has the ability to dissolve organic and inorganic tissues and has a low toxicity effect.[8]

Sodium hypochlorite (NaOCl) is currently the golden standard for root canal irrigation. NaOCl at concentrations of 5.25% to 6% can kill *E. faecalis*, but these concentrations can increase the risk of toxic effects.[7] The disadvantages of NaOCl are that it is only effective for cleaning organic debris components, is toxic to periapical tissue, can

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cause cell damage, degrades the micromechanical properties of dentin in root canal walls, has an unpleasant odor and taste, corrodes endodontic appliances, and cannot remove resistant bacteria such as *Actinomyces spp*.[9,10,11]

Cyanobacteria spirulina have shown effective antimicrobial properties. The antimicrobial activity found in *Cyanobacteria spirulina* extract was obtained from the content of linolenic acid.[12] Several preliminary studies on the algae *Cyanobacteria spirulina* have shown effective antimicrobial properties. Antimicrobial activity by *Spirulina* should be investigated in depth to develop antibiotics against multiple drug-resistant pathogens.[13]

In previous studies, *Cyanobacteria spirulina* extract can be used as an antibacterial agent against gram-positive bacteria, one of which is *Staphylococcus aureus*. Based on research conducted by El-Sheekh et al, pure antimicrobial substances produced by *Spirulina platensis* against *Staphylococcus aureus* showed the minimum inhibition concentration (MIC) of 65 mg/ml and the minimum bactericidal concentration (MBC) of 90 mg/ml.[12] Winahyu et al also conducted a study which showed that *Spirulina platensis* can inhibit the growth of *Staphylococcus aureus* with an inhibition zone diameter of 14.88 at a concentration of 25%, 10.39 mm at a concentration of 50%, 16.43 mm at a concentration of 75%, and 16.97 mm at a concentration of 100%. *Staphylococcus aureus* has similar characteristics to *Enterococcus faecalis*, namely facultative anaerobic gram-positive cocci bacteria and one of the microorganisms that play a role in infecting root canal treatment.[14] This study was conducted to determine the effective concentration of *Cyanobacteria spirulina* solution to eliminate *Enterococcus faecalis* biofilm compared to sodium hypoclorite 5.25%.

MATERIALS AND METHODS

This research was conducted based on approval from the Research Ethics Commission of Faculty of Dentistry, Hang Tuah University with number EC/021/KEPK-FKGUHT/VII/2022. This research was a true experiment. The groups were divided into 6, namely the negative control group (*E. faecalis* 0.1 mL + 0.1 mL distilled water (K-)), the first positive control group (*E. faecalis* 0.1 mL + NaOCl 5% (K+)), the treatment group that was given *Cyanobacteria spirulina* at a concentration of 60 mg/ml (P1), the treatment group given *Cyanobacteria spirulina* at a concentration of 70 mg/ml (P2), the treatment group given *Cyanobacteria spirulina* at a concentration of 80 mg/ml (P3), and the treatment group given *Cyanobacteria spirulina* at a concentration of 90 mg/ml (P4).

The materials used in this research were suspension of *Enterecoccus faecalis*; Blood agar; Congo red agar; green microalgae *Cyanobacteria spirulina* at concentrations of 60mg/ml, 70 mg/ml, 80 mg/ml, and 90 mg/ml; calcium hydroxide 60%; crystal violet 0.1%; Tween 80 at concentration of 2%; and sterile distilled water. *Cyanobacteria spirulina* powder was diluted to concentrations of 60 mg/ml, 70 mg/ml, 80 mg/ml, and 90 mg/ml with sterile distilled water. *Cyanobacteria* powder is an over-the-counter product with batch number 51120319.

Biofilm formation and antibiofilm performance assays were done using the congo red method agar and microtiter plate assays. *Enterococcus faecalis* were cultured on Tryptic soy broth (TSB) medium overnight then diluted up to 1:100 on TSBglu. Then 0.1 ml of bacteria *Enterococcus faecalis* that had been cultured on the TSB media at a concentration

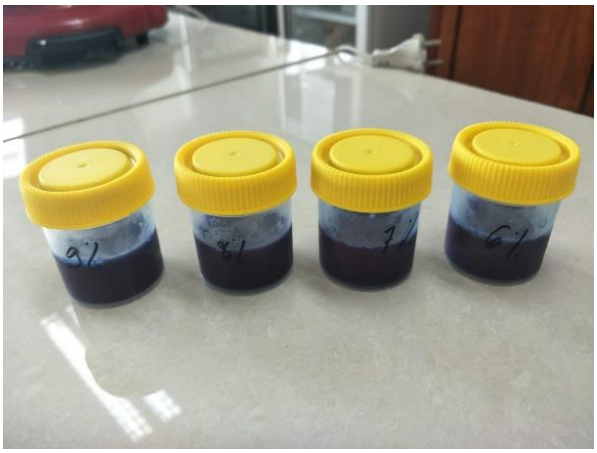

Figure 1. *Cyanobacteria spirulina* dissolution with distilled water in concentration of 60 mg/ml, 70 mg/ml, 80 mg/ml, and 90 mg/ml

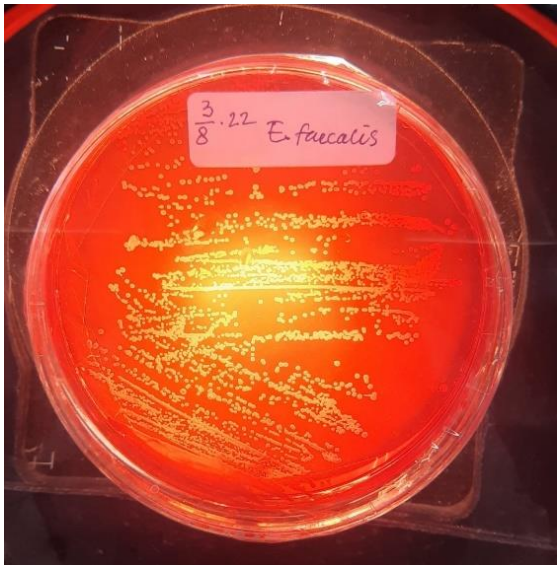

Figure 2. *Enterococcus faecalis* biofilm growth in Congo red agar

of 106 bacteria/ml was filled in a 96-well flat-bottom plastic tissue culture plate (microtiter plate). The *Enterococcus faecalis* bacteria medium was subcultured on blood agar and incubated for 24 hours at 37 °C. The formation of *E. faecalis* biofilm on Congo red media was tested using the streaking method. Observations were made by looking at the color changes of the growth of bacterial colonies on the surface of the Congo Red Agar. Black and brown colonies indicate that bacteria form biofilms, whereas reddish colonies indicate that biofilms are not formed.[15] *Enterococcus faecalis* biofilm examination procedure using Congo red agar and Microtiter Plate Assay (MPA) was the main standard used. Colonies of *E. Faecalis* were made into bacterial suspension according to the standard of 0.5 McFarland. In the case of intact biofilms, the biofilms were allowed to set for 24 hours at 37 °C. The wells were gently washed three times with 1 ml of PBS, to remove loosely adherent and planktonic cells.

Each of the wells in the 96 flat-bottom polystyrene microtiter plate was filled with 180 l of *Cyanobacteria spirulina* extract solution at concentrations of 60 mg/ml, 70 mg/ml, 80 mg/ml, and 90 mg/ml. Furthermore, 20 l of *E. faecalis* suspension was added into the wells, then the plate was closed and incubated at 37 °C for 24 hours. After that, the bacterial suspension in the wells was removed and washed with 300 l of phosphate buffered saline (pH 7.2) 3 times and dried in an inverted position. The biofilm formed by the bacteria would stick to the well wall, fixed with 150 l of methanol in each well and left for 20 minutes. The microtiter was dried by tapping and placed in an inverted position then stained with 0.1% crystal violet for 5 minutes; the excess dye was washed with deionized water and the plate was dried. Optical density (OD) of bacteria was bound by staining and differentiated by ELISA auto reader at a wavelength of 595 nm (OD 595 nm). Biofilm (+) in MPA shows optical density between 0.066-0.132 for medium scale and > 0.132 for strong scale, while biofilm (-) in PA shows optical density between 0.033 and < 0.066. The percentage of inhibition was the inhibition of *Cyanobacteria spirulina* against the growth of *Enterococcus faecalis* bacteria calculated by the formula:

%Inhibition = 1-(OD K(-) - OD P) / OD K (-) x 100%

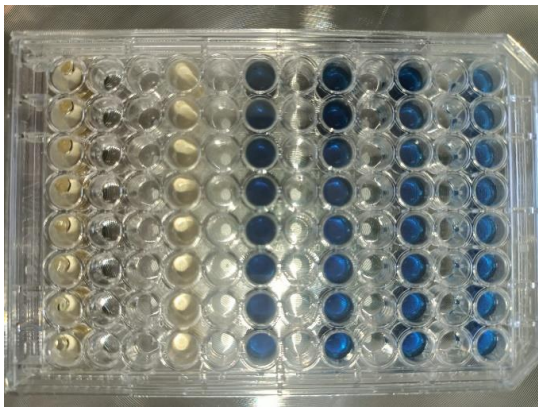

Figure 3. Microtiterplate assay with control groups K(-), K(+), P1, P2, P3, P4

The initial step of all the data obtained through the results of reading the ELISA Reader on the *Enterococcus faecalis* biofilm was carried out with a one-way analysis of variance (ANOVA) test followed by the LSD test. The homogeneity of all the samples was tested using the Levene's Test. For samples with 50 replications, the Shapiro-Wilk normality test was used.

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RESULTS

The purpose of this study was to determine the antibiofilm power of *Cyanobacteria spirulina* against *Enterococcus faecalis* biofilm as a root canal irrigation material. The research data were analyzed descriptively to obtain an overview of the distribution and summary of the data in order to clarify the presentation of the results.

The normality test used in this study was the Shapiro-Wilk test because this study used less than 50 samples. Data are normally distributed if the significance is $p > 0.05$, but if the significance is $p < 0.05$, then data are abnormally distributed. The results of the Shapiro-Wilk normality test showed that all the groups were normally distributed ($p > 0.05$), indicating that the percentage of biofilm inhibition was normally distributed. Normally distributed data have many values close to the average value and do not have extreme differences in values. A homogeneity test is one of the requirements before performing a parametric test. The homogeneity test aims to find out whether the variation of some data from the sample have the same variance. The homogeneity of the results of this study was tested using the Levene's Test.

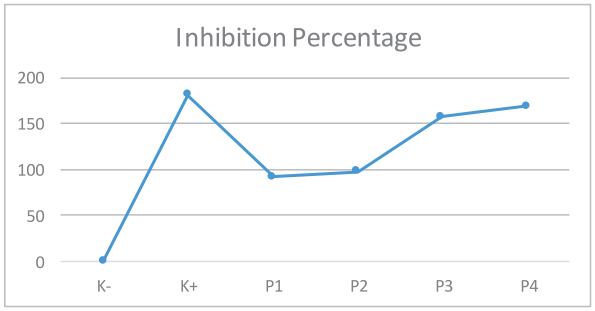

Figure 4. *Enterococcus faecalis* biofilm inhibition precentage

The data obtained from the Shapiro-Wilk test and Levene's test were normally distributed and homogeneous, followed by a parametric statistical test using the One-Way ANOVA test because this study included a comparative test with >2 independent samples. The ANOVA analysis was used to test whether the three samples had the same average. The Least Significance Different (LSD) test was conducted to find out which sample groups had significant differences in sealer radiopacity. The LSD test is a multiple comparison test to determine whether the mean values differ significantly in the number of analysis of variance. To test whether there was a difference in the average of the three sealers, the significance of the SPSS output should be greater or less than 0.05.

The results of the LSD test showed that the significance between K+ and P1, P2, P3 was < 0.005, meaning that there was a significant difference between the groups. Meanwhile, the significance between K+ and P4 was 0.129,

Table 1. Mean and standard deviation of the percentage of *Enterococcus faecalis* biofilm inhibition

	N	Minimum	Maximum	Mean	Std. Deviation
K(-)	5	.00	.00	.0000	.00000
K(+)	5	168.10	189.22	181.5680	8.49242
P1	5	69.23	106.56	91.6560	16.10929
P2	5	73.25	113.53	97.7760	15.99223
P3	5	151.92	161.27	157.9500	3.93273
P4	5	152.60	183.96	169.0180	13.49457
Valid N (listwise)	5				

Notes :
K(-) : Negative control group (Biofilm *E.faecalis* + aquadest 0.1 mL)
K(+) : Positive control group (Biofilm *E.faecalis* + NaOCl 5%)
P1 : Biofilm *E. faecalis* + *Cyanobacteria spirulina* solution 60 mg/ml
P2 : Biofilm *E. faecalis* + *Cyanobacteria spirulina* solution 70 mg/ml
P3 : Biofilm *E. faecalis* + *Cyanobacteria spirulina* solution 80 mg/ml
P4 : Biofilm *E. faecalis* + *Cyanobacteria spirulina* solution 90 mg/ml

Table 2. Post HOC LSD Test

	K+	P1	P2	P3	P4
K+		0.000*	0.000*	0.007*	0.129
P1			0.449	0.000*	0.000*
P2				0.000*	0.000*
P3					0.178
P4					

Notes : * Significant data

showing no significant difference. Between groups P1 and P2, the significance was 0.449, meaning there was no significant difference in the two groups. Meanwhile, the significance between the P3 and P4 groups was 0.178, indicating that there was no significant difference between the two groups.

DISCUSSION

Enterococcus faecalis is a versatile pathogen that plays a major role in the etiology of treated and untreated root canal infections and is strongly associated with treatment failure. One way to eliminate bacteria from the root canal is by irrigation. An irrigation material that has become the golden standard in root canal treatment is 5.25% sodium hypochlorite.[16] Sodium hypochlorite has some disadvantages, including inability to remove the smear layer.[17]

A biofilm can be defined as a microbial population containing organic and inorganic substrates covered by extracellular microbial products that form an intermicrobial matrix. Biofilm formation in root canals can occur at the first invasion of planktonic microorganisms. The most common microbial community found in root canals is the *Enterococcus faecalis* biofilm. Bacteria that have formed biofilms have higher resistance to antimicrobial agents and body defense mechanisms.[18] Biofilm formation in *Enterococcus faecalis* is regulated by a well-defined quorum sensing system, the fsr (faecal streptococcal regulator) locus. This locus consists of three genes, namely fsrA, fsrB, and fsrC, located adjacent to two genes encoding virulence factors: one encoding gelatinase (gelE) and the other serine protease (sprE). GelE expression is regulated by the fsr locus.[19]

This study compared 5% sodium hypochlorite as the positive control group and *Cyanobacteria spirulina* solution at concentrations of 60 mg/ml, 70 mg/ml, 80 mg/ml, and 90 mg/ml. The results showed that the *Cyanobacteria spirulina* solution at a concentration of 90 mg/ml had an antibiofilm ability that was almost equivalent to that of 5% sodium hypochlorite.

Spirulina is a blue-green microalgae classified as unicellular and spiral *Cyanobacteria*. *Spirulina* has high nutrition and is rich in vitamin B12. *S. platensis* biomass contains compounds needed by the human body, including 55-70% protein, 4-6% lipids, 17-25% carbohydrates, polyunsaturated fatty acids such as linoleic acid (LA) and gamma linolenic acid (GLA), some vitamins such as nicotinic acid, riboflavin (vitamin B2), thiamin (vitamin B1), cyanocobalamin (vitamin B12), minerals, amino acids, carotenoids, chlorophyll, and phycocyanins.[20]

Cyanobacteria are prokaryotic organisms that carry out oxygenic photosynthesis, belonging to the kingdom *Monera* and the division *Cyanophyta.*[21] *Cyanobacteria* are also known as blue-green algae due to the formation of pigments phycobilin and phycocyanin which at certain concentrations will produce a bluish color in this species. *Cyanobacteria* have three main classification systems: Geitler, Drouet, and Stanier. *Cyanobacteria* range in size from 1-10 µm.[22] *Cyanobacteria* cellular structure resembles plants, i.e., simple prokaryotes and performs photosynthesis, but *Cyanobacteria* do not have

a cell wall like plants where it resembles primitive bacteria. *Cyanobacteria* do not have membrane-bound nuclei or an internal membrane system. The cell membrane of *Cyanobacteria* resembles that of gram-negative bacteria, that is, it has an outer peptidoglycan layer, which is a polymer consisting of two sugar derivatives, namely Nacetylglucosamine (NAG), N-acetylmuramic acid (NAM), and several different amino acids. *Cyanobacteria* do not reproduce sexually, but asexually by cell division and plate formation, separating the two cells.[22] *Cyanobacteria spirulina* is a blue-green microalgae that is widely cultivated commercially. *Cyanobacteria spirulina* has high nutritional content such as protein, fatty acids, vitamins, and antioxidants, so it has the potential to be developed as natural feed ingredients and industrial raw materials used for manufacturing supplements, pharmaceuticals, and cosmetics.[23]

Cyanobacteria are known to produce several secondary metabolites that have antimicrobial activity against gram-positive and gram-negative bacteria.[24] The active antimicrobial secondary metabolites identified in *Cyanobacteria* include fatty acids, acrylic acid, halogenated aliphatic compounds, sulfur-containing heterocyclic compounds, terpenes, carbohydrates, alkaloids, flavonoids, pigments, phenols, saponins, steroids, tannins, and vitamins.[25] *Cyanobacteria* also contain many biologically active molecules that have been known to exhibit antioxidant activity, such as polyunsaturated fatty acids (PUFA), -carotene, pro-vitamins, and phenolic compounds that can act in combination and induce antimicrobial and cytotoxic activity.[26]

Cyanobacteria spirulina are known to produce intracellular and extracellular metabolites with diverse biological activities such as antibacterial, antifungal, cytotoxic, immunosuppressive, and antiviral activities.[12] *Cyanobacteria spirulina* has the potential as a strong natural antioxidant because it contains phenolic compounds, carotenoids, phycobiliproteins, and chlorophyll which are able to donate hydrogen atoms to free radicals. One of the bioactive compounds contained in *Spirulina plantesis* is beta carotene which is a carotenoid group.[23] *Cyanobacteria spirulina* is able to produce compounds that are beneficial to other living things, including proteins and other compounds for medicines. *Cyanobacteria spirulina* contains bioactive compounds as natural antibacterials.[14] The results of the qualitative analysis of the active components on the dry biomass of *S. platensis* in the study of Afriani, et al. showed positive results on flavonoid compounds, phenols, steroids, and saponins, but no alkaloid compounds were detected after reacting with Dragendorf, Meyer, and Wagner reagents. Alkaloids are compounds containing nitrogen atoms that are distributed in a limited manner in plants and are mostly found in higher plants, Angiosperms.[27]

Phytochemical substances that inhibit the growth of bacteria in algae are steroids. The mechanism of action of steroids in inhibiting microbes is by damaging the plasma membrane of microbial cells, causing leakage of the cytoplasm out of the cell which in turn causes cell death. This is suspectedly because steroid molecules have nonpolar (hydrophobic) and polar (hydrophilic) groups so they have a surfactant effect that can dissolve the phospholipid components of plasma membranes; phospholipids are the dominant components that make up the plasma membrane of microbial cells.[8]

Thus, the use of microalgae, as well as *Cyanobacteria,* is of increasing importance to humans due to their potential antimicrobial properties, among other things. The compounds associated with this bioactivity are usually produced in small quantities from microalgae because most of them are secondary metabolites. However, lipids are one of the main metabolites of microalgae and *Cyanobacteria*, which enrich their uses in the form of food, medicine, and fuel. *Cyanobacteria* and microalgae produce a wide range of lipids with antibiotic and antibiofilm activity against the most important pathogens which cause severe infections in humans.[28]

The limitation in this research is that *Cyanobacteria spirulina* liquid causes a lot of precipitate. It is necessary to conduct further research on *Cyanobacteria spirulina* as a root canal irrigation material. *Cyanobacteria* extract

should be made into nanoparticles and then tested using a Scanning Electron Microscope to see the cleaning power of *Cyanobacteria spirulina*. The antimicrobial substances of *Cyanobacteria spirulina* involved may target various kinds of micro-organisms, prokaryotes as well as eukaryotes. The properties of secondary metabolites in nature are not completely understood.[29]

CONCLUSION

Cyanobacteria spirulina solution at a concentration of 9% (90 mg/ml) had the greatest inhibition effect against *Enterococcus faecalis* biofilm formation and the inhibition effect was close to that of 5%sodium hypoclorite. *Cyanobacteria spirulina* extract can be used as an antibacterial agent against gram-positive bacteria. This research could support the development of dental materials from marine-based materials.

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CONFLICT OF INTEREST

There is no conflict of interest between authors.

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