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Research

STEVIA LEAF EXTRACT *(STEVIA REBAUDIANA BERTONI M.)* INHIBITS *STREPTOCOCCUS SANGUINIS* ATCC 10556 BIOFILM FORMATION *IN VITRO*

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ABSTRACT

Background: Oral biofilms play a significant role in oral diseases such as caries and periodontal diseases. *Streptococcus sanguinis* is a key component in the development of oral biofilms. Biofilm formation can be inhibited by using anti-adhesion and antibacterial substances contained in plants. Stevia leaves (*Stevia rebaudiana* Bertoni M.) contain flavonoids, alkaloids, and tannins, which may possess anti-adhesion and antibacterial properties.

Objective: The purpose of this study was to determine the effect of a stevia leaf extract on the inhibition of *S. sanguinis* ATCC 10556 biofilm formation.

Methods: The inhibition of biofilm formation was examined using a 96-wells microplate. Various concentrations of stevia leaf extract (5.36, 2.68, 1.34, and 0.67%), 0.2% chlorhexidine gluconate (positive control), and PBS (negative control) were incubated with *S. sanguinis* in BHI broth medium for 24 h. The biofilm was then stained with 0.1% crystal violet and the absorbance was measured using a microplate reader (λ = 540 nm).

Results: One-way ANOVA showed a significant difference among the groups in the inhibition *of S. sanguinis* biofilm formation. The LSD Post-Hoc test showed that 5.36% stevia leaf extract had the same effectiveness as 0.2% chlorhexidine gluconate.

Conclusions: This study demonstrated that S. sanguinis ATCC 10556 biofilms were inhibited by stevia leaf extract *in vitro*. In addition, 5.36% of the stevia leaf extract was as effective as 0.2% chlorhexidine gluconate in inhibiting the formation of *S. sanguinis* ATCC 10556.

Keywords: *Streptococcus sanguinis*, stevia leaf extract, inhibition of biofilm formation

INTRODUCTION

The Basic Health Research in 2018 recorded that the proportion of oral health problems experienced by the Indonesian population was 57.6%.[1] One of the oral health issues is periodontal disease, accounting for 70%, which ranks it as the second major health problem in the community.[2]

Dental plaque is a community of microorganisms that develops as a biofilm on the tooth surface, embedded in a polymer matrix derived from bacteria and saliva.[3] Based on its pathogenic effects, dental plaque was classified into three types: cariogenic plaque, plaque associated with periodontal disease, and calculogenic plaque.[4]

Streptococcus sanguinis is a gram-positive, facultative anaerobic bacterium that acts as a pioneer in bacterial colonization within the oral cavity.[4,5] *It* can adhere to the surface of teeth and initiate the biofilm formation process in the oral cavity. The attachment process of S. sanguinis to the tooth surface begins with the involvement of fimbriae, which mediate the adhesion of S. sanguinis to hydroxyapatite through interactions with salivary glycoproteins in the form of the acquired pellicle.[6] *Streptococcus sanguinis* forms attachments with other oral microorganisms, leading to the formation of dental plaque and contributing to the development of caries and periodontal diseases. Other studies have indicated that *S. sanguinis, Streptococcus mutans,* and *Actinomyces viscosus* are pioneer bacteria in dental plaque formation.[2] *Streptococcus sanguinis* adheres to the tooth surface, initiating biofilm formation in the oral cavity. The high aggregation ability with other bacteria enables *Streptococcus sanguinis* to play an important role in the maturation of dental plaque on tooth surfaces and in the calcification process of plaque into dental calculus. The attachment process of *S. sanguinis* to the tooth surface began with the involvement of fimbriae that mediate *S.*

sanguinis adhesion to hydroxyapatite through interactions with saliva glycoproteins in the form of acquired pellicle.[6]

A biofilm is a collection of microorganisms that grow on a surface, organized both structurally and functionally, and interact with each other.[7] Dental biofilm or plaque is an etiological factor in oral health problems.[8] The formation of a biofilm involves several stages, that is, formation of acquired pellicle on the tooth surface, followed by the initial adhesion stage, which includes the adhesion of pioneer bacteria such as *S. sanguinis* to the tooth surface. This process is supported by adhesive forces, such as hydrogen bonding, hydrophobic interactions, van der Waals forces, acid-base interactions, and electrostatic interactions.[9] Subsequently, co-aggregation occurs among plaque bacteria and a maturation stage, where bacteria continue to reproduce within the extracellular polymeric substance (EPS) matrix. The final stage is the release of bacteria or dispersal from the biofilm, allowing bacterial cells to form new microcolonies on other substrates in response to specific physiological or environmental conditions.[10,11]

One way to prevent oral diseases is to control plaque. The primary method of plaque control is regular tooth brushing, ensuring proper brushing techniques, adhering to the recommended duration.[2] Additionally, plaque control can also be achieved by using mouthwash, including those containing chlorhexidine (CHX). However, long-term use of CHX-containing mouthwash leads to several side effects such as taste disturbances, burning sensation, and tooth discoloration.[12] Therefore, there is a need for research on alternative substances that could minimize the adverse effects of mouthwash.

Stevia (*Stevia rebaudiana Bertoni M.*) is a native plant of Paraguay that grows in various locations in Indonesia, such as Bandung and Tawangmangu, with suitable temperature and soil humidity conditions.[13,14] Stevia leaves contain stevioside and rebaudioside, which can be used as natural sweeteners with a sweetness level 300 times greater than that of sucrose found in sugarcane.[13] These sweetening compounds cannot be fermented by bacteria in the oral cavity and are non-carcinogenic.[28] In addition, stevioside content in stevia leaves has been declared non-toxic.[16] Stevia leaves possess antibacterial, antiviral, anti-inflammatory, antifungal, antidiabetic, antioxidant, and antimicrobial properties.[15] Additionally, stevia leaves contain substances such as alkaloids, tannins, and flavonoids that exhibit antiplaque and antibacterial activities. Alkaloids and tannins work by inhibiting bacterial cell wall synthesis, while flavonoids affect the bacterial cell surface structure, deactivate adhesins, and inhibit attachment abilities.[14,15] Stevioside in stevia leaves is non-toxic, making it a safe alternative sweetener with potential health benefits.[16] The results of the study showed that, quantitatively, the stevioside content in the leaf extract was 1.2 times higher than that in the stem extract.[29]

This study aimed to investigate the effect of stevia leaf extract on the inhibition of *S. sanguinis* biofilm formation *in vitro*.

MATERIAL AND METHODS

Ethical clearance for the study was obtained from the Ethics and Advocacy Unit of the Faculty of Dentistry, Universitas Gadjah Mada (reference number 0126/KE/FKG-UGM/EC/2022). Plant determination was conducted at the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada in the Natural Plant Analysis section (reference number 0116/S). Tb./VII/2022.

Stevia leaves were obtained from Tawangmangu, Central Java, and their extract was prepared using the maceration method with 70% ethanol as solvent.[17] The process, after which 1 kg of stevia leaves was washed, cut into small pieces, and dried under sunlight until the moisture content reached approximately ±10%. The dried simplicia were finely ground using a chopper blender, filtered through a 70-mesh sieve, and extracted by soaking in 70% ethanol at a ratio of 1:7 for 24 h at a temperature of 55°C. Subsequently, the extract was filtered using a Buchner funnel and vacuum, and the resulting solution was allowed to settle. The filtrate was evaporated using a rotary evaporator at 50°C, and the concentrated extract was transferred to a stainless steel pan in a water bath, stirred, and dried with a fan. This process yielded a pure stevia leaf extract in a gel form.

The preparation of *S. sanguinis* ATCC 10556 suspension began with inoculation of a pure culture into Brain Heart Infusion Broth (BHI-B) medium. The culture was incubated for 24 h at 37°C. After the incubation period, bacteria from the BHI-B medium were collected using a micropipette and transferred into tubes containing the PBS solution. PBS was used in this study because it is a physiological solution that is isotonic and nontoxic to cells, and its primary purpose is to maintain pH levels. This ensures that the observed effects are not attributed to changes in pH or ion composition unrelated to the tested substance.[30] The concentration of the bacterial suspension was then adjusted to the 0.5 McFarland standard or equivalent to 1.5 x 10^8 CFU/mL.

The Minimum Inhibitory Concentration (MIC) was the lowest concentration of the stevia leaf extract capable of inhibiting the growth of *S. sanguinis* ATCC 10556. The MIC test was conducted using serial dilutions from 11 reaction tubes. A 100% stevia leaf extract was prepared by dissolving 100 g of the extract in 100 mL of distilled water. In tube 1, 15 mL of 100% stevia leaf extract was added, followed by serial dilutions in tubes 2-6 to achieve concentrations of 50%, 25%, 12.5%, 6.25%, and 3.125%. A blank was used as a control at the same concentrations. Subsequently, 1 mL of *S. sanguinis* bacterial suspension was added to tubes 2-6, resulting in final concentrations of 42.86%, 21.43%, 10.71%, 5.36%, and 2.68%, respectively. Incubation was performed for 24 h at 37°C, and the results were visually observed. The MIC was determined by the first tube showing turbidity, and the concentration of stevia leaf extract in that tube was used to test the inhibition of *S. sanguinis* ATCC 10556 bacterial biofilm formation.

The inhibition test for biofilm formation was conducted using a 96-well microplate with six sample groups: stevia leaf extract concentrations at 2 MIC, MIC, 1⁄2 MIC, 1⁄4 MIC, negative control (PBS), and positive control (chlorhexidine gluconate 0.2%). The use of 0.2% CHX is considered to be the gold standard for daily mouthwash use. Chlorhexidine has antibacterial properties against both aerobic and anaerobic bacteria, and acts as an antifungal agent. The choice of 0.2% CHX represents the optimal dosage for mouthwash use, as reported by Sajjan et al. (2016). Antibiotics were not used in this study; instead, 0.2% CHX was selected because this study aimed to compare the effectiveness of stevia extract with 0.2% CHX, a commonly used daily mouthwash. Each well in the test group was filled with 40 µL BHI-B, 50 µL stevia leaf extract suspension at concentrations of 2 MIC, MIC, 1⁄2 MIC, 1⁄4 MIC, and 10 µL bacterial suspension at a concentration of 0.5 McFarland. The blank control consisted of media and test substances without bacteria. The microplate was then incubated anaerobically at 37°C for 24 h using an anaerobic jar. After incubation, the microplate was washed once with PBS to remove unattached media and cells during the incubation process. The remaining cells were stained with 125 µL of 0.1% crystal violet. The microplate was washed twice with PBS and air-dried. The PBS washes were aimed at eliminating unabsorbed color. Subsequently, 200 µL 96% ethanol was added to each well and incubated again at room temperature for 15 min. Next, 150 µL of the solution was transferred to a 96-well flat-bottom microplate and the optical density (OD) was measured using a microplate reader at a wavelength of 540 nm. The percentage of inhibition of biofilm formation was calculated using the following formula:

$$\%\ Inhibition\ = \left(1 - \left|\frac{OD\ Sample - OD\ Blank\ Sample}{OD\ Control - OD\ Blank\ Control}\right|\right) x\ 100\%$$

In this study, three repetitions were performed for each group to obtain the data**.** The research data were first tested for homogeneity using Levene's test and for normality using the Shapiro-Wilk test. Subsequently, the data were statistically analyzed using One-way ANOVA followed by Post-Hoc LSD analysis.

RESULTS

The results indicated inhibition of biofilm formation by *S. sanguinis* ATCC 10556 after exposure to stevia leaf extract at concentrations of 0.67%, 1.34%, 2.68%, and 5.36%. The average percentage of inhibition of biofilm formation is presented in Table 1.

This study used a sample size of less than 50; thus, the Shapiro-Wilk normality test was conducted, and the results are presented in Table 2. The Shapiro-Wilk normality test results indicated that the treatment groups of 5.36%, 2.68%, 1.34%, and 0.67%, and the positive control (chlorhexidine gluconate 0.2%) had normally distributed data with p-values > 0.05. The next step involved testing the homogeneity of data using Levene's test.

The results of the homogeneity test using Levene's test for all test solutions showed a p-value of 0.451 (p > 0.05), indicating that all the test solutions were homogeneous (Table 3). It can be concluded that with a 95% confidence interval, the treatment data have equal variances (homogeneous).

Table 1. Mean and standard deviation of the percentage inhibition of biofilm formation by Streptococcus sanguinis ATCC 10556

Treatment	Mean and Standard Deviation
0.67% Extract	51.84±2.96
1.34% Extract	67.48±3.48
2.68% Extract	78.53±6.13
5.36% Extract	84.97±3.23
Chlorhexidine gluconate 0,2%	91.10±5.07

Table 2. Shapiro-Wilk Normality Test Result

Treatment Group	Statistics	df	Sig.
0,67% Extract	0,871	3	0,298
1,34% Extract	0,855	3	0,253
2,68% Extract	0,953	3	0,583
5,36% Extract	0,993	3	0,843
Chlorhexidine gluconate 0,2%	0,824	3	0,174

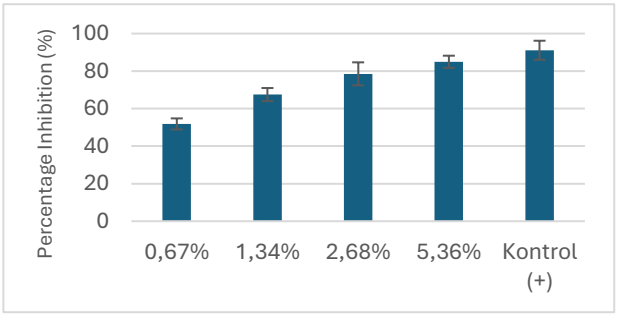

Figure 1. Histogram of the mean and standard deviation of the percentage inhibition of biofilm formation of *Streptococcus sanguinis* ATCC 10556

The data were further tested using One-Way ANOVA (Table 4). Based on the results of the One-Way ANOVA, a p-value of 0.000 was obtained (p < 0.05). It can be concluded that there is a significant difference between the groups with a 95% confidence interval. These findings suggest that the stevia leaf extract inhibits biofilm formation by *S. sanguinis* ATCC 10556. Subsequently, a Post-Hoc Least Significant Difference (LSD) Fisher test was conducted to determine the differences among the groups (Table 4).

The results of the Post-Hoc LSD test (Table 5) indicated significant differences in the mean percentage inhibition (p<0.05) between the chlorhexidine gluconate 0.2% treatment group and the 2.68%, 1.34%, and 0.67% extract groups. This suggests that 0.2% chlorhexidine gluconate has a higher ability to inhibit biofilm formation compared to the concentrations of 2.68%, 1.34%, and 0.67% extract. There was no significant difference (p>0.05) between the 5.36% and 2.68% extract-treated groups. The

LSD test results also showed that there was no significant difference (p>0.05) between the 5.36% extract and the positive control (chlorhexidine gluconate 0.2%). This suggests that 5.36% the stevia leaf extract has the same effectiveness as chlorhexidine gluconate 0.2% in inhibiting biofilm formation by *S. sanguinis* ATCC 10556.

Table 3. Levene's Test Homogeneity Test Result

		Levene Statistic	**df1**	**df2**	**Sig.**
Percentage Inhibition	*Based on Mean*	1,002	4	10	0,451

Table 4. One Way ANOVA Test Result

	Sum of Square	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	2891,377	4	722,844	38,219	0,000
Within Groups	189,130	10	18,913		
Total	3080,507	14			

Table 5. Post-Hoc LSD Test Result

Treatment Group	**0,67%**	**1,34%**	**2,68%**	**5,36%**	**CHX**
0,67%		0,001*	0,000*	0,000*	0,000*
1,34%			0,011*	0,001*	0,000*
2,68%				0,100	0,005*
5,36%					0,115
CHX					

*p<0.05

DISCUSSION

Based on the results of the present study, it is evident that stevia leaf extract inhibits biofilm formation by *S. sanguinis*. This is probably attributable to the presence of compounds in stevia leaf extract, such as flavonoids, alkaloids, and tannins, which possess antibacterial properties and can inhibit bacterial attachment.[15]

Flavonoids can inhibit biofilm formation by targeting the intercellular adhesion mechanisms (IcaA and IcaD) present in *S. sanguinis*. The Ica genes mediate the formation of polysaccharide intercellular adhesin (PIA) by activating the Ica promoter, leading to the loss of the ability of the bacteria to form biofilms.[18] In addition to their anti-adhesive properties, flavonoids also act as antibacterial agents by inhibiting the activity of glycosyltransferase enzymes (GTF) due to their phenolic compounds. Phenolic compounds exert antibacterial effects by damaging the cell walls and deactivating GTF enzymes. Disruption of GTF enzyme activity results in reduced extracellular polymeric substance (EPS) production, thereby inhibiting the formation of *S. sanguinis* biofilms.[19]

Alkaloids are known for their ability to disrupt the components of bacterial peptidoglycan, preventing the formation of the cell wall layer in its entirety.[20] The nitrogen content in the basic alkaloid groups can denature bacterial cell proteins and react with amino acids that make up the bacterial cell membrane and DNA. This reaction leads to structural changes and alterations in the amino acid composition, disrupting the genetic balance in the DNA chain and resulting in damage and promotion of lysis. Increased permeability and damage to the cell membrane cause the bacteria to lose essential components, leading to cell death.[21]

Tannins exhibit antibacterial activity and inhibit bacterial adhesion by damaging the bacterial cell membranes. This is because tannins can form complex bonds with proteins, thereby inactivating bacterial adhesins.[22] The disturbance of bacterial cell wall activity is believed to occur when tannins attach to the bacterial cell wall. The cell wall undergoes morphological changes, becoming thicker and altering the space between the cell wall and the plasma membrane.[23] Disruption and increased permeability of the bacterial cell wall membrane are followed by intracellular leakage, causing cellular components to exit. This results in decreased physiological activity of the bacterial cell, leading to lysis and preventing bacterial growth.[22,24] Tannins, as anti-adhesives, also reduce bacterial hydrophobicity, disrupting bacterial adhesion to host surfaces.[2]

Based on the results of the Post-Hoc LSD test, the higher the concentration of the used stevia leaf extract, the more effective the inhibition of biofilm formation. This is because at higher concentrations of stevia leaf extract, there is a likelihood of a higher content of anti-adhesive and antibacterial substances, such as flavonoids, alkaloids, and tannins, making its ability to inhibit biofilm formation more effectively. This aligns with the theory that the larger the concentration of stevia leaf extract, the more active substances are present in the extract, thereby increasing its ability to inhibit biofilm formation.[15] The concentrations of 2.68% and 5.36% of the extract exhibit equivalent abilities in inhibiting biofilm formation.

The results of the Post-Hoc LSD test indicate that chlorhexidine gluconate 0.2% and a concentration of 5.36% extract had the same effectiveness in inhibiting the formation of *S. sanguinis* biofilms. The ability of chlorhexidine gluconate 0.2% to inhibit biofilm formation aligns with the theory that states chlorhexidine gluconate 0.2% has antibacterial benefits against both aerobic and anaerobic bacteria.[25] Chlorhexidine is bactericidal, increasing cell membrane permeability and causing the loss of intracellular components, including nucleotides due to bacterial cell lysis.[26] Chlorhexidine can inhibit the activity of GTF enzymes, thus impeding biofilm formation.[27]

CONCLUSION

Based on this research, it was concluded that *Stevia rebaudiana Bertoni* M. leaf extract at concentrations of 5.36%, 2.68%, 1.34%, and 0.67% inhibited the formation of *S. sanguinis* ATCC 10556 biofilm. The higher the concentration of Stevia rebaudiana leaf extract, the more effective it was in inhibiting biofilm formation. *Stevia rebaudiana* leaf extract at a concentration of 5.36% had the same effectiveness as 0.2% chlorhexidine gluconate in inhibiting *S. sanguinis* ATCC 10556 biofilm formation.

LIMITATIONS

The limitation of this study is the absence of biocompatibility testing, such as cytotoxicity assays, which are important for evaluating the cytotoxic potential of stevia leaf extract. Without these data, it is difficult to determine an appropriate and safe dosage for practical application.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this study. This study was conducted with utmost integrity, and there are no financial, personal, or professional relationships that could compromise the objectivity, integrity, or validity of the research findings.

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