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Antibiofilm Potential of *Stevia Rebaudiana* Bertoni Leaf Extract Irrigated Solution against The Bacteri *Enterococcus Faecalis* ATCC 29212

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ABSTRACT

Disinfection of dental root canals 2.5% NaOCL is still a challenge related to its toxicity and the virulence of *Enterococcus faecalis* which is able to form biofilms in dental root canals. There is no evidence on the antibiofilm ability of *Stevia rebaudiana* Bertoni leaf extract against *Enterococcus faecalis.*

Objective: To evaluate the efficacy of Stevia rebaudiana Bertoni leaf extract irrigant in preventing Enterococcus faecalis biofilm formation.

Materials and Methods: This in vitro study utilized a *Post-Test Only Control Group Design*. *Stevia rebaudiana* Bertoni leaf extract irrigants were serially diluted to concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56%. 2.5% NaOCl and Brain Heart Infusion-Broth (BHI-B) media with 2% sucrose were employed as the positive and negative controls, respectively. *Enterococcus faecalis* ATCC 29212 bacterial suspension was equated with Mc Farland 0.5 standard. Biofilm growth media using BHI-B + 2% sucrose. Antibiofilm potential using crystal violet assay. Measurement of optical density (OD) value at 490 nm.

Results: Significant differences were identified using the Kruskal-Wallis test ($p=0.000$) between the mean experimental OD values. Mann-Whitney U test showed the mean experimental OD values of all *Stevia rebaudiana* Bertoni leaf *extract* irrigants were significantly smaller than the negative control ($p<0.05$). The 100% Stevia rebaudiana Bertoni leaf extract irrigants did not exhibit a significantly different mean optical density (OD) value compared to the positive control ($p > 0.05$), while 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% *Stevia rebaudiana* Bertoni leaf extract irrigants significantly outperformed the positive control ($p<0.05$).

Conclusion: All irrigation solutions of *Stevia rebaudiana Bertoni* leaf extract proved to have antibiofilm potential of *E.faecalis* significantly greater than the negative control. The antibiofilm potential of 50%; 25%; 12.5%; 6.25%; 3.125% and irrigation solution containing 1.56% *Stevia rebaudiana Bertoni* leaf extract was significantly less than NaOCL 2.5% while 100% *Stevia rebaudiana* Bertoni leaf *extract* irrigant was not significantly different from NaOCL 2.5%.

KEYWORD: *Enterococcus faecalis*, irrigation solution, dental root canal, *Stevia rebaudiana* Bertoni leaf extract, antibiofilm.

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INTRODUCTION

Dental root canal (endodontic) treatment is performed to prevent and treat the pulp and root canal system of a tooth contaminated by microorganisms.[1] Bacteria and their products have been known to be contributing factors in dental pulp and *peri-radicular* tissue inflammation that need to be eliminated to achieve successful endodontic treatment.[2] *Enterococcus faecalis* bacteria as gram-positive opportunistic pathogenic bacteria, which are facultative anaerobes, are The predominant bacterial species in dental biofilms with instances of root canal treatment failure[2,3]. Researched by Zhang C *et al* cited by Liu Y *et al* in 2020, it was explained

that *Enterococcus faecalis* bacteria were detected 77% in cases of persistent periapical periodontitis.[4] Analysis using *Polymerase Chain Reaction* by Sedgley et al cited by Yang S *et al* in 2020 has also proven that *Enterococcus faecalis* bacteria were detected 67.5% in cases of primary infection and 89.6% in cases of secondary infection in the tooth root canal.[5] *Enterococcus faecalis* may thrive to survive in an environment with high alkaline levels, limited nutrients and oxygen after chemomechanical action on the tooth root canal.[6] According to Sedgley CM *et al* cited by Liu Y *et al* in 2020, these bacteria are also very resistant to endodontic medicaments and have the ability to form biofilms that are difficult to remove. The biofilm can adhere firmly along the main root canal including lateral canals, isthmuses and apical deltas.[5].The ability of *Enterococcus faecalis* to form biofilms, tolerate a high alkaline environment and invade deep dentinal tubular areas, allows bacteria to thrive for a lengthy time in the tooth root canal. and avoid the host immune response.[7,8].

Biofilm is a complex three-dimensional structure formed by various bacteria and fungi that are protected in a matrix of secreted extracellular polymers (Extracellular Polymeric Substance) and attached to biotic or abiotic surfaces.[9,10] Extracellular Polymeric Substance (EPS) as a biofilm matrix consists of *lipids*, proteins, *extracellular* DNA (eDNA) and polysaccharide components.[10] Resistance of microorganisms in biofilms to antimicrobial agents is known to increase 1000-1500 times compared to bacteria in a free-living (planktonic) state.[11] Biofilm resistance to antimicrobial agents can be caused by the contribution of several factors, including the presence of extracellular polymeric substance (EPS) which acts as a barrier / barrier to diffusion and interferes with the penetration of antimicrobial agents into biofilms. Another factor is the availability of oxygen and different nutrients in the inner layer of the biofilm, which will encourage microbial cells to grow more slowly so that they are more resistant to exposure to antimicrobial agents.[12]

Disinfection to eliminate or inhibit the activity of microorganisms in the dental root canal, can be done among others through irrigation procedures, namely rinsing the cavities and root canals with medicinal solutions. Irrigation solution materials must be highly effective against anaerobic and facultative microorganisms in both single and *biofilm* forms.[1,13]The most commonly used irrigation solution material and the most effective method for root canal treatment to date is sodium hypochlorite (NaOCL) because It has antibacterial capacity and can disintegrate necrotic tissue, important pulp tissue, dentin, and biofilm components.[1,5,13] The recommended concentration of NaOCL for irrigation solution material in root canal treatment ranges from 0.5%-5.25% [1, 13]. However, NaOCL irrigation solution still has problems because it can cause allergic reactions, is cytoxic, has the potential to irritate the *periapical* region of the tooth, and has an unpleasant odor and taste.[1, (5,)(14] Based on the obstacles that are still encountered in the use of NaOCL irrigation solution materials, it is necessary to find alternative natural irrigation solution materials based on herbs that are easily available, safe, *biocompatible* and effective in inhibiting the formation of bacterial biofilms in the tooth root canal, especially against *Enterococcus faecalis bacteria.* [15] Extract of *Stevia rebaudiana Bertoni* leaves as one of the herbal ingredients, has been known from various research results, has antimicrobial activity against both bacteria and fungi.[16,17] Deviyanti S et al. (2022) reported the phytochemical composition of alkaloids, saponins, flavonoids, steroids and tannins which are antibacterial in *Stevia* leaf *extract* and proved that all concentrations of extracts tested were able to form a growth inhibition zone against *Enterococcus faecalis* bacteria.[18] Researched further by Deviyanti S *et al* in 2024 has reported a minimum inhibitory concentration (KHM) of 6.25% *Stevia* leaf extract irrigant with the turbidimetric method and a concentration of 1.56% with the *total plate count* method. so that it can reduce the number of *Enterococcus faecalis* bacterial colonies. [19] The minimum kill concentration (KBM) was shown by 50% *Stevia* leaf extract irrigant. The bactericidal effectiveness of 100% and 50% Stevia leaf extract irrigants was found to be equivalent to that of NaOCL 2.5%[19]. Hastuty A's research in 2019 has also proven the antimicrobial and antibiofilm efficacy of *Stevia* leaf extract but against *Bacillus sp* and *Enterobacter sp* bacterial *strains*.[20] Related research to the antibiofilm efficacy of *Stevia* leaf extract irrigant against *Enterococcus faecalis* bacteria has never been reported. The purpose of this study with Post-Test Only with Control Group Design is to assess the efficacy *of Stevia* leaf extract irrigation solution in inhibiting biofilm formation by *Enterococcus faecalis* ATCC 29212.

MATERIAL ANDMETHODS

Plant Classification

The National Agency for Research and Innovation's Botanical Characterization Laboratory in Cibinong, Bogor, conducted the identification of *Stevia rebaudiana* Bertoni plants harvested from Ciputri Village, Pacet District, Cianjur Regency.

Tool sterilization

All equipment (glass, including eppendorf tubes and centrifuge tubes) were sterilized using an autoclave at 121^0C for 15 minutes before being used in all research procedures.

Stevia rebaudiana Bertoni leaf extraction

Stevia rebaudiana Bertoni leaves that were dried without direct contact with sunlight were chopped using a *blender* and size uniformity was carried out using a 40 *mesh* sieve.[21] A total of 175 grams of *Stevia* leaf simplisia was macerated at room temperature (25^0C) for 72 hours using 96% ethanol solvent with a simplisia and solvent ratio of 1:10 divided into 4 bottles (Schott Duran® , Germany) while shaking every 15

minutes.[21,22,23] The results of maceration were then filtered using Whatman filter paper (Cytiva™, China)[2)(4,22] The filtrate from the filtration was evaporated with a rotary vacuum evaporator (Buchi, Switzerland) to obtain a thick extract of *Stevia* leaves.[2)(2,25] The residue was then macerated again 1x in the same way. The thick extract of *Stevia* leaves obtained was then weighed and put into a *centrifuge* tube and stored in a frezzer at -20 ^{0} C until ready to be used for *Enterococcus faecalis* bacteria antibiofilm test.[16]

Preparation of *Enterecoccus faecalis* bacteria culture and suspension [26,27,28]

Pure culture of *E. faecalis strain* ATCC (American Type Culture Collection) 29212 was removed from cryo storage and thawed at room temperature. Bacterial culture medium was prepared by weighing 3.7 g of BHI-B (*Brain Heart Infusion*) powder (Himedia, India) broth dissolved with 100 mL sterile distilled water in an erlenmeyer flask and then stirred until homogeneous with a glass stirring rod. The surface of the mouth of the erlenmeyer tube was covered with aluminum foil and tied tightly to be sterilized in an *autoclave* for 15 minutes at 121 ^{0} C then left at room temperature. Bacterial culture was carried out by taking 10 µl of pure culture of bacteria to be put into 1.7 ml of BHI-B media, then homogenized with a vortex (DLAB MX-S, USA) incubated in an anaerobic jar in an incubator (JISICO, South Korea) for 24 hours at 37° C. Bacterial suspension of *E. faecalis* was prepared by dissolving the bacterial culture in BHI-B medium that had been incubated for 24 hours, and homogenized with a *vortex*. The bacterial suspension was equated to the *Mc Farland* standard of 0.5 so that the total bacterial density used, was equivalent to 1.5 x 10^8 CFU/ml.

Preparation of *Enterococcus* biofilm media

E. faecalis bacterial biofilm growth medium was prepared by weighing 3.7 g of BHI-B + 2 g of 2% sucrose then put in an erlenmeyer tube and dissolved in 100 ml of sterile distilled water and stirred until homogeneous. The surface of the erlenmeyer tube was covered with aluminum foil and tied and then sterilized in an autoclave for 15 minutes at 121 ^{0} C then left at room temperature. A total of 1.7 ml of BHI-B + 2% sucrose as a biofilm growth medium was taken using a micropipette and put into a centrifuge tube. The *centrifuge* tube was then vortexed and incubated in an incubator for 24 hours at 37^{0}C.

Preparation of *Stevia rebaudiana* Bertoni leaf extract irrigant and Control Solution

The irrigation solution containing extract of *Stevia rebaudiana* Bertoni leaves was prepared using the serial dilution method. A total of 5 ml of *Stevia* leaf extract was put into a centrifuge tube containing 5 ml of sterile distilled *water* to make 100% *Stevia* extract irrigation solution. 5 ml of *Stevia* leaf extract solution from the first tube was then transferred to the 2nd tube containing 5 ml of sterile distilled water to make 50% *Stevia* extract irrigation solution. The same thing was done serially in the next tube until all test concentrations of *Stevia* extract irrigation solution were obtained. Positive control solution of 2.5% NaOCL was made by putting 3 ml of 5.2% NaOCL solution (Bayclin, Indonesia) into a centrifuge tube and then adding 3.24 ml of sterile distilled *water* solution

Antibiofilm Test (*Crystal Violet Assay*)[22,27,29,30,31]

A total of 100µL of *Stevia* leaf extract irrigant of each test concentration, 100µL of 2.5% NaOCL solution as positive control and 100µL of 2% BHI-B + sucrose media solution as negative control were introduced using a micropipette into each 96-well polystyrene flat-bottom microplate (Biologix®, Germany) according to the design. A total of 100µL of bacterial suspension in BHI-B + 2% sucrose medium was added into each microplate wells. Blank microplate wells were also made by adding 200µL of each concentration of *Stevia* leaf extract test solution, 2.5% NaOCL solution (positive control), and BHI-B + 2% sucrose media (negative control) without exposure to *E. faecalis* bacteria to each wells. Microplate wells were incubated under anaerobic conditions for 24 hours at 37°C. After the incubation period, the contents of the Microplate wells were removed by rinsing using sterile distilled *water*, then dried. Microplate wells were fixed by passing over the fire. A total of 200µL of 0.5% *crystal violet* solution as a biofilm coloring agent, inserted into each *microplate well* using a micropipette, incubated at room temperature for 30 minutes. The 0.5% *crystal violet* solution in each *microplate well was* then discarded, the biofilm was rinsed three times under running tap water. Microplate wells were dried. Each *microplate well* was then filled with 200 µL of 96% *ethanol* solution and the *optical* density (OD) of the biofilm was measured using a microplate reader (Safas MP96, Monaco) at a wavelength of 490 nm. The percentage inhibition of *E.faecalis* biofilm formation was calculated using the formula: [22,32,23,31,32]

$$\% \text{ Biofilm Inhibition} = \frac{\text{OD negative control} - \text{OD Experimental}}{\text{OD of negative control}} \times 100\%$$

RESULTS

***Stevia rebaudiana* Bertoni plant determination test results**

The plant used in this study was identified from the determination test as *Stevia rebaudiana Bertoni* from the *Asteraceae* tribe.

***Stevia rebaudiana* Bertoni leaf extraction results**

Maceration of *Stevia rebaudiana* Bertoni leaf simplisia followed by filtration and evaporation of the filtrate and

remaceration 1 time, produced a thick extract weighing 95.2 grams.

Antibiofilm test results

The average value of the optical density (OD) of the experiment derived from the average value of the OD of each concentration of *Stevia* leaf extract irrigant and the control group that has been reduced by the OD value of the blank can be seen in table 1.

Table 1. Average *Optical Density* (OD) Values of *E. faecalis* Bacterial Biofilm Experiments of *Stevia rebaudiana* Bertoni Leaf extract irrigant

No.	Sample	*Mean* OD± Standard Deviation	*P*
1.	100% Stevia Extract	0.05060 ± 0.051525	0.000*
2.	Stevia Extract 50%	0.14140 ± 0.048335	
3.	25% Stevia Extract	0.27340 ± 0.099961	
4.	Stevia extract 12.5%	0.32180 ± 0.053025	
5.	Stevia extract 6.25%	0.45200 ± 0.088927	
6.	Stevia extract 3.125%	0.55680 ± 0.071942	
7.	Stevia extract 1.56%	1.28300 ± 0.087937	
8.	NaOCL 2.5% (positive control)	0.04960 ±0.035949	
9.	BHI-B medium + 2% sucrose (negative control)	1.81960 ± 0.025745	

*p<0.05= significant by Kruskal-Wallis test, N=5

Data normality testing using the Shapiro-Wilk test showed that the data of the experimental OD variable of 6.25% *Stevia* extract irrigation solution was not normally distributed (*p<0.05*), so that inferential data processing was carried out with non-parametric statistical tests.The results of the Kruskal-Walis test on the average value of *optical* density (OD) of *E.* faecalis bacterial biofilm experiments from various variations of *Stevia* leaf extract concentration irrigation solutions tested, as well as positive control (NaOCL 2.5%) and negative control (BHI-B media + 2% sucrose) showed significant differences.*faecalis* from the different variations of *Stevia* leaf extract concentration irrigation solutions tested, as well as the positive control (NaOCL 2.5%) and negative control (BHI-B medium + 2% sucrose) showed significant differences (*p=0.000*) between the mean experimental OD variables studied. A diagram of the average *Optical Density* (OD) values of the *E. faecalis* bacterial biofilm experiments from the various concentrations of *Stevia* leaf extract irrigants tested, as well as the positive control (NaOCL 2.5%) and negative control (BHI-B Media + 2% sucrose), can be seen in Figure 2.

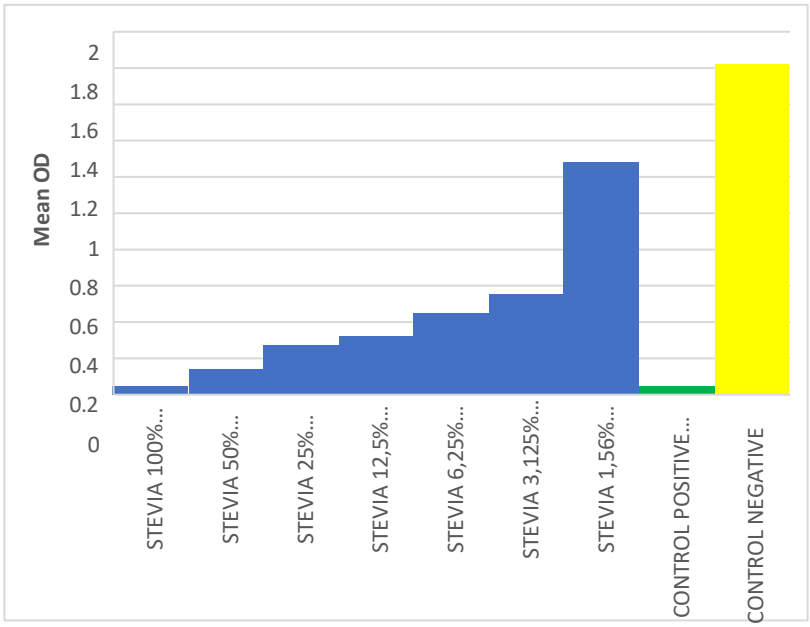

Diagram of average *optical* density (OD) values *E.faecalis* bacterial biofilm experiments of leaf extract irrigant *Stevia rebaudiana* Berton

The results of the Mann-Whitney U test showed that the average experimental OD values of the irrigation solutions of 100% *Stevia* leaf extract, 50% *Stevia*, 25% *Stevia*, 12.5% *Stevia*, 6.25% *Stevia*, 3.125% Stevia and 1.56% Stevia had a significant difference (significantly smaller) when compared to the average OD value of the negative control experiment, namely BHI-B media solution + 2% sucrose ($p<0.05$). The average experimental OD value of the 100% *Stevia* leaf extract irrigant did not have a significant difference when compared to the average positive control experimental OD value of the 2.5% NaOCL solution ($p>0.05$). The average experimental OD values of 50% *Stevia* leaf extract irrigant, 25% *Stevia*, 12.5% *Stevia*, 6.25% *Stevia*, 3.125% *Stevia* and 1.56% *Stevia* had significant differences (significantly greater) when compared to the average positive control experimental OD value of 2.5% NaOCL solution ($p<0.05$). The results of the Mann- Whitney U test on the average experimental OD value of *E.faecalis* bacterial biofilm by irrigation solution of *Stevia leaf extract* and the control group, can be seen in the table 2 below.

Table 2 Results of Mann-Whitney U Test on Mean OD Value of *E. faecalis* Bacteria Biofilm Experiment by *Stevia rebaudiana* Bertoni Leaf extract irrigant and Control Group

	100% Stevia	Stevia 50%	Stevia 25%	Stevia 12.5%	Stevia 6.25%	Stevia 3.125%	Stevia 1.56%	K (+)	K(-)
100% Stevia	-	0,028*	0,009*	0,009*	0,009*	0,009*	0,009*	0,753	0,009*
Stevia 50%	0,028*	-	0,028*	0,009*	0,009*	0,009*	0,009*	0,016*	0,009*
Stevia 25%	0,009*	0,028*	-	0,347	0,028*	0,009*	0,009*	0,009*	0,009*
Stevia 12.5%	0,009*	0,009*	0,347	-	0,075	0,009*	0,009*	0,009*	0,009*
Stevia 6.25%	0,009*	0,009*	0,028*	0,075	-	0,075	0,009*	0,009*	0,009*
Stevia 3.125%	0,009*	0,009*	0,009*	0,009*	0,075	-	0,009*	0,009*	0,009*
Stevia 1.56%	0,009*	0,009*	0,009*	0,009*	0,009*	0,009*	-	0,009*	0,009*
K(+)	0,753	0,016*	0,009*	0,009*	0,009*	0,009*	0,009*	-	0,009*
K(-)	0,009*	0,009*	0,009*	0,009*	0,009*	0,009*	0,009*	0,009*	-

*p<0.05= significant with Mann-Whitney test

The average value of the percentage inhibition of biofilm formation of *E. faecalis* bacteria by various concentrations of *Stevia* leaf extract irrigant tested can be seen in table 3 and figure 3.

Table 3 Average values of percentage inhibition of biofilm formation of irrigation solution *Stevia rebaudiana* Bertoni Leaf Extract Against *E.faecalis* Bacteria

No.	Sample	% Biofilm Inhibition
1.	100% Stevia Extract	97,22
2.	Stevia Extract 50%	92,23
3.	25% Stevia Extract	84,97
4.	Stevia extract 12.5%	82,31
5.	Stevia extract 6.25%	75,16
6.	Stevia extract 3.125%	69,39
7.	Stevia extract 1.56%	29,49
8.	NaOCL 2.5% (positive control)	97,27
9.	BHI-B medium + 2% sucrose (negative control)	0

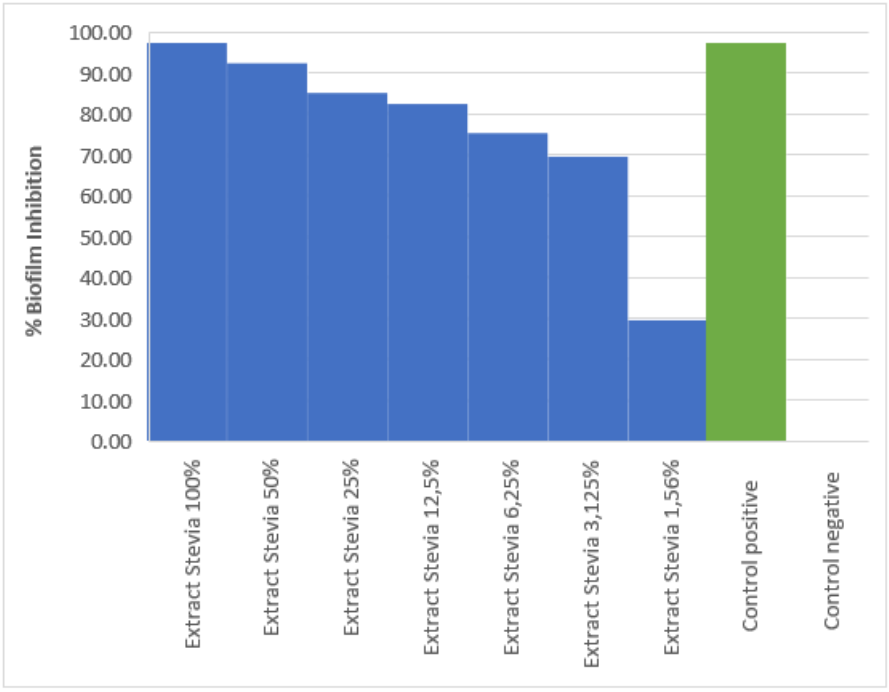

Figure 3 Diagram of the percentage value of biofilm formation inhibition *E.faecalis* from *Stevia rebaudiana* Bertoni leaf extract irrigant

The results of the average percentage value of biofilm *inhibition* of *E. faecalis* bacteria in this study were then continued with the determination of the IC_{50} value (Inhibition Concentration 50)which was determined from the linear regression equation between the sample concentration and the percentage of biofilm inhibition using calculator [33]The IC $_{50}$ value is the concentration value of the *Stevia* leaf extract solution which is able to inhibit *E. faecalis* bacterial biofilm by 50%.[37] The IC $_{50}$ value was obtained is 2.21 (Figure 4 and Table 4).

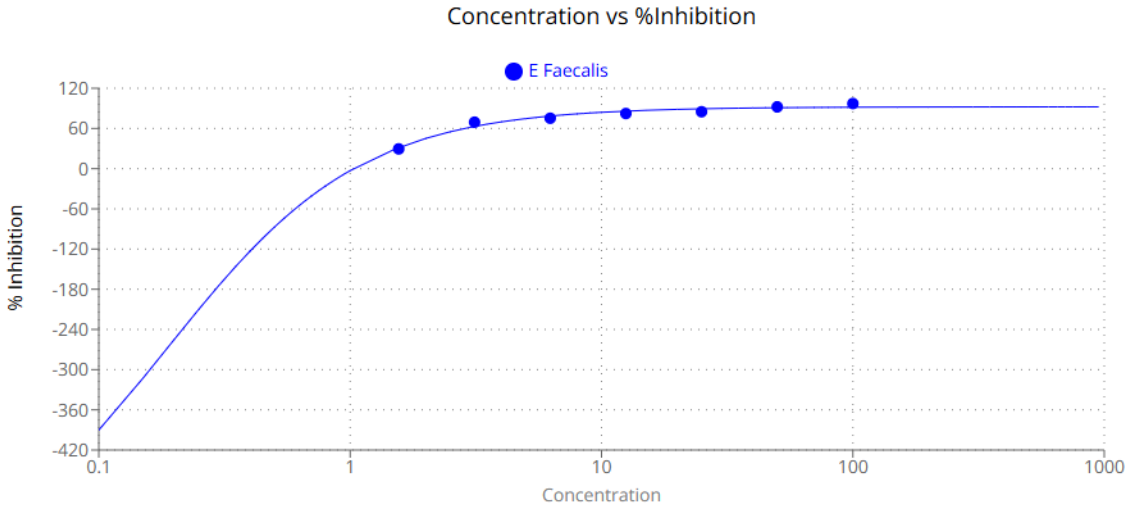

Diagram of IC_{50} value of leaf extract irrigant *Stevia rebaudiana* Bertoni Against *E.faecalis* Biofilms

Table 4. IC_{50} value of *Stevia rebaudiana* leaf extract irrigant Bertoni at a concentration of 2.21%.

IC_{50} Regression Results [E Faecalis]

Parameter	Value
IC_{50}	0.1861
Equations	(show alternative)
Equation	$Y = -630.6543 + \frac{92.3209 + 630.6543}{1 + (\frac{X}{0.1861})^{-1.1216}}$
Equation Form	$Y = Min + \frac{Max - Min}{1 + (\frac{X}{IC50})^{Hill\ coefficient}}$

IC_{50} Regression Calculator [E Faecalis]

Bulk Calculate Copy Table

X	Y
2,214763797624202	50

DISCUSSION

The results of the determination test of the test plants in this study, based on the identification of fresh plant samples by the Botanical Characterization Laboratory of the National Research and Innovation Agency in Cibinong, Bogor, were declared as *Stevia rebaudiana Bertoni* plants from the *Asteraceae* tribe. This is in accordance with the taxonomic classification of the test plant which is generally known as sweet leaf or honey leaf with species *rebaudiana Bertoni*, *genus Stevia*, *family Asteraceae*, *order Asterales*, *class Magnoliopsida*, *division Magnoliophyta* and *kingdom Plantae*.[3(4] The study of the antibiofilm potential of dental root canal irrigation solution from *Stevia* leaf extract against *Enterococcus faecalis* bacteria was carried out considering that irrigation solution plays an important role in the process of cleaning and disinfecting dental root canals, especially in dental root canal areas with complex anatomy, accessory root canals, apical ramifications and the presence of bacteria in deep dentin tubules in the walls of dental root canals. All these areas, which are difficult to access during mechanical cleaning of the root canal by endodontic instruments, may harbor the presence of bacteria in the form of biofilm structures adhering to the dentin surface of the root canal wall, thus requiring the role of antimicrobial and antibiofilm irrigation solutions.[12,26] Microbial biofilms, which consist of many species in heterogeneous communities, are known to be a common cause of infection in root canals because they have higher resistance to antimicrobial agents.[5,12] Biofilms can also support bacterial growth and reproduction and increase bacterial viability through the exchange of nutrients and signaling molecules that aid in bacterial attachment and adaptation to specific environments.[5] Biofilm formation of *E.faecalis* bacteria can occur because *E.faecalis* bacteria are known to have virulence factors that are significantly involved in adhesion and biofilm formation, including *E.faecalis regulator B* (fsrB), adhesion of collagen (ace), extracellular surface protein (esp), *gelatinase* (geIE) and aggregation substance (asa).[(3)(5)] Other literature also explains that the virulence factors of *E.faecalis* bacteria so that they are able to cause dental root canal infections and induce an inflammatory response in the periradicular area are bacterial surface adhesins, sex pheromones, lipoteichoic acid, extracellular superoxide production, gelatinase enzymes and hyaluronidase enzymes.[2]

One strategy to overcome infections related to *E. faecalis* biofilm is to utilize *phytoterapic* agents from plants to inhibit bacterial biofilm formation[5]. The utilization of *Stevia* leaf extract as one of the herbal plants has been studied to have a promising ability to be used as a root canal irrigation solution because it contains bioactive components that are antibacterial with bacteriostatic and bactericidal abilities against *E.faecalis*.[18,19] *In vitro* studies of the *E.faecalis* antibiofilm potential of *Stevia* leaf extract irrigant conducted to date, have shown that all variations in concentration of *Stevia rebaudiana* Bertoni leaf *ethanol* extract irrigation solution tested, proved to have the ability to inhibit the formation of *E.faecalis* ATCC 29212 bacterial biofilm. The ability to inhibit biofilm formation was assessed based on the results of measuring the OD value using a *microplate reader* at a wavelength of 490 nm.[29] Measurement of the OD value indicates the population density of the bacterial biofilm or biofilm mass consisting of bacterial cells and biofilm extracellular matrix.[(35)] The mean experimental OD values of the *Stevia* 100%, *Stevia* 50%, *Stevia* 25%, *Stevia* 12.5%, *Stevia* 6.25%, *Stevia* 3.125% and *Stevia* 1.56% leaf extract irrigants which appeared significantly lower than the mean experimental OD value of the negative control which was 2% BHI-B + sucrose media solution ($p<0.05$) proved that the ability to inhibit biofilm formation of all concentrations of *Stevia* leaf extract irrigants tested was significantly greater than the negative control. The average experimental OD

value of the 100% *Stevia* leaf extract irrigant did not have a significant difference when compared to the average experimental OD value of the positive control, namely 2.5% NaOCL solution ($p>0.05$), proving that the 100% *Stevia* leaf extract irrigant has the ability to inhibit biofilm formation of *E. faecalis* bacteria which is not significantly different from the 2.5% NaOCL solution as a positive control and *gold standard* irrigation solution. The average experimental OD values of 50% *Stevia* leaf extract irrigant, 25% *Stevia* , 12.5% *Stevia*, 6.25% *Stevia*, 3.125% Stevia and 1.56% *Stevia* which appeared significantly greater when compared to the average experimental OD value of the positive control, namely NaOCL 2.5% solution ($p<0.05$) proved that its ability to inhibit biofilm formation of *E.faecalis* bacteria was significantly lower than NaOCL 2.5% solution as a positive control. The percentage value of *Enterococcus faecalis* biofilm formation inhibition from the irrigation solution of *Stevia* leaf extract at concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% in this study sequentially increased in line with the increase in the concentration of *Stevia* leaf extract irrigant tested. The highest percentage value of *Enterococcus faecalis* biofilm formation inhibition of 97.22% was shown by the irrigation solution of *Stevia* leaf extract with a concentration of 100% while the irrigation solution of *Stevia* leaf extract with a concentration of 1.56% showed the lowest percentage value of *Enterococcus faecalis* biofilm formation inhibition of 24.49%. The positive control showed a percentage inhibition value of *Enterococcus faecalis* biofilm formation of 97.27% while the negative control showed a percentage inhibition value of *Enterococcus faecalis* biofilm growth of 0%. The percentage of *Enterococcus faecalis* biofilm inhibition by *Stevia* leaf extract irrigant in this study seems to be directly proportional to the increase in the amount of bioactive compounds it contains, so that the greater the concentration of *Stevia* leaf extract solution tested, the greater its ability to inhibit *Enterococcus faecalis* biofilm. The ability of *Stevia* leaf extract to inhibit the formation of bacterial biofilms has also been proven from the results of other studies by Hastuti A in 2019 which explained that a solution of *Stevia* leaf extract at concentrations of 45%, 75% and 90% under incubation temperature conditions of 30 ^{0}C and 37 ^{0}C and pH conditions of 6, 7 and 9, was able to inhibit the formation of biofilms *of Bacillus s.strain* SA 1-3 and *Enterobacter sp.strain* SA 1-5.[20] Research by Escobar E *et al* 2020 also proved the ability of *Stevia* leaf extract to reduce the number of biofilms *of Streptococcus mutans bacteria.*[36] Further research by Theophilus PAS *et al* in 2015 has also proven the antibiofilm ability of *Stevia* leaf extract significantly against *Borrelia burgdorferi* bacteria as gram-negative bacteria that cause lyme disease.[37]

The antibiofilm potential of *Stevia* leaf extract irrigant inhibit biofilm formation of *Enterococcus faecalis* bacteria can occur, among others, due to the contribution of secondary metabolite compounds. Based on previous research by Deviyanti S et al in 2022, qualitative phytochemical analysis on ethanol extract solution of *Stevia rebaudiana Bertoni* leaves has proven the content of secondary metabolite compounds alkaloids, terpenoids, flavonoids, tannins, saponins and steroids from *Stevia* leaf extract with antibacterial activity.[18] Research by Deviyanti S et al further in 2024 has also explained that the irrigation solution of *ethanol* extract of *Stevia rebaudiana Bertoni* leaves has been shown to have bacteriostatic and bactericidal properties against *Enterococcus faecalis*.[19] The MIC value against *Enterococcus faecalis* bacteria was shown by the irrigation solution of *Stevia* leaf ethanol extract with a concentration of 6.25% in the turbidimetric method and the irrigation solution of *Stevia* leaf *ethanol* extract with a concentration of 1.56% in the *total plate count* method, while the MBC value was shown by the irrigation solution of *Stevia* leaf ethanol extract with a concentration of 50%.[19]

The antibiofilm mechanism of the *ethanol* extract irrigation solution of *Stevia rebaudiana Bertoni* leaves evaluated in this study, can occur through various mechanisms.[27] The antibiofilm mechanism of terpenoid compounds by damaging *planktonic* cells in biofilms and disrupting the integrity of the bacterial cell membrane.[23] Alkaloid bioactive compounds play a role in biofilm inhibition through inhibiting cell communication activity. [38] Bioactive tannin and flavonoid compounds inhibit biofilms through binding to one of the bacterial adhesin proteins used as bacterial surface receptors resulting in a decrease in bacterial attachment and inhibition of protein synthesis for cell wall formation.[22] These tannin and flavonoid compounds can also inhibit the expression of icaA and icaD genes that synthesize Polysaccharide Intercellular Adhesion (PIA) so that the impact causes inhibition of the formation of extracellular polymeric substances (EPS) as a biofilm matrix.[(2)(2),(38)]EPS, which consists of protein components, polysaccharides, glycoproteins, extracellular nucleic acids and phospholipids, plays an important role in the formation, growth, stability and maintenance of biofilms.[9]

The antibiofilm ability of NaOCL 2.5% as a positive control with an experimental OD value that has no significant difference with 100% *Stevia* leaf extract irrigant and has an *E. faecalis* biofilm formation inhibition value of 97.27% in our current research, seems to be in line with the results of research by Frough-Reyhani M *et al* in 2016 which proves that NaOCL 2.5% and NaOCL 5.2% irrigation solutions have a great ability to eliminate all *E. faecalis* biofilms aged 4.6 and 10 weeks to produce colony forming unit calculations of 0.[11] The antibiofilm mechanism of action of NaOCL 2.5% occurs through the reaction between oxidized hypochlorite (OCL $^{-)}$ and extracellular polymeric materials from the biofilm matrix (such as proteins and polysaccharides). The rapid chemical reaction will form bubbles with the main gas content being carbon dioxide and chloroform components.

This chloroform component is likely a reaction product of the oxidation of polymeric content of the biofilm matrix and/or peptidoglycan (a component of the cell wall of gram-positive bacteria) by hypochlorous acid ($HOCL^-$) from NaOCL. Bubble formation can lead to the breakdown of the biofilm structure allowing for biofilm dissolution or mechanical biofilm removal at different stages.[6]

Calculation of the IC_{50} value based on the linear regression equation between the sample concentration and the percentage of biofilm inhibition. In our current study after knowing the percentage value of biofilm inhibition, shows the IC_{50} value of 2.21 which means that the concentration of *Stevia* leaf extract irrigant that is able to inhibit biofilm of *E. faecalis* bacteria up to 50% is 2.21% concentration. The IC50 value is the concentration value of the *Stevia* leaf extract solution that is able to inhibit the biofilm of *E. faecalis* bacteria by 50%.[22] The IC_{50} value is often used in biofilm inhibition tests, the smaller the IC_{50} value, the more effective the tested sample is in inhibiting biofilm formation.[(2(7)]

Although *Stevia* leaf extract has been shown to have antibiofilm potential against *E.faecalis* bacteria, *in vivo* studies related to the utilization of irrigation solution from *Stevia* leaf extract as an antibiofilm agent still need to be conducted to confirm its safety and efficiency aspects for use in the field of dentistry so that it can help improve clinical success in overcoming persistent infections in dental root canals caused by biofilms of *E. E.faecalis* bacteria.

CONCLUSION

All *Stevia rebaudiana* Bertoni leaf extractirrigation solutions tested had antibiofilm potential against *E. faecalis* ATCC 29212 bacteria that was significantly greater than the negative control. *Stevia* leaf extract irrigants of 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% had significantly less antibiofilm potential than NaOCL 2.5%. The *E.faecalis* antibiofilm potency of 100% *Stevia* leaf extract irrigant was not significantly different from that of 2.5% NaOCL solution.

The percentage of *E. faecalis* biofilm inhibition by *Stevia rebaudiana* Bertoni leaf extract irrigant increased in line with the increase in concentration of *Stevia* leaf extract irrigant tested, which ranged from 29.49%-97.22%, while the percentage of *E. faecalis* biofilm inhibition by 2.5% NaOCL solution as a positive control was 97.27%.

The IC_{50} value of the *Stevia* leaf extract irrigant, which showed its ability to inhibit the biofilm of *E. faecalis* bacteria by 50%, was shown by the concentration of 2.21% *Stevia* leaf extract irrigant.

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REFRENCES

I. Hargreaves KM, Berman LH,. *Cohen's Pathway of the Pulp*.11th ed. St.Louis: Elsevier.2016:209- 260.
II. Alghamdi F, Shakir M. The influence of Enterococcus faecalis as a Dental Root Canal Pathogen on Endodontic Treatment: A Systematic Review.*Cureuc*.2020.:1-10.
III. Deng Z, Lin B, Liu F, Zhao W. Role of Enterococcus faecalis in Refractory apical Periodontits:from Pathogenicity to Host Cell Reponse. *Journal of Oral Microbiology*.2023;15:1-15.
IV. Liu Y, Ping Y, Xiong Y, Zhou R, Xu F, Wang J. Genotype, Biofilm Formation Ability and Specific Gene Transcripts Characteristics of Endodontic Enterococcus faecalis Under Glucose Deprivation Condition. *Archives of Oral Biology*.2020;118:1-8.
V. Yang S, Meng X, Zhen Y, Baima Q, Wang Y, Jiang X, Xu Z. Strategies and Mechanisms Targeting Enterococcus faecalis Biofilms Associated with Endodontic Infections: A Comprehensive Review. *Front.Cell.Infect.Microbiol*.2024:1-15.
VI. Gao Y, Jiang X, Lin D, Chen Y, Tong Z. The Starvation Resistance and Biofilm Formation of Enterococcus faecalis in Coexixtence with Candida albicans, Streptococcus gordonii, Actinomyces viscousnor Lactobacillus acidophilus. *Journal of Endodotic*.2016;42(8):1233-1238.
VII. Fan W, Huang Z, Fan B. Effect of Prolonged Exposure to Moderate Static Magnetic Field and Its Synergistic Effects with Alkaline pH on Enterococcus faecalis. *Microb Pathog*.2018;115:117- 122
VIII. Ponce JB, Midena RZ, Pinke KH, et al. In Vitro Treatment of *Enterococcus faecalis* with Calcium Hydroxide Impairs Phagocytosis by Human Macrophages.*Acta Odontol Scan*.2019;77(2):158-163.
IX. Husaini IM, Oyewole OA, Sulaiman MA, Dabban AI, Sulaiman AN, Tarek R. Microbial-Antibiofilms: Types and Mechanism of Action. *Research in Microbiology*.2023:1-11.
X. Jamal M, Ahmad W, Andleeb S, Jalil F, Imran M, Nawaz M, et al.2018. Bacterial Biofilm and Associated Infection. *J.Chin.Med.Assoc*.2018;81:7-11.
XI. Frough Reyhani M, Ghasemi N, Soroush Barhaghi M, Amini M, Gholizadeh Y. Antimicrobial efficacy

of different concentrations of sodium hypochlorite on the biofilm of Enterococcus faecalis at different stages of development.*J Clin Exp*.*Dent*.2016;8(5): e480-484.

XII. Boutsioukis C, Aias-Moliz MT, Paz LEC,. A Critical analysis of Research Methods and Experimental Models to Study Irrigants and Irrigation Systems. *International Endodontic Journal*.2021:295-329.

XIII. Torabinejad M, Walton RE, Fouad AF.*Endodontics Principles and Practice*. 5th ed. St.Louis: Elsevier Saunders.2015 : 273-283,349.

XIV. Rashad HR, Al Haidar AH. Antimicrobial Efficacy of A Novel Herbal Endodontic Irrigant Against Enterococcus faecalis in Root Canals of Permanent Teeth: An In Vitro Study.*SEEJPH*.2024;602-611.

XV. Daga P, Asrani H, Farista S, Mishra P. Comparative Evaluation of Antimicrobial Efficacy of Neem, Miswak, Propolis and Sodium Hypochlorite Against Enterococcus faecalis using Endovac. *International Journal of Prosthodontics and Restorative Dentistry*.2017;7(2):60-65.

XVI. Arambula MM, Alvarado MO, Sanchez ML. Antibacterial Activity of Extract of Stevia rebaudiana Bertoni Against *Staphylococcus aereus, Staphylococcus epidermis and Pseudomonas aeruginosa*. *Journal of Medicinal Plant Research.*2017;11(25):414-418.

XVII. Nied MM.Influence of Stevia rebaudiana on Oral Health-Literature Review.*Revista da Faculdade Odontogia de Porto Alegre*.2019;2:83-90.

XVIII. Deviyanti S, Abraham S, Pasiga DP. Antibacterial potential of Stevia rebaudiana Bertoni leaf extract on the growth of Enterococcus faecalis bacteria. *Azerbaijan Medical Journal*.2022;62(10):5671-5680.

XIX. Deviyanti S, Abraham S, Pasiga DP. Effectivity of irrigation solution from Stevia rebaudiana Bertoni leaf extract on the growth of Enterococcus faecalis bacteria. *International Journal of Phamaceutical and Bio Medical Scienc*.2024;4(3):176-181.

XX. Hastuty A. Antibiofilm and antimicrobial activities of papaya (*Carica papaya L*.) and stevia *(Stevia rebaudiana Bertoni*) leaf extracts against three biofilm-forming bacteria. *Journal of Microbial Systematics and Biotechnology*, 2019;*1*(1): 19–29. https://doi.org/10.37604/jmsb.v1i1.18

XXI. Sumaryono and Sinta MM. *Stevia Plant Cultivation*. Bogor: Indonesian Biotechnology and Bioindustry Research Center; 2015:1-2.

XXII. Tobi CHB, Saotarini O, Rahmawati I. Antibiofilm Activity of Extracts and Fractions of Areca nut (Areca catechu L.) against *Staphylococcus aureus* ATCC 25923.*Journal of Pharmaceutical Science and Clinical Research*.2020;01:56-70.

XXIII. Suhartono S, Soraya C, Shabira P. Antibiofilm Activity of Neem Leaf (Azadirachta indica A.Juss)) Ethanolic Extract Against Enterococcus faecalis In Vitro. *Dental Journal*.2023;56(2):98- 103.

XXIV. Pandey A, Tripathi S. Concept of Standardization, Extraction, and Phytochemical Screening Strategies for Herbal Drug. *Journal of Pharmacognosy and Phytochemistry*.2014;2(5):115-119.

XXV. Chandra A. Initial Study of Batch Extraction of Stevia rebaudiana Leaves with variable Solvent Types and Extraction Temperatures. *Pros Sem Nasy Mas Biodiv Indon*.2015;1(1):114-115.

XXVI. Zand V, Lotfi M, Soroush MH, Abdollahi AA, Sadeghi M, Mojadadi A. Antibacterial efficacy of different concentrations of sodium hypochlorite gel and solution on *Enterococcus faecalis* biofilm. *Iranian Endodontic Journal*.2016;11(4):315-319.

XXVII. Besan EJ, Rahmawati I, Saptarini O. Antibiofilm Activity of Telang Flower (*Clitoria ternatea L*) Extract and Fractions Against *Stapyhlococcus aureus.Pharmaceutical Journal of Indonesia*.2023;20(01):1-11.

XXVIII. Pant DR, Pant ND, Saru DB, Yadav UN, Khanal DP. Phytochemical Screening and Study of Antioxidant, Antimicrobial, Antidiabetic, Anti-inflamatory and Analgesic Activities of Extracts From Stem Wood of *Pterocarpus marsupium* Roxburgh. *Journal of Intercultural Ethnopharmacology*.2017.

XXIX. Maulina SA, Soulissa AG, Widyarman AS. Antibiofilm Effect of Rambutan Leaf Extract (Nephelium lappaceum L.) on Selected Periodontal Pathogens. *Journal of Indonesian Dental Association*.2023;5(2):57-61.

XXX. Balhaddad AA, AlSheih RN. Effect of Eucalyptus Oil on Streptococcus mutans and Enterococcus faecalis.*BDJ Open*. 2023;9(26).

XXXI. Fathi M, Ghane M, Pishkar L. Phytochemical Composition, Antibacterial and Antibiofilm Activity of Malva sylvestris Against Human Pathogenic Bacteria. *J Nat Pharm Prod*.2022;17(1):e114164

XXXII. Fouad AF. *Endodontic Microbiology*.2nd ed. Singapore:John Wiley & Sons.2017:129-170.

XXXIII. Angestia W, Ningrum V, Lee TL, Lee SC, Bakar A. Antibacterial Activities of Moringa Olifiera Freeze Dried Extract on *Staphylococcus aureus*. *Journal of Dentomaxillofacial Science*.2020;5(3):154-157.

XXXIV. Kazmi A, Khan MA, Mohammad S, Ali A, Ali H. Biotechnological production of natural calorie free steviol glycosides in *Stevia rebaudiana*: an update on current scenario.*Current Biotechnology*. 2019;8(2):70-84.

XXXV. Mira P, Yeh P, Hall BG. Estimating Microbial Population Data From Optical Density. *PLoS One*.2022;17(10):1-8.

XXXVI. Escobar E, Piedrahita M, Gregory RL. Growth and Vialbility of Streptococcus mutans in Sucrose with Different Concentrations of *Stevia rebaudiana* Bertoni. *Clinical Oral Investigation.*2020:1- 6.

XXXVII. Theophilus PASVictoria MJ, Socarras KM, Filush KR, Gupta K, Luecke DF, Sapi E. Effectiveness of *Stevia rebaudiana* Bertoni Whole Leaf Extract Against Various Morphological Forms of Borrelia Burgdorferi In Vitro. *European Journal of Microbiology and Immunology*.2015;5:268-280.

XXXVIII. Dewi MA, Gumilar A, Indah WS. Antibiofilm Activity of Papaya Leaf Extract (Carica papaya L.) against *Staphylococcus aureus. Pharmacoscript*; 7(1):122-133.