

Research Report

Inhibition effect of cashew stem bark extract (*Anacardium Occidentale* L.) on biofilm formation of *Streptococcus sanguinis*

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ABSTRACT

Background: Biofilm is communities of microorganisms attached to solid surface and enclosed in extracellular matrix that protected microorganisms from antibacterial agents and host defense. One of bacteria might have a role in initial colonization of biofilm formation is *Streptococcus sanguinis* (*S. sanguinis*). Previous studies showed that cashew stem bark extract (*Anacardium occidentale* L.) can inhibit the growth of *Streptococcus* strains. **Purpose:** The purpose of this study was to determine the inhibition effect of cashew (*Anacardium occidentale* L.) stem bark ethanol extract on biofilm formation of *S. sanguinis*. **Methods:** *Streptococcus sanguinis* grown in Brain Heart Infusion (BHI) + 2% sucrose medium by using microplate polystyrene 96 wells. The samples were divided into 3 groups, 5% polyethyleneglycol (PEG) as negative control, cashew stem bark extract (concentration 3.125 mg/ml, 6.25 mg/ml, 9.375 mg/ml, and 12.5 mg/ml), and 0.12% chlorhexidine (as positive control). Biofilm was stained by 1% crystal violet. Afterwards, optical density (OD) of samples were measured by microplate reader λ 595 nm. The data of biofilm formation inhibition percentage were analyzed by one way ANOVA and then continued by Least Significant Difference (LSD) test. **Results:** The result of one way ANOVA showed that there were significant differences in inhibition of *S. sanguinis* biofilm formation ($p < 0.05$). LSD test showed that concentration extract 3.125 mg/ml had significant difference with concentration 9.375 mg/ml and 12.5 mg/ml. Reciprocally, concentration 6.25 mg/ml had significant difference with concentration 9.375 mg/ml and 12.5 mg/ml. **Conclusion:** Cashew stem bark extract was able to inhibit biofilm formation of *S. sanguinis*.

Key words: Inhibition of biofilm formation, *Streptococcus sanguinis*, cashew stem bark

ABSTRAK

Latar belakang: Biofilm merupakan sekumpulan mikroorganisme yang melekat pada permukaan solid dan diselubungi oleh matriks ekstraseluler yang melindungi mikroorganisme dari bahan-bahan antibakteri dan sel-sel pertahanan tubuh. Salah satu bakteri yang berperan pada awal pembentukan biofilm adalah *Streptococcus sanguinis* (*S. sanguinis*). Beberapa penelitian menunjukkan bahwa ekstrak kulit batang jambu mete (*Anacardium occidentale* L.) dapat menghambat pertumbuhan bakteri strain *Streptococcus*. **Tujuan:** Penelitian ini bertujuan untuk mengetahui pengaruh ekstrak etanol kulit batang jambu mete (*Anacardium occidentale* L.) terhadap pembentukan biofilm *S. sanguinis*. **Metode:** Media pertumbuhan *S. sanguinis* menggunakan Brain Heart Infusion (BHI) + 2% sukrosa yang ditumbuhkan pada microplate polystyrene 96 wells. Kelompok perlakuan dibagi menjadi tiga kelompok yaitu PEG 5% (kontrol negatif), ekstrak kulit batang jambu mete (konsentrasi 3,125 mg/ml, 6,25 mg/ml, 9,375 mg/ml, dan 12,5 mg/ml), dan klorheksidin 0,12% (kontrol positif). Biofilm yang terbentuk diwarnai dengan crystal violet 1%. Kemudian optical density (OD) sampel diukur menggunakan microplate reader λ 595 nm. Data berupa persentase penghambatan pembentukan biofilm dianalisis menggunakan uji one way ANOVA dan dilanjutkan dengan uji Least Significant Difference (LSD). **Hasil:** Uji one way ANOVA menunjukkan terdapat perbedaan daya hambat pembentukan biofilm *S. sanguinis* yang signifikan ($p < 0,05$). Hasil uji LSD menunjukkan konsentrasi 3,125 mg/ml memiliki perbedaan yang signifikan dengan konsentrasi 9,375 mg/ml dan konsentrasi 12,5 mg/ml. Begitu juga dengan konsentrasi 6,25 mg/ml

memiliki perbedaan yang signifikan dengan konsentrasi 9,375 mg/ml dan konsentrasi 12,5 mg/ml. **Kesimpulan:** Ekstrak kulit batang jambu mete dapat menghambat pembentukan biofilm *S. sanguinis*.

Kata kunci: Daya hambat pembentukan biofilm, *Streptococcus sanguinis*, kulit batang jambu mete

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INTRODUCTION

The bacteria can form dental plaque and cause periodontal disease.¹⁻² Since 1996, dental plaque is not only recognized as the etiologic factor of periodontal disease, but also considered as a biofilm.³ Biofilms are a community of bacteria that have extracellular matrix, circulation, and communication system. Biofilms grow in moist area and attach to solid surface such as tooth, dental restoration, prosthesis, and dental implant.²⁻³ The formation of biofilm begins with the attachment of bacteria such as *Neisseria* and *Streptococcus*, at over tooth surface which dominated by mitis groups such as *S. sanguinis*.⁴ The bacteria in biofilm will grow and become mature, then it form microcolonies.^{2,5}

An extracellular polymeric matrix is a thick layer surrounded the cells that form biofilm.⁴ This layer is a biofilm barrier against antibiotics, antimicrobials, and immunity cells. The main component is exopolysaccharides (EPS).⁶ Exopolysaccharides is mostly formed by bacteria that produced glucosyltransferase (GTF), such as *S. sanguinis*.⁶⁻⁷ This bacteria plays an important role in initial colonization of biofilm formation.⁶ *S. sanguinis* have some adhesin that bind to tooth surface that was layered by saliva. Beside that, IgA1 protease that is produced by colony of *S. sanguinis*, enable this bacteria grow and proliferate over tooth surface.⁴

The cashew excessively have been widely used such as from its wood, bark, leaf, fruit, and seed.⁸ Cashew was reported might have antidiabetic, antibacterial, and antiinflammation.⁹ Cashew's leaf could have inhibited bacterial growth such as *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, *Escherichia coli*, and *Candida albicans*.¹⁰ The stem bark was also used for medication. From previously report, the stem bark could inhibit *Staphylococcus aureus* growth.¹¹ The aim of the research was to determine the effect of cashew stem bark ethanol extract (*Anacardium occidentale* L.) on inhibition biofilm formation of *S. sanguinis*.

MATERIALS AND METHODS

Cashew stem bark (*Anacardium occidentale* L.) was taken from cashew trees at Kasiutri Plantation, Imogiri. Plant identification and extraction processed at Unit II laboratory of Pharmaceutical Biology, Faculty of

Pharmacy, Universitas Gadjah Mada. One kilogram of cashew stem bark was dried using a drying cabinet at 40-50°C for 48 hours. After drying, the bark was made into powder using machine pollinators. The 300g of powdered cashew stem bark was then extracted using 3L of 70% ethanol by maceration method then stirred for 30 minutes and allowed to stand for 24 hours. Powdered bark that was extracted then filtered using a Buchner funnel. Ethanol in filtrate was evaporated using stove for 3-4 hours until the stiff extract obtained.

The materials used for the inhibition of biofilm formation test were 2.5% polyethyleneglycol (PEG), 0.12% chlorhexidine (as positive control), BHI media containing 2% sucrose, *S. sanguinis* McFarland standard V (15×10^8), 1% crystal violet and 96% ethanol. Culture of *S. sanguinis* was from Balai Laboratorium Kesehatan (BLK), Yogyakarta.

Bacteria were prepared in McFarland V suspension (15×10^8 cfu/ml) using Densicheck. Plate was divided into two, one test plate and one blank plate. The test plate was containing extract solution/PEG and BHI + 2% sucrose with the addition of 10% v/v suspension of bacteria, while the blank plate containing extract solution/PEG and BHI + 2% sucrose with the addition of 10% v/v saline. According to Pereira et al.,¹⁴ cashew stem bark extract was starting inhibit *S. sanguinis* growth in concentration 3.12% (mg/ml), the antimicrobial activity was carried out on solid media plates by a diffusion method for the screening and determination of minimum inhibitory concentration (MIC) of the extract on bacterial. Whereas in this study, biofilm inhibition assay did with microdilution method. Extract concentrations was 3.125 mg/ml, 6.25 mg/ml, 9.375 mg/ml, and 12.5 mg/ml and BHI + 2% sucrose added to the polystyrene U bottom microplate with a total volume of each well 90 µL.

Chlorhexidine is the most effective antibacterial agent for oral use.⁴ As a positive control using 0.12% chlorhexidine (v/v) each well while the negative control using 5% PEG. Afterward, the microplate was incubated for 24 hours at 37°C. After the incubation, the microplate was washed with water three times. Then a solution of 1% crystal violet in 125 µL each well was added, and incubated at room temperature for 15 minutes, and then microplate was washed with water three times. Two hundred µL 96% ethanol added to each well using a 50-200 µL micropipette and incubated at room temperature for 15 minutes. Furthermore, as each 150 µL solution was transferred into a microplate flat bottom polystyrene 96 wells. OD readings

used Biorad Benchmark® microplate reader with 595 nm wavelength at the Laboratory of Parasitology Faculty of Medicine, Universitas Gadjah Mada.

Based on Quave *et al.*¹² research modified by Ardani *et al.*¹³ the percentage inhibition of biofilm formation was calculated using the formula:

$$\% \text{ Inhibition} = \left(1 - \left(\frac{OD_{\text{sample}} - OD_{\text{blank sample}}}{OD_{\text{vehicle}} - OD_{\text{blank vehicle}}} \right) \right) \times 100\%$$

OD sample: Optical density extract and bacterial suspension; OD blank sample: Optical density extract and saline; OD vehicle: Optical density PEG and bacterial suspension; OD blank vehicle: Optical density PEG and saline.

The data of biofilm inhibition percentage assay were tested for normality and homogeneity. To determine the mean difference between groups statistically analyzed using one way ANOVA test and then proceed using LSD test.

RESULTS

The inhibition of biofilm formation was done by calculating the percentage of inhibition of *S. sanguinis* biofilm formation after adding cashew stem bark extract. Biofilms will absorb color crystal violet. The percentage

Table 1. Mean and standard deviation of the inhibition of biofilm formation in 5% PEG, cashew stem bark extract (3.125 mg/ml, 6.25 mg/ml, 9.375 mg/ml and 12.5 mg/ml), and 0,12% chlorhexidine

Concentration	Mean (%) ± SD
5% PEG	-27.35 ± 1.11
Cashew stem bark extract 3.125 mg/ml	67.22 ± 4.80
Cashew stem bark extract 6.25 mg/ml	73.22 ± 1.45
Cashew stem bark extract 9.375 mg/ml	87.24 ± 9.51
Cashew stem bark extract 12.5 mg/ml	94.20 ± 5.71
0,12% Chlorhexidine	89.55 ± 4.85

SD: Standard deviation

of inhibition of biofilm formation values obtained with the four values substituted into the equation OD percentage inhibition. The results of the calculation of the percentage of inhibition of biofilm formation can be seen in Table 1.

Table 1 showed that 5% PEG did not inhibit biofilm formation because it had a negative mean. As the increasing concentrations of cashew stem bark extract, the inhibition of biofilm formation also increased. Cashew stem bark extract concentration 12.5 mg/ml was the highest average inhibition biofilm formation if compared with concentration 3.125 mg/ml, 6.25 mg/ml, 9.375 mg/ml and 0.12% chlorhexidine. To make clearing up the results in Table 1, the mean of each group would presented in Figure 1.

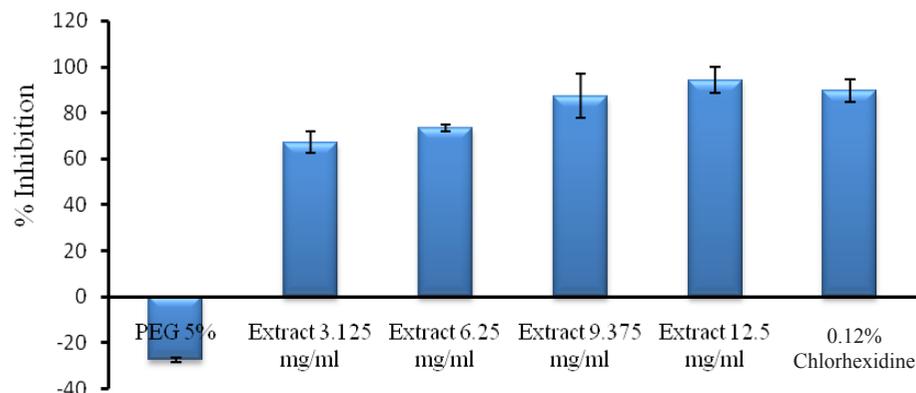


Figure 1. Mean of the inhibition of biofilm formation PEG 5%, cashew stem bark extract (3.125 mg/ml, 6.25 mg/ml, 9.375 mg/ml, and 12.5 mg/ml), and chlorhexidine 0.12%.

Table 2. LSD test results 5% PEG, cashew stem bark extract, and 0.12% chlorhexidine

Groups	Control (-)	3.125 mg/ml	6.125 mg/ml	9.375 mg/ml	12.5 mg/ml	0.12% Chlorhexidine
Control (-)	-	0.001*	0.001*	0.001*	0.001*	0.001*
3.125 mg/ml	-	-	0.131	0.001*	0.001*	0.001*
6.125 mg/ml	-	-	-	0.002*	0.001*	0.001*
9.375 mg/ml	-	-	-	-	0.084	0.552
12.5 mg/ml	-	-	-	-	-	0.236
0.12% Chlorhexidine	-	-	-	-	-	-

Control -: 5% PEG

* There were significant differences

Based on one way ANOVA result, the treatment between groups had significant difference ($p < 0.05$). Furthermore, to find a group that has a significant difference, the LSD test was using with a level of 95%. Least Significant Difference (LSD) analysis results can be seen in Table 2.

Based on LSD test, the inhibition of formation 5% PEG groups had a significant differences with the cashew stem bark extract concentration of 3.125 mg/ml, 6.25 mg/ml, 9.375 mg/ml, and 12.5 mg/ml. This was due to cashew stem bark extract had substances that act as an antibacterial, while PEG did not have antibacterial substances. The results were consistent with studied of Pereira *et al.*,¹⁴ who showed that cashew stem bark extract had antibacterial power against *Streptococcus mutans*, *S. sanguinis*, and *Streptococcus mitis*.

DISCUSSION

Biofilm is a bacterial community that attach to surface that covered by extracellular matrix. This matrix has a main component, that is exopolysaccharides (EPS). Exopolysaccharides is formed by bacterial that produce GTF such as *S. sanguinis*.^{6,15} This study used BHI with 2% sucrose as growth medium. Addition 2% sucrose in BHI because of sucrose is substrate that will breakdown by *S. sanguinis* to form EPS.⁶

In this study, biofilm of *S. sanguinis* attached to wall of microplate polystyrene wells. According to Ge *et al.*,¹⁶ this surface is one of abiotic surface that biofilm can attach. Biofilm that have attached, was stained with crystal violet. Assessment of crystal violet-biofilm bonding quantitatively was using an ELISA reader (microplate reader).¹⁷

The result showed that cashew (*Anacardium occidentale* L.) stem bark extract capable of inhibiting biofilm formation of *S. sanguinis*. *Anacardium occidentale* L. stem bark extract had antibacterial activity against *S. sanguinis*.¹⁴ *Anacardium occidentale* L. had some antibacterial agent such as tannin and flavonoid that can disturb metabolism of *S. sanguinis*. Beside that, these bacterial agent can also to deactivated enzyme.^{18,19}

The high concentration of *Anacardium occidentale* L. extract more inhibit biofilm formation than others. This is due to the high concentration has more antibacterial agent, so that make an effect to number of percentage of inhibiting biofilm formation. Increase in the concentration of cashew stem bark extract will increase the antibacterial of *S. sanguinis*.¹⁴

In this study, chlorhexidine had antibacterial power greater than the concentration of 3.125 mg/ml, 6.25 mg/ml, and 9.375 mg/ml and lower than the cashew stem bark extract concentration 12.5mg/ml. Chlorhexidine against some bacteria and fungi because of this agent increased cell membrane permeability then followed by cytoplasm macromolecule coagulating. Chlorhexidine against some bacteria and fungi because of this agent increased cell

membrane permeability then followed by cytoplasm macromolecule coagulating.²⁰ However, the results of our study showed that chlorhexidine 0.12% inhibition of biofilm formation was lower than the cashew stem bark extract concentration of 12.5 mg/ml. This might be due to the concentration of 12.5 mg/ml was too thick so it settles on the surface of the base well and stained with crystal violet. This caused the difference between the OD test well (12.5 mg/ml + BHI + sucrose 2% + *S. sanguinis*) to the OD blank sample well (extract 12.5 mg/ml + BHI + sucrose 2% + saline) very small compared with the difference in OD control vehicle (2.5% PEG). Therefore, the inhibition of biofilm formation was intense. In addition, the antibacterial microdilution test required agent in small volume.²¹ Cashew stem bark extract concentration 12.5 mg/ml with a volume 2.5 mL per well seem too many that are less appropriate to be used in the microdilution test.

Broadly, cashew bark extract can inhibit the formation of biofilms that was formed by *S. sanguinis*. This might be caused by the phenolic compounds such as tannins and flavonoids contained in cashew stem bark extract. Phenolic compounds can provide an antibacterial effect by disrupting the cell wall and membrane, precipitate proteins, and deactivate the enzyme.²² One of enzyme activity might be inhibited by phenolic compounds was GTF enzymes. Some phenolic compounds could inhibit enzyme activity GTF.²³ If the GTF enzyme activity was interrupted, as a matrix of EPS biofilm formation also declined. Thus, the formation of biofilm *S. sanguinis* can be inhibited. In conclusion, cashew stem bark extract (*Anacardium occidentale* L.) can inhibit *Streptococcus sanguinis* biofilm formation.

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