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EFFECTIVITY OF IRRIGATION SOLUTION FROM *Stevia rebaudiana* Bertoni LEAF EXTRACT ON THE GROWTH OF *Enterococcus faecalis Bacteries*

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ABSTRACT

Disinfection of *Enterococcus faecalis bacteria* in dental root canal treatment using NaOCL 0.5-2.5% is still a challenge. There is still very little data on the effectvity of *Stevia rebaudiana* Bertoni leaf extract irrigation solution against the growth of *Enterococcus faecalis*. This study evaluated the effectiveness of *Stevia rebaudiana* Bertoni leaf extract irrigation solution against *Enterococcus faecalis* ATCC 29212 through MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericide Concentration) values. A number of 100 μl of *E. faecalis* suspension was inoculated into 96 microtitration wells, each containing 100 μl of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56% of *Stevia* leaf extract irrigation solutions, positive (NaOCL 2.5%) and negative (distilled water) control. Incubation was carried out for 24 hours at 37°C then optical density was measured with a microplate reader at 600 nm. Five μl of 10^{-9} dilution of each group was dripped and spread onto nutrients agar plate. The number of *Enterococcus faecalis* colonies was counting manually. There was no significant difference in average colony count of *Enterococcus faecalis* between the 100% and 50% *Stevia* leaf extract irrigation solution groups and the positive control ($p>0.05$). However, the 25%, 12.5%, 6.25%, 3.125%, and 1.56% *Stevia* leaf extract irrigation solution groups showed significant differences ($p<0.05$). *Stevia* leaf extract irrigation solution suppressed growth at MIC 6.25% by turbidimetry and MIC 1.56% by total plate count method using nutrient agar and than killed *Enterococcus faecalis* at MBC 50%. The bactericidal ability of 100% and 50% *Stevia* leaf extract irrigation solution is equal to NaOCL 2.5%.

Keywords: *Stevia rebaudiana* Bertoni, irrigation solution, *Enterococcus faecalis*, MIC, MBC

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INTRODUCTION

One part of the cleaning and shaping stage is the use of irrigation solutions to disinfect the root canal system which plays an important role in achieving successful endodontic treatment. The biological purposed of using an irrigation solution in root canal treatment, related to the antimicrobial effect and the capacity of the irrigation solution to get rid or lessen bacteria in the root canal effectively. The basic principles for achieving the biological objectives of a root canal irrigation solution are that it must have "high efficacy against anaerobic and facultative microorganisms in their planktonic state and biofilms, inactive endotoxin, be nontoxic when they come in contact with vital tissues, and not cause *anaphylactic* reaction."[1] The regular used solution for root canal irrigation is NaOCl solution with 0.5% to 5.25% concentration and is the gold standard "because of its antibacterial capacity and the ability to dissolve necrotic tissue, vital pulp tissue and organic components of dentin and biofilms." in the root canal system.[1,2,3,4] However, NaOCl irrigation solution still has disadvantages until now, namely it is cytotoxic to intra oral soft tissues and peri-radicular tissues, has an unpleasant taste and odor, does not have the ability to dissolve inorganic materials in the root canal, can cause corrosion of metal objects and can cause hypersensitivity reactions [1,3,4] Through root canal disinfection is still a challenge because of root canal anatomy complexity.[5] The bacterium *Enterococcus faecalis*, which is known to cause 80-90% of cases of endodontic secondary infection (infection after endodontic treatment failure), is known to evade the action of endodontic instruments and irrigation solutions during the chemomechanical preparation stage of root canal treatment.[1,4] Related to the obstacles that are still found in NaOCl irrigation solutions and in order to find alternative irrigation solutions that are effective against *Enterococcus faecalis* bacteria, research on alternative irrigation solutions sourced from herbal ingredients, one of which is from *Stevia rebaudiana* Bertoni leaf extract, is now starting to be developed.[6] Shrub plant named *Stevia rebaudiana* Bertoni is originating from South American and has antibacterial activity.[7] Research by Deviyanti S et al in 2022 has proven that all test solutions of 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% concentrations of extract *Stevia rebaudiana Bertoni* leaf have ability to form inhibition zone towards *Enterococcus faecalis* ATCC 29212 with the largest inhibition zone diameter of 23.07 mm at 100% *Stevia* leaf extract concentration and the smallest inhibition zone diameter of 10.34 mm at 1.56% *Stevia* leaf extract concentration.[6] The inhibition activity to this bacteria growth seems to be related to the phytochemical content of flavonoids, steroids, tannins, alkaloids and saponins which are proven to be contained in this extract.[6] This in vitro research was purposed to

evaluate the effectiveness of irrigation solution from *Stevia rebaudiana* Bertoni leaf extract on the growth of *Enterococcus faecalis* bacteria by evaluating the MIC (Minimum inhibitory Concentration), MBC (Minimum Bactericide Concentration) and measuring the colony count of *Enterococcus faecalis* bacteria.

METHOD

Plant extract

The dried *Stevia* leaves were minced using a blender and sieved with a 100 *mesh* sieve. A total of 150 g of Stevia leaf fine powder (simplisia) was put into 3 Duran bottles containing 50 g in each bottle and 1200 ml of 96% ethanol solvent was added which was divided into three Duran bottles so that each bottle contained 400 ml. The Duran bottles were then tightly closed and shaken until well mixed. and left for 3 days at 27°C. During the maceration process, each Duran bottle was jiggled in 15 minutes with interval 8 hours every day until the soluble ingredients could be dissolved.[10] The sample was filtered through *Whatman* paper in a glass funnel and evaporated using a rotary evaporator at 72°C until a thick *Stevia* leaf extract was obtained, then stored in centrifuge tubes at -20°C for later use.

Bacterial strain culture

Reference strain of *Enterococcus faecalis* (ATCC 29212) was used. To culture *E. faecalis* strain ATCC 29212, 20 μl of bacteria were added to 5 ml of Nutrient broth medium (Sigma-Aldrich, St. Louis, USA) and incubated at 37°C for 24 hours. The optical density (OD) of the bacterial suspension was determined at 600 nm using a microplate reader (MP 96 SAFAS, Monaco), and adjusted to match the McFarland standard of $1.5x10^{-8}$ (CFU/ml)

Preparing irrigation solution with *Stevia rebaudiana* Bertoni leaf extract.

Eppendorf tubes were used to prepare 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56% *Stevia* leaf extract irrigation solutions using the serial dilution method. To prepare a 100% *Stevia* leaf extract solution in tube 1, 1 g *Stevia* leaf extract was dissolved in 1 ml of sterile distilled water. To make a 50% *Stevia* leaf extract solution, 500 μl of extract was transferred from tube 1 to tube 2 with 500 μl of sterile distilled water. Similar technique was repeated until a solution with 1.625% *Stevia* leaf extract was achieved.

Media preparation

Nutrient Agar (NA) media was prepared by weighing 2.6 g of Bacteriological Agar No.1 powder (Oxoid) plus 3 g of Nutrient broth No.3 powder (Sigma-Aldrich, St.Louis, USA) then dissolved with 200 ml of sterile distilled water in an erlenmeyer tube. Boiled and stirring using hot plate until dissolved homogeneous. The surface of the erlenmeyer tube was then tightly encased in aluminum foil and subjected to sterilization via autoclaving at 121°C for 15 minutes then stirred again and poured as much as 20 μl into each petri dish (petri disc). After media cool and solidify.then incubated for 24 hours at 37°C to ensure the media was not contaminated before being used for the total plate count assay.

MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericide Concentration) Determination

The MIC and MBC values determinated to see the antibacterial effectiveness of *E. faecalis* from *Stevia rebaudiana* Bertoni leaf extract irrigation solution in this in vitro research was carried out based on the broth microdilution method using 96-well microtitration plates followed by the total plate count method on *Nutrient Agar* medium in petri dishes.[11,12,13,16] The broth microdilution procedure began by inserting 100 μl of each concentration of *Stevia rebaudiana* Bertoni leaf extract irrigation solution tested (100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56%) and 100 μl NaOCL 2.5% and 100 μl of distilled water into the wells of 96-well microtitration plate in the 1st row in columns 1, 2, 3, 4, 5, 6, 9 and 11 respectively. A total of 100 μl of *E. faecalis* bacterial suspension that has been equalized with Mc Farland standard 0.5 was then inoculated into each well of 96-well microtitration plate that has contained each concentration of *Stevia* leaf extract irrigation solution tested as well as into the control group solution, then incubated at 37°C for 24 hours. Blank solution (serial dilutions of *Stevia* leaf extract irrigation solution and control solution without *E. faecalis*) in the 5th row in columns 1, 2, 3, 4, 5, 6, 9 and 11. Three replications (triplo) were performed for all measurements. Optical Density (OD) measurement using a microplate reader at 600 nm, after 24 hours incubation for turbidity assessment. Dilution using sterile PBS (*Phosphate Buffer Saline-Oxoid*) up to 10^{-9} was performed on each test concentration of *Stevia rebaudiana* Bertoni *leaf* extract irrigation solution as well as the control group on 96-well microtitration plate after incubation at 37°C for 24 hours. A total of 9 Petri dishes containing *Nutrient Agar* media were prepared and confirmed to be free of contaminants after previous incubation for 24 hours. Each Petri dish was divided into 3 parts for one type of concentration of *Stevia* leaf extract irrigation solution tested as well as for each positive and negative control tested. The results of 10^{-9} dilutions of

each sample concentration of *Stevia rebaudiana* Bertoni leaf extract irrigation solution tested and the control solution were then vortexed until homogeneous and taken as much as 5 μl to be dripped (subculture) onto each part of the *Nutrient Agar* surface in a petri dish and then spreaded on the surface of the *Nutrient Agar* medium in a petri dish. All Petri dishes were then incubated for 24 hours in an anaerobic jar at 37°C and the number of bacterial colonies that grew was counted. The formula for calculating the number of colonies (CFU or colony forming unit) of the sample:[13]

$$\text{CFU/ml} = \xi \frac{\text{Colonies per dish}}{\text{Dilution factor}}$$

Percentage inhibition of bacterial growth, calculated by the formula: [12]

$$\%\ \text{inhibition} = \frac{\text{OD of Bacterial Control-(OD of TEST Solution-OD of } \textit{Stevia} \text{ Control) x100\%}}{\text{OD of Bacterial Control}}$$

Statistical analysis

The SPSS program version 26 was used for statistical analysis, including the Kruskal Wallis and post-hoc Mann-Whitney tests.

RESULTS

Stevia rebaudiana Bertoni Leaves Maceration Extraction Results

The maceration of 150 grams of *Stevia rebaudiana* Bertoni leaf simplisia resulted in a total of 22.47 grams of thick extract, which was then filtered and evaporated for 1.5 hours using a rotary vacuum evaporator.

Effectivity of *Stevia* Leaf Extract Irrigation solution against Colony Count of *E.faecalis* Bacteries

The total plate count *Stevia rebaudiana* Bertoni method results of leaf extract irrigation solution concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% and the control group against *E. faecalis* bacteria (Figure 1A to 1I).

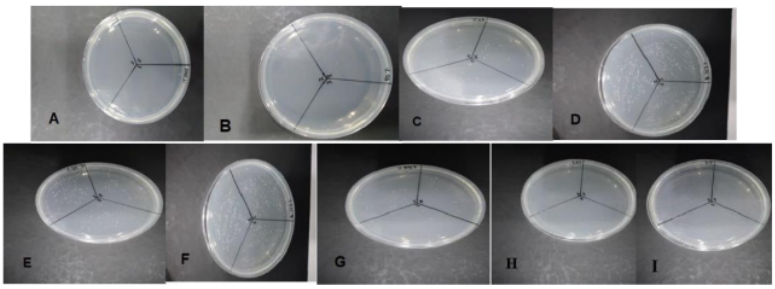

Figure 1 Formed colony of *E.faecalis* bacteria by *Stevia* leaf extract (A) 100%, (B) 50%, (C) 25%, (D) 12.5%, (E) 6.25%, (F) 3.125%, (G) 1.56%, (H) Control +, (I) Control -.

The mean colony count of *E.faecalis* bacteria (CFU/ml) in the *Stevia rebaudiana* Bertoni leaf extract irrigation solution group in various test concentrations as well as the control group can be seen in table 1.

Table 1. MEAN OF COLONY COUNT *Enterococcus faecalis* BACTERIES

NO	CONCENTRATE TYPE	FIRST TEST (CFU/ml)	SECOND TEST (CFU/ml)	THIRD TEST (CFU/ml)	MEAN
1	Extract Stevia 100%	0	0	0	0
2	Extract Stevia 50%	0	0	0	0
3	Extract Stevia 25%	2.00E+07	3.80E+07	4.20E+07	3.33E+07
4	Extract Stevia 12.5%	4.38E+08	7.60E+07	1.54E+08	2.23E+08
5	Extract Stevia 6.25%	1.70E+08	8.00E+07	1.00E+08	1.17E+08
6	Extract Stevia 3,125%	3.92E+08	4.76E+08	3.32E+08	4.00E+08
7	Extract Stevia 1,56%	7.40E+07	4.00E+07	3.40E+07	4.93E+07
8	Control + (NaOCL 2,5%)	0	0	0	0
9	Control - (Aquadest)	2.00E+09	2.00E+09	2.00E+09	2.00E+09

The diagram of mean colony count of *E. faecalis* bacteria (CFU:Colony Form Unit/ml) in the treatment groups of *Stevia rebaudiana* Bertoni leaf extract irrigation solution in various concentrations tested as well as positive control group (NaOCL 2.5%) and negative control (aquadest) can be seen in figure 2.

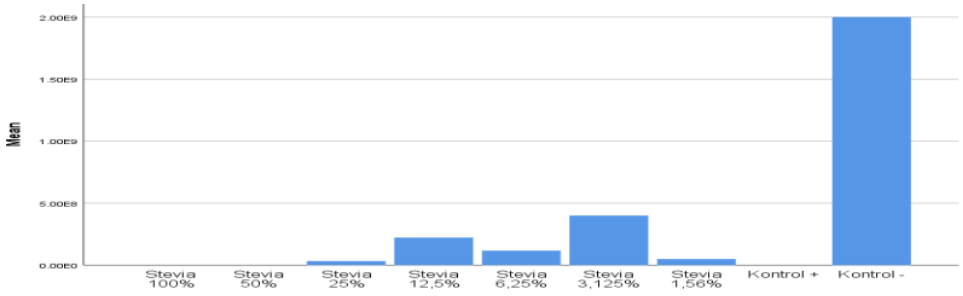

Figure 2. Diagram of mean colony count of *Enterococcus faecalis* bacteria (CFU:Colony Form Unit) in 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% *Steviarebaudiana* Bertoni leaf extract groups and K+ (NaOCL 2.5%) and K- (aquadest*)* groups

The results of the Shapiro-Wilk normality test (n<50) for 25%, 12.5%, 6.25%, 3.125% and 1.56% *Stevia rebaudiana* Bertoni leaf extract irrigation solution showed that the data were normally distributed (p>0.05), except for 100% and 50% *Stevia rebaudiana* Bertoni leaf extract irrigation solution, positive control and negative control (p<0.05). The results of the Kruskal-Wallis test of various concentrations of *Stevia* leaf extract and the control group on the mean colony count of *E.faecalis* bacteria, obtained a value of p=0.001 (p < 0.05), indicating that there is a difference in the mean colony count of *E.faecalis* bacteria at various concentrations of *Stevia* leaf extract irrigation solution and the control group so that it is continued with the Mann-Whitney post hoc test at the 95% confidence level. The results of the Mann-Whitney post hoc test showed that the 100% and 50% *Stevia* leaf extract irrigation solution groups did not show a significant difference in mean colony count of *E. faecalis bacteria* with the positive control group (p>0.05). The 25%, 12.5%, 6.25%, 3.125% and 1.56% *Stevia* leaf extract irrigation solution groups showed significant differences in mean colony count of *E.faecalis* bacteria with the positive control group (p<0.05). The 100%,50%, 25%, 12.5%, 6.25%, 3.125%, 1.56% *Stevia* leaf extract irrigation solution groups and the positive control group showed significant differences in mean colony count of *E.faecalis* bacteria with the negative control group (p<0.05). The percentage value of *E.faecalis* growth inhibition can be seen in table 2.

TABLE2. PERCENTAGE VALUE OF GROWTH INHIBITION OF *E.faecalis* BACTERIES

	A	B	C	D	E	F	G
K	100	50	25	12.5	6.25	3.125	1.56
% KB	112.8205	195.3846	-124.359	-126.154	-4.35897	43.84615	138.2051

K: concentration of *Stevia* leaf extract irrigation solution*;* % KB: percentage of growth inhibition of bacteria

DISCUSSION

The utilization of irrigation solutions from biological resources of *Stevia rebaudiana* Bertoni leaf extract as an effort to find alternative materials for dental root canal irrigation solutions against *Enterococcus faecalis* bacteria in this study, has proven effective in inhibiting and killing the growth of *Enterococcus faecalis* strain ATCC 2921 bacteria with MIC (Minimal Inhibitory Concentration) value at a concentration of 6.25% by turbidimetry method using microplate reader and MIC value at a concentration of 1.56% by total plate count method using nutrient agar and MBC (Minimal Bactericide Concentration) value at a concentration of 50%.

The determination of the MIC value is obtained from the smallest concentration of *Stevia* leaf extract irrigation solution samples that are able to show inhibition of the growth of *E.faecalis* bacterial colonies , which gives a negative value from the calculation of the percentage formula of inhibition of *E.faecalis* bacterial growth in turbidimetry methode and which exhibited the lowest total average of colony forming unit in total plate count methode. Results on Table 2 showed the percentage of growth inhibition of *E.faecalis* bacteria at a concentration of 6.25% there is the first negative value which means that growth inhibition (death of bacteria) begins to occur. Sample irrigation solution of *Stevia* leaf extract with the smallest concentration that isable to kill 99.9% of *E.faecalis* bacteria on Nutrient Agar in petri dishes (from the results of total colony count assay) in this study, designated as MBC. [11,14,15]

The average number of colony growth of *E.faecalis* bacteria in the *Stevia* leaf extract irrigation

solution group with a concentration of 6.25% as the MIC value in this study by turbidimetry is 1.17 x10^8 CFU/ml and 1.56% as the MIC value by total plate count is 4.93 x10^7. Based on the results of the Mann-Whitney post hoc test analysis, the average number of colony growth of *E.faecalis* bacteria in the *Stevia* leaf extract irrigation solution group with 6.25% concentration have no significant difference with the *Stevia* leaf extract irrigation solution groups with 25%, 12.5%, 3.125% and 1.56% concentration. Similarly, the average number of colony growth of *E.faecalis* bacteria in the *Stevia* leaf extract irrigation solution group with 1.56% concentration have no significant difference with the *Stevia* leaf extract irrigation solution groups with 25%, 12.5%, 6.25% and 3.125% concentration, although there were differences in the average number of colony growth of *E.faecalis* bacteria in the five groups of *Stevia* leaf extract irrigation solution. (table 1 and figure 2). In contrast to the negative control group which showed the highest growth of *E.faecalis* bacterial colonies, namely 2.000 x 10^9 CFU/ml, the *Stevia* leaf extract irrigation solution groups with concentrations of 25%, 12.5%, 6.25%, 3.125% and 1.56% have shown bacteriostatic activity which causes a significant decrease in the number of *E.faecalis* bacterial colony growth (table 1 and figure 2). *Stevia* leaf extract irrigation solution with concentrations of 100% and 50% showed the best antibacterial effectiveness equivalent ($p>0.05$) to the positive control group (NaOCL 2.5%) because it has the ability to kill bacteria (bactericidal) so that no growth of *E.faecalis* bacterial colonies was found from the total plate count assay results.

The total plate count assay *was* used in this study because it is the most sensitive way to calculate the number of viable live bacteria in the range of 30-300 CFU/ml.[16,17] The choice of dilution factor in this study was carried out based on considerations in order to obtain calculation results according to the standard range of the number of viable bacterial colonies. Measurement of the number of bacterial colonies in each petri dish in this study was replicated 3 times (triplo) to see the accuracy of the calculation results.[18] The difference of MIC value

from those two methods are caused by the number of bacterial cells counted. Turbidimetry method will include every bacterial cell in medium, viable or not viable cell. In contras only viable cell of bacteria will count in the total plate count method. [16,17]

The ability of *Stevia* leaf ethanol extract in various test concentrations to inhibit or kill the growth of *E. faecalis* bacterial colonies in this study can occur due to the content of bioactive substances with antibacterial activity in the form of phytochemical flavonoids, tannins, saponins, steroids, terpenoids, and alkaloids.[6,19] "Flavonoids cause damage to bacterial cell wall permeability and inhibit bacterial motility" [20] Tannins are known to cause bacterial cell wall damage through the mechanism of attacking bacterial cell wall polypeptides.[20] Saponins have hydrophobic and hydrophilic molecules that can reduce cell surface tension which results in the destruction of bacterial cells.[20] Steroids can cause leakage in liposomes resulting in a decrease in bacterial cell membrane integrity and changes in bacterial cell membranes that cause bacterial cells to become brittle and lysed.[20] Terpenoid phytochemicals exhibit antibacterial action, according to Griffin SG et al cited by Mahizan MA et al[21] happens through inhibition of the oxygen uptake process that limits bacterial respiration and inhibition of the oxidative phosphorylation process in bacterial cellular respiration. Alkaloid phytochemicals exhibit antibacterial action via the destruction of bacterial cell membranes.[22]

The results of this *Stevia* leaf extract irrigation solution research, however, cannot be compared with the results of other studies because there is no recent research that discusses the MIC and MBC values of *Stevia rebaudiana* Bertoni leaf extract irrigation solution with 96% ethanol solvent against *Enterococcus faecalis* ATCC 29212. Recent research related to the effectiveness of *Stevia* against the growth of *Enterococcus faecalis* bacteria, has been conducted by Badra H *et al* in 2023 in vitro, but evaluated the effect of intracanal medicinal use of *Stevia* gel mixed with chitosan on observation for 24 hours, 48 hours, 72 hours and after one week through the diffusion test method. His study reported evidence that Stevia gel

mixed with chitosan medicated was able to improve antimicrobial activity (in forming a zone of bacterial growth inhibition) against *Enterococcus faecalis* ATCC 29212 when compared to *Stevia gel* medicated and chitosan solution medicated in single dosage form.[23]

The bactericidal ability NaOCl 2.5% irrigation solution as a positive control against *Enterococcus faecalis* bacteria in this study, can occur through the mechanism of action of saponification reaction, neutralization reaction, hypochlorous acid formation, solvent action and high pH.[1] The content of hypochlorous acid (HOCl) in NaOCl irrigation solution which acts as an oxidizer, plays a role in activating bacteria because it causes degradation of amino acid and hydrolysis.[1,3] Mode of action from NaOCl which is able to release chlorine, is also known to inhibit crucial bacterial enzymes through irreversible oxidation of sulfhydryl (SH) groups [1] Hydroxyl ion action from NaOCl solutionas a strong base with high pH (pH>11), is also effective as an antimicrobial.

CONCLUSION

Stevia rebaudiana Bertoni leaf extract irrigation solution effectively inhibited and killed the growth of *Enterococcus faecalis* bacteria at MIC value of 6.25% by turbidimetry method using microplate reader and 1.56% by total plate count method using nutrient agar and MBC value of 50%. The bactericidal ability of 100% and 50% *Stevia* leaf extract irrigation solution is equivalent to NaOCl 2.5%.

Advice

Additional research is required to examine the capacity of stevia leaf extract to inhibit *Enterococcus faecalis* biofilms and confirm its efficacy in vivo.

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