AMJ
ISSN: 0005-2523

ISSN: 0005-2523
Volume 62, Issue 10, December, 2022

Antibacterial Potential of Stevia rebaudiana Bertoni Leaf Extract on The Growth of Enterococcus faecalis Bacteries

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Keywords:
Stevia rebaudiana Bertoni, Enterococcus faecalis, antibacterial, irrigation solution, dental root canal

ABSTRACT

Enterococcus faecalis bacteria are often found in cases of root canal treatment failure. Information on the antibacterial potential of Stevia rebaudiana Bertoni herbs against E.faecalis, is still very limited. To determine the antibacterial potential of Stevia rebaudiana Bertoni leaf extract against Enterococcus faecalis. In vitro laboratory experimental research with post test only design with control group. Stevia leaf extraction using maceration method. The antibacterial activity of Stevia leaf extract against Enterococcus faecalis was tested by agar well diffusion method. A total of 50 µl of Stevia leaf extracts in different concentration with positive control NaOCL 2.5% and negative control distilled water, were put into the wells on Nutrient agar plates that have been inoculated with E.faecalis. Incubate at 37°C for 24 hours. Zone of inhibition (ZOI) was measured using a caliper. The ZOI of all test concentrations of Stevia leaf extract were varied, with a maximum diameter of 23.07 mm and a minimum of 10.34 mm, significantly different ($P<0.05$) from the negative control. The growth inhibition of E. faecalis bacteria by 100% and 50% Stevia leaf extracts was not significantly different ($P>0.05$) compared to the positive control while 25%, 12.5%, 6.25%, 3.125% and 1,56% Stevia leaf extracts were significantly different ($P<0.05$) compared to the positive control. All test concentrations of Stevia leaf extract have the potential to inhibit the growth of *Enterococcus faecalis* bacteria. Stevia leaf extracts of 100% and 50% have the ability to inhibit *E.faecalis* bacteria which is equivalent to NaOCl 2.5%.

1. Introduction

Enterococcus faecalis is a facultative anaerobic gram-positive bacterium that is often found in dental pulp infections and 80-90% are found in cases of infection due to failure of root canal treatment (secondary endodontic infection) [1], [2]. Enterococcus faecalis is also known to be the most virulent bacteria and resistant to antibiotics (such as penicillin G, vancomycin) and antiseptics. This bacterium is difficult to

eradicate because it is able to survive (persistence) in dentinal tubules in the root canal as an area with limited access to antibiotics and antiseptics.1 These bacteria can also survive in the root canals of obturated teeth for at least 6 months even without the availability of nutrients from outside [3]. The ability of these bacteria to survive in the root canal system and cause secondary endodontic infections because they have the ability to adhere to dentin and invade dentinal tubules, are able to live in high pH and low pH environments, and are able to form biofilms, which contribute to resistance and persistence after antimicrobial procedures in the root canal [1], [4]. The pathogenicity of these bacteria ranges from causing life-threatening disease in compromised individuals to less severe conditions such as infection in the root canals of obturated teeth with chronic apical periodontitis.4 The virulence factors of this bacterium most associated with endodontic infection and peri-radicular inflammatory response are aggregation substance, surface adhesion, sex pheromones, lipoteichoic acid, extracellular superoxide production, the lytic enzymes gelatinase and hyaluronidase and the toxin cytolysin [4].

Medicinal plants are becoming very popular for the treatment of different diseases all over the world. Stevia rebaudiana Bertoni is one of the herbs known to have more than 100 types of phytochemical content, has been used in ancient medical systems for antimicrobial, antifungal, anti-oxidative, antitumor, anti-hypertention, hepatoprotective, hypoglicemic, and antiviral activity. Stevia rebaudiana Bertoni is a species of plant from the Asteraceae family.

This plant is known as honey leaf or sweet leaf because it contains Steviol glycoside component as a natural non-caloric sweetener that causes a sweet taste in its leaves. This plant originates from the highland areas of northeastern Paraguay and currently the Stevia plant has been recognized in various countries including Indonesia [5].

Various studies have proven that Stevia rebaudiana Bertoni leaf extract has antibacterial activity against various types of bacteria and fungi, such as Streptococcus mutans, Streptococcus sobrinus, Streptococcus salivarius, Streptococcus mitis, Lactobacillus acidophilus, Lactobacillus casei, Staphylococcus aereus, Staphylococcus epidermis, Pseudomonas aeruginosa and Candida albicans [6- 8]. However, information on research results related to the antibacterial activity of Stevia rebaudiana Bertoni leaf extract against the growth of Enterococcus faecalis bacteria, is still very limited until now. The purpose of this study was to determine the antibacterial potential of Stevia rebaudiana Bertoni leaf extract against the growth of Enterococcus faecalis bacteria.

2. Materials and Methods

2.1 Determination assay of Stevia rebaudiana Bertoni plant

Determination tests of *Stevia rebaudiana Bertoni* plants purchased from plantations in Ciputri village, Pacet sub-district, Cianjur district in West Java, were carried out at Herbarium Bogoriense, Botany Division-BRIN Biological Research Center in Cibinong, Bogor.

2.2 Phytochemical screening of Stevia rebaudiana Bertoni leaf extract

Detection of terpenoids (*Salkowski's test*) is done by adding 2 ml of chloroform (CHCL3) and 3ml of concentrated sulfuric acid (H2SO4) to 1ml of *Stevia* leaf extract solution. If a reddish brown color is formed, it indicates the content of terpenoid phytochemicals [9], [10]. Flavonoid detection is done by adding 5 ml of liquid ammonia (NH3) and 1 ml of concentrated sulfuric acid (H2SO4) into 1 ml of Stevia leaf extract solution. If a yellow color is formed, it indicates the content of flavanoid phytochemicals. [10]Steroid detection is done by adding 1 ml of anhydrous acetic acid (CH3 COOH) and 1 ml of concentrated

ISSN: 0005-2523
Volume 62, Issue 10, December, 2022

sulfuric acid (H2SO4) into 1 ml of *Stevia* leaf extract solution. If a green color is formed, it indicates the content of steroid phytochemicals [10]. Detection of *tannin* is done by adding 1 ml of *hydrogen chloride (*HCL) and 1ml of Dragendorff's reagent (potassium bismuth solution) into 1 ml of *Stevia* leaf extract solution. If a reddish brown or reddish orange color is formed, it indicates the content of alkaloid phytochemicals [10], [11]. Alkaloid detection is done by adding 1 ml of ferric chloride (FeCL3) into 1 ml of *Stevia* leaf extract solution. When formed green or purplish green color, indicating the content of alkaloid phytochemicals [9], [10]. Detection of saponins (foam test) is done by putting 0.5 g of *Stevia* leaf extract into 2 ml of water and then shaken. If foam is formed that lasts for 10 minutes, it indicates the content of saponin phytochemicals [12].

2.3 Preparation of extraction of Stevia rebaudiana Bertoni leaf with maseration methods

Dry leaf samples of *Stevia rebaudiana Bertoni* were pulverized with a blender and filtered using a mesh (100 mesh) to obtain 150 gr of simplisia which was poured into 3 *Duran* bottles so that each bottle contained 50 gr of *Stevia* leaf simplisia. The preparation of *Stevia* leaf extract was carried out through the maceration method by means of each Duran bottle then filled with 96% ethanol solvent as much as 400 ml and soaking for 3x24 hours at room temperature (25^0 C). The maceration solution was shaken periodically every 8 hours for 15 minutes each day. Furthermore, filtering was carried out with a glass funnel lined with Whatman paper and evaporation of the macerated solution using a rotary evaporator at a temperature of 72^0 C with a rotation speed of 60 rpm and a vacuum pump pressure of 900 mBar until a thick extract of *Stevia* leaves was obtained [12- 14]. *Stevia* leaf extract was stored in a -20^0 C freezer before being used for testing.

2.4 Preparation of E.faecalis culture bacteria

E. faecalis strain ATCC 29212 was cultured by growing 20 µl of bacteria on 5 ml of Nutrient broth medium and incubated for 24 hours at 37°C. Optical Density (OD) measurement of the bacterial suspension was carried out using a microplate reader at a wavelength of 600 nm and equalized the amount of bacterial density according to the Mc Farland standard of $1.5x10^8$ CFU/ml (Mc Farland 0.5).

2.5 Preparation of Stevia rebaudiana Bertoni leaf extract

The preparation of 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% *Stevia* leaf extract solution was done through serial dilution method in eppendorf tubes. A total of 1 g *Stevia* leaf extract was dissolved in 1 ml sterile distilled water to obtain 100% *Stevia* leaf extract solution in tube 1. The percentage of 50% *Stevia* leaf extract solution was made by transferring 500 µl of extract in tube 1 to tube 2 containing 500 µl sterile distilled *water*. The solution of 25% *Stevia* leaf extract was made by transferring 500 µl of extract in tube 2 to tube 3 containing 500 µl of sterile distilled water. The serial dilution procedure was continued until a solution of 1.625% *Stevia* leaf extract was obtained.

2.6 Detection of antibacterial activity of Stevia rebaudiana Bertoni leaf extract against E.faecalis

The *E. faecalis* antibacterial activity of *Stevia* leaf extract *was* tested using agar well diffusion method [15]. Sterile *Nutrient agar* media on 7 Petri dishes were prepared and incubated for 24 hours at 37^0 C. A total of 20 µl of fresh suspension of *E. faecalis* bacteria that has been equalized with Mc Farland 0.5 standard was streaked onto the surface of solid *Nutrient Agar* media in Petri dishes. Five *wells* in each Petri dish were made using a 6 mm diameter cork borer. A total of 3 wells in each petri dish (*triplo*), each filled with 1 type of specific concentration of *Stevia* leaf extract solution as much as 50 µl. The other two wells were each filled with positive control (NaOCL 2.5%) and negative control (sterile distilled *water*) by 50 µl in each petri dish. A total of 7 Petri dishes were then incubated under anaerobic conditions at 37^0 C for 24 hour. The mean ZOI formed was measured using a caliper in 3 directions (vertical, horizontal, diagonal) for each sample [16].

2.7 Statistical analysis

Statistical analysis was performed by Kruskal Wallis and post hoc Mann-Whitney test using the SPSS software version 26.

3. Results

Stevia rebaudianan Bertoni Plant Determination Test Results

The results of the determination test stated that the tested plant is the species *Stevia rebaudiana Bertoni* from the Asteraceae family.

3.1 Results of Maceration Extraction of Stevia rebaudiana Bertoni Leaves

The results of the extraction of 150 grams of *Stevia rebaudiana Bertoni* leaf simplisia by maceration method for 3x24 hours using 1200 ml of 96% ethanol solvent, followed by filtration then the filtrate is evaporated with a rotary vacuum evaporator for 1.5 hours at a temperature of 72^0 C can produce a thick extract weighing 22.47 grams.

3.2 Phytochemical Screening Result of Stevia rebaudianan Bertoni Leaf Extract

Phytochemical screening results of *Stevia rebaudiana Bertoni* leaf extract in this study showed the presence of bioactive compounds terpenoids, flavonoids, steroids, tannins, alkaloids and saponins (Table 1).

Table 1. Phytochemical screening results of ethanol extract of *Stevia rebaudiana Bertoni* leaves

PHYTOCHEMICAL TEST	*ETHANOLIC* EXTRACT OF *Stevia rebaudiana Bertoni* LEAVES	INDICATOR
Terpenoids	+	A reddish-brown color is formed,
Flavonoids	+	Yellow color formed
Steroids	+	Green color formed
Tannins	+	Forms a green or purplish green color
Alkaloids	*	Reddish brown or reddishorange color is formed
Saponins	+	Forms a foam that lastsfor 10 minutes

3.3 Antibacterial activity of Stevia leaf extract irrigation solution against E.faecalis:

The antibacterial activity test results of *Stevia rebaudiana Bertoni* leaf extract concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% and the control group against *E. faecalis* bacteria by agar well diffusion method (Figure 1a, 1b, 1c, 1d, 1e, 1f, 1g, 1h).

AMJ
ISSN: 0005-2523
ISSN: 0005-2523
Volume 62, Issue 10, December, 2022

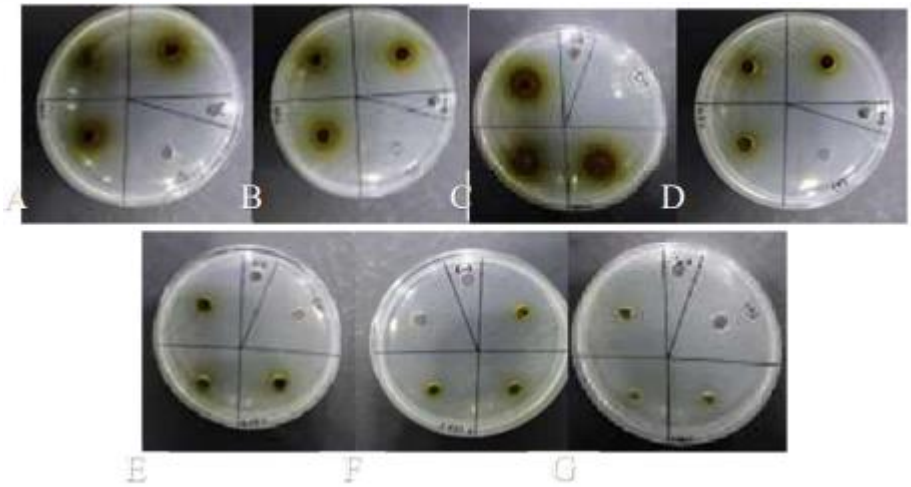

Figure 1 Zone of inhibition (ZOI) of *E.faecalis* bacteria by *Stevia* leaf extract
(A) 100%, K+ and K-, (B) 50%, K+ and K-, (C) 25%, K+ and K-,(D)12.5%, K+ and K-,
(E)6.25%, K+ and K-, (F)3.125%, K+ and K-, (G)1.56%, K+ and K-

The mean ZOI of *E.faecalis* bacteria (mm) in the *Stevia rebaudiana Bertoni* leaf extract solution group in various test concentrations as well as the control group can be seen in table 2.

Table 2. The mean ZOI of *E.faecalis* bacteria (mm) in the *Stevia* leaf extract groups in various test concentrations as well as the control group

		Stevia 100%	Stevia 50%	Stevia 25%	Stevia 12.5%	Stevia 6.25%	Stevia 3.125%	Stevia 1.56%	Control +	Control -
N	Valid	3	3	3	3	3	3	3	7	7
	Missing	0	0	0	0	0	0	0	0	0
Mean		23.0667	22.8433	21.0667	17.7900	16.4433	13.5567	10.3433	25.8714	.0000
Std. Deviation		1.51767	.80600	2.72015	.91995	1.83538	.87552	1.02788	2.04834	.00000
Minimum		21.70	22.10	18.13	17.00	14.63	12.63	9.73	22.40	.00
Maximum		24.70	23.70	23.50	18.80	18.30	14.37	11.53	29.00	.00

The diagram of mean ZOI of *E. faecalis* bacteria (mm) formed in the treatment groups of *Stevia rebaudiana Bertoni* leaf extract in various concentrations tested as well as the positive control group (NaOCL 2.5%) and negative control (aquadest) can be seen in figure 2.

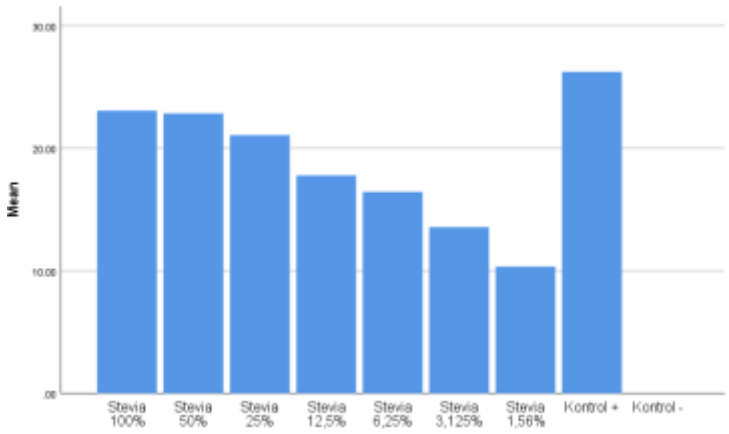

Figure 2. Diagram of mean ZOI of *Enterococcus faecalis* bacteria (CFU/ml) in 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% *Stevia rebaudiana Bertoni* leaf extract groups and K+ (NaOCL 2.5%) and K- (aquadest) groups.

The results of the Shapiro-Wilk normality test (n<50) for 100%, 50%, 25%, 12.5%, 6.25% and 3.125% *Stevia rebaudiana Bertoni* leaf extract showed that the data were normally distributed (p>0.05), except for 1.56% *Stevia rebaudiana Bertoni* leaf extract, 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 ZOI of *E.faecalis* bacteria, obtained a value of p = 0.000 (p < 0.05), indicating that there is a difference in the *mean* ZOI of *E.faecalis* bacteria at various concentrations of *Stevia* leaf extract 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 (table 3) showed that the 100% and 50% *Stevia* leaf extract solution groups did not show a significant difference in *mean* ZOI 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 solution groups showed significant differences in mean ZOI of E.*faecalis bacteria* with the positive control group (p<0.05). The 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% *Stevia* leaf extract solution groups showed significant *mean* differences in ZOI of E.*faecalis bacteria* with the negative control group (p<0.05).

Table 3. Results of Post Hoc Mann-Whitney ZOI Test of *E.faecalis* Bacteria Growth

	Stevia 100%	Stevia 50%	Stevia 25%	Stevia 12.5%	Stevia 6.25%	Stevia 3.125%	Stevia 1.56%	Control +	Control -
Stevia 100%		.827	.275	.050	.050	.050	.050	.051	**.003**
Stevia 50%	.827		.275	.050	.050	.050	.050	.051	**.003**
Stevia 25%	.275	.275		.127	.127	.050	.050	**.029**	**.003**
Stevia 12.5%	.050	.050	.127		.275	.050	.050	**.016**	**.003**
Stevia 6.25%	.050	.050	.127	.275		.050	.050	**.016**	**.003**
Stevia 3.125%	050	.050	.050	050	.050		.050	**.016**	**.003**
Stevia 1.56%	.050	.050	.050	.050	.050	050		**.016**	**.003**
Control +	.051	.051	**.029**	**.016**	**016**	**.016**	**.016**		**.001**
Control -	**.003**	**.003**	**.003**	**.003**	**.003**	**.003**	**.003**	**.001**	

Notes: Significant at p<0.05

4. Discussion

ISSN: 0005-2523
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Determination of Stevia rebaudiana Bertoni plants was carried out to ensure the correct identity of plant samples to be used in this study [17]. The results of the Stevia plant sample determination test used in this study, have shown the suitability of the taxonomic hierarchy of the Stevia rebaudiana Bertoni plant which states that the plant tested is the species Stevia rebaudiana Bertoni from the Asteraceae family [5].

The antibacterial activity test of Stevia rebaudiana Bertoni leaf extract against E.faecalis ATCC 29212 bacteria in this study was carried out using agar well diffusion method because it has the advantage of being able to accommodate more antibacterial agents tested compared to disc diffusion method so that it is expected that the inhibition zone formed is larger [18]. All variations of concentration of *Stevia rebaudiana Bertoni* leaf ethanol extract tested in this study, proved to be able to inhibit the growth of *E.faecalis* bacteria which was significantly different when compared to the negative control group ($p<0.05$). Mean ZOI appeared to be greater in line with the increase in concentration with the largest and smallest mean ZOI values of *Stevia rebaudiana Bertoni* leaf extract solutions of 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% were 23.07 mm, 22.84 mm, 21.07 mm, 17.79 mm, 16.44 mm, 13.56 mm and 10.34 mm respectively. The mean ZOI of the positive control (NaOCL 2.5%) was 25.87 mm while the negative control (aquadest) showed no ZOI. The ability of all concentration variations of *Stevia rebaudiana Bertoni* leaf extract solution tested in this study to inhibit the growth of E. faecalis bacteria is possible because it contains various bioactive compounds with antibacterial activity. Phytochemical testing in this study proved that Stevia rebaudiana Bertoni leaf extract contains bioactive compounds (phytochemicals) including terpenoids, flavonoids, steroids, tannins, alkaloids and saponins. The content of phytochemical compounds detected in Stevia rebaudiana Bertoni leaf extract in this study seems to be in line with the results of several other researchers. Research by [19] has reported that the phytochemical content in ethanol and methanol extracts of *Stevia rebaudiana Bertoni* leaves are phenols, tannins, saponins, glycosides and flavonoids. Research by [8] has also proven the phytochemical content of alkaloids, steroids, tannins, saponins and flavanoids in Stevia rebaudiana Bertoni leaves. The antibacterial activity of tannin phytochemicals against E.faecalis occurs through the mechanism of inhibiting bacterial cell wall synthesis by forming irreversible complexes with proline-rich proteins. The antibacterial activity of saponin phytochemicals occurs through its ability to break down proteins and enzymes from bacterial cells. The antibacterial activity of terpenoid phytochemicals, occurs through the dissolution of bacterial cell walls. Furthermore, flavonoid phytochemicals play an effective role in forming complexes with soluble proteins and bacterial cell walls. Steroidal phytochemicals have the ability of bacterial activity through the mechanism of liposome breakdown [20]. The antibacterial activity of alkaloid phytochemicals, occurs through inhibition of bacterial cell wall synthesis and disruption of the formation of peptidoglycan components in bacterial cells resulting in failure of bacterial cell wall layer formation [21]. Mean ZOI of *E.faecalis* bacteria growth of *E.faecalis* bacteria from 100% and 50% *Stevia rebaudiana Bertoni* leaf extract solution which is not significantly different ($p>0.05$) from the positive control group from the results of this study, indicating that both concentrations of *Stevia* leaf extract have antibacterial activity that is as good as the positive control (NaOCL 2.5%). The growth inhibition response category of *E faecalis* bacteria from 100% and 50% *Stevia rebaudiana Bertoni* leaf extract is classified as very strong according to Davis W and Stout TR (1971) cited by [22]. The group of *Stevia rebaudiana Bertoni* leaf extract with concentrations of 25%, 12.5%, 6.25%, 3.125% and 1.56% showed a difference in the mean ZOI of *E.faecalis* bacteria when compared to the positive control group ($p<0.05$) with the response category of *E faecalis* bacteria growth inhibition classified as strong according to Davis W and Stout TR (1971) cited by [22].

The ability of the entire *Stevia rebaudiana Bertoni* leaf extract in ethanol solvent to inhibit the growth of *E.faecalis* bacteria from the results of this study, seems to be in line with the research of Ghosh S *et al* cited by [6] states that the concentration of *Stevia rebaudiana Bertoni* leaf extract in ethanol solvent

concentration of 250 µg/ml and various other types of solvents, able to inhibit the growth of various bacteria and fungi including *E.faecalis* bacteria. The diameter of the inhibition zone of ethanol extract of *Stevia rebaudiana Bertoni* leaves against *E.faecalis* MTCC 2729 bacteria by disc diffusion method in ethanol, water, chloroform, cyclohexane, acetone and petroleum ether solvents from the results of the study in order are 5 mm, 5 mm, 6mm, 8.3 mm and 13 mm. The positive control (streptomycin) in the study produced an inhibition zone diameter of 18 mm.

The use of sodium hypochlorite (NaOCL) 2.5% as a positive control in this study because it has a very strong antimicrobial capacity [3], [23]. This is evidenced by the mean ZOI NaOCL 2.5% which is the greatest against the growth of *E.faecalis* bacteria compared to all groups of *Stevia rebaudiana Bertoni* leaf extract solutions and negative controls. The antimicrobial activity of NaOCL is based on high pH conditions as a strong base (pH> 11) which can disrupt the integrity of the cytoplasmic membrane of bacterial cells due to irreversible enzyme inhibition, cause biosynthetic changes in cellular metabolism and cause phospholipid degradation [23].

5. Conclusion

All *Stevia rebaudiana* leaf extract tested in this study were proven to have antibacterial potential of *Enterococcus faecalis* ATCC 29212 because they were able to form ZOI of bacterial growth.

Stevia rebaudiana Bertoni leaf extract 100% and 50% have a very strong inhibition response category with antibacterial potential to inhibit the growth of *Enterococcus faecalis* ATCC 29212 equivalent to NaOCL 2.5%.

Stevia rebaudiana Bertoni leaf extract 25%, 12.5%, 6.25%, 3.125% and 1.56% have a strong inhibitory response category and have antibacterial potential to inhibit the growth of *Enterococcus faecalis* ATCC 29212 which is significantly different from the positive control group.

The antibacterial ability of 25%, 12.5%, 6.25%, 3.125% and 1.56% *Stevia rebaudiana Bertoni* leaf extracts because they are proven to contain phytochemicals including terpenoids, flavonoids, steroids, tannins, alkaloids and saponins.

5.1 Advice

Further research is needed to test the effectiveness and toxicity of *Stevia rebaudiana Bertoni* leaf extract against *E. faecalis* bacteria so that its clinical utilization can be more optimal, among others, as an alternative solution for dental root canal irrigation.

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