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EVALUATION THE ANTIBACTERIAL ACTIVITY OF NEWLY INNOVATED MIXTURE OF STEVIA AND CHITOSAN AS AN INTRACANAL MEDICAMENTS ON ENTEROCOCCUS FAECALIS

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ABSTRACT

Purpose: This study aimed to evaluate the antibacterial effect of mixture of stevia and chitosan as an intracanal medicaments on Enterococcus Faecalis.

Materials and method: The agar diffusion method was used to evaluate the antibacterial effect of three intracanal medicaments (stevia gel by Carbopol, chitosan solution and mixture of stevia gel by chitosan) against Enterococcus faecalis. The zone of inhibition around each medicament was measured in millimetres after 24, 48, 72 hours and 1 week of incubation at 37°C. The antibacterial effect of each medicament against Enterococcus faecalis was measured using One way ANOVA and Tukey`s Post Hoc tests.

Results: After 24, 48 and after 72 hrs., Stevia gel by Carbopol group was significantly the lowest, while mixture of stevia gel by chitosan group was the highest with insignificant difference with Chitosan solution group . After 1 week, there was a significant difference between all groups as, Stevia gel by Carbopol group was significantly the lowest, followed by Chitosan solution and Stevia gel by Chitosan were significantly the highest.

Conclusion: Mixture of stevia gel by chitosan demonstrated the strongest antibacterial activities among tested medicaments.

KEYWORDS: Stevia, Chitosan, zone of inhibition, Enterococcus faecalis.

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INTRODUCTION

A gram-positive facultative anaerobic bacterium called Enterococcus faecalis (E.faecalis) is frequently detected in dental pulp infections and is responsible for 80–90% of secondary endodontic infections that develop when root canal therapy fails[1,2].

Enterococcus faecalis, is also resistant to antiseptics and antibiotics like penicillin G and vancomycin. Because it can persist in dentinal tubules in the root canal, which have restricted access to antibiotics and antiseptics, this bacterium is challenging to get rid of it [1].Even without the availability of nutrients from the outside, these bacteria can endure for at least 6 months in the root canals of obturated teeth [3] .

Because of their capacity to adhere to dentin and invade dentinal tubules, their ability to live in both high and low pH environments, and their capacity to form biofilms, which contribute to resistance and persistence after antimicrobial procedures in the root canal, these bacteria are able to survive in the root canal system and cause secondary endodontic infections [1,4].

Antimicrobial medications have secondary role to completely sterilise root canals and produce improved treatment results[5,6]. In endodontics, calcium hydroxide is frequently utilised as an intracanal medication in between appointments to treat necrotic permanent teeth[7,8]. Some bacterial strains, including Faecalis, are unaffected by it [8–10]. Therefore, faecalis is one of the most abundant and resistant bacteria species in both deciduous and permanent root canals, and it is linked to infections in the primary root canals along with a wide range of other microorganisms [11–13] .

Chitosan is a naturally occurring polysaccharide that exhibits bioadhesion, biodegradability, biocompatibility, and lacks toxicity [14]. Chitosan is a cationic biopolymer with long-lasting antibacterial effects and affordable manufacturing processes [15]. Chitin, which is present in crab and prawn shells, is deacetylated to produce chitosan [16].

Chitosan has a variety of biological qualities, including those that make it antibacterial, antifungal, biodegradable, and biocompatible. Pharmaceutical drug delivery[17], tissue engineering[18], implants[19], genetic engineering[20], vaccine adjuvants [21] and wound healing [22] are only a few of the therapeutic applications for these features.

Worldwide, the use of medicinal plants to treat various diseases is growing in popularity. One herb with more than 100 different types of phytochemicals is Stevia rebaudiana Bertoni, which has been used in traditional medicine for thousands of years for its antibacterial, antifungal, antioxidant, anticancer, antihypertension, hepatoprotective, hypoglicemic, and antiviral properties. A species of plant belonging to the Asteraceae family is called Stevia rebaudiana Bertoni [23] .

Because it contains steviol glycoside, a naturally occurring non-caloric sweetener that imparts a sweet flavour to its leaves, this plant is also known as honey leaf or sweet leaf. This plant comes from the highland regions of northeastern Paraguay, and it is now accepted in many nations, including Indonesia [24]

The purpose of this study was to determine the antibacterial potential usage of Stevia rebaudiana Bertoni leaf extract mixed with chitosan as an intracanal medication against the growth of Enterococcus faecalis bacteria.

The null hypothesis of this study was the presence of antibacterial effect of stevia against the Enterococcus Faecalis.

MATERIALS AND METHODS

Materials:

- Chitosan low molecular weight (LMW) (50,000-190,000 Da) was purchased from Sigma Aldrich Chemie GmbH company – Iceland Country.
- Acetic acid (96%) ($C_2H_4O_2$, M=60.05g/mol was purchased from El-Nasr Pharmaceutical Chemicals Company – Egypt.
- Stevia powder.
- Carbopol 940 polymer.

Methods:

1. Ethical Approval

The study had been approved by the Research Ethics Committee, faculty of Dentistry, October 6 University; Research number: RECO6U/13-2023.

2. Sample size calculation:

Sample size calculated depending on a previous study (Ballal et al.) [25] as reference. According to this study, the minimally accepted sample size was 5 per group, when mean ± standard deviation 11±0.707, the estimated mean was 12.5, with 2.12 effect size, when the power was 80 % & type I error probability was 0.05. Total sample size increased to 6 to compensate 20 % drop out. Sample size was calculated by using Independent t test that was performed by G.Power 3.1.9.7.

3. Preparations of Materials:

*** Minimal Inhibition zone (MIC) of Stevia Extract:**

MIC method was done using serial dilutions of a solution of stevia extract (5, 10, 25, 50, 100) to determine the lowest concentration of material that would show antibacterial properties. Stevia extract of concentration of 10gm in 100ml was found to be the lowest concentrationsthat showed an antibacterial effect and used in this study [26,27].

*** Preparation of Stevia gel by Carbopol 940 polymer (National Research Centre):**

Dissolve 10 g of stevia powder in 100 ml of double-distilled water [26, 27] in a glass beaker on a magnetic stirrer for 5–15 minutes. Add Carbopol 940 powder (0.4 g) to the above stevia solution with continuous stirring for 2–3 hours. We notice that the solution has become like a gel and is very thick.

*** Preparation of Chitosan LMW solution 2% (National Research Centre):**

Dissolve Chitosan LMW powder (2 g) in 100 ml of acetic acid solution (1%) in a glass beaker on a magnetic stirrer for 2–3 hours.

*** Preparation of Stevia gel by Chitosan LMW (National Research Centre):**

Dissolve stevia powder =10gm in 100ml Acetic acid solution (1%)[26,27] in glass beaker on magnetic stirrer for 5-15 min. - Add Chitosan low molecular weight (LMW) powder = 2gm on the above stevia solution with continuous stirring for 2-3 hours.

1. Antibacterial activity against Enterococcus Faecalis:

In our study, the antibacterial activity of Stevia gel, Chitosan solution ,Stevia gel by Chitosan were determined by Agar Diffusion Test (ADT) [28].

Enterococcus faecalis ATCC29212 (American Type Culture Collection 29212) was cultured 72 -hour in agar BHI (brain heart infusion) then 3-4 colonies were collected and injected in 3 mL of BHI broth under the same condition. The culture was diluted in sterile saline to obtain 0.5 McFarland.

On brain heart infusion agar plates (30g dehydrated culture media "Thermoscientific, UK"/1L distilled water in 50 mL sheep blood), the antibacterial activity was attained. The produced bacterial suspension was then inoculated into 15 sterile petri plates after the agar had been added. Each agar plate was punched out to a diameter of 6 mm, and the ingredients under test were then distributed among the 5 plates as follows:

1. **Group 1 (Stevia gel by Carbopol) :** The punch hole of the 5 plates was filled with stevia gel by Carbopol
2. **Group 2 (Chitosan solution) :** The punch hole of the 5 plates was filled with chitosan solution.
3. **Group 3 (Stevia gel by Chitosan):** The punch hole of the 5 plates was filled with stevia gel by chitosan

The agar plates were incubated at 37°C, and the diameters of the inhibition growth zones were measured after 24-, 48,72 hours and 7-days (1 week) with a vernier calliper.

2. Statistical analysis

The antimicrobial activity was rated based on the diameters of the inhibition growth zones; Comparison between different groups was performed using One Way ANOVA test which revealed significant difference between all groups at different intervals, followed by Tukey`s Post Hoc test for multiple comparisons.

RESULTS

Effect of material (comparison between different groups):

Minimum, maximum, mean and standard deviation of antibacterial activity of all groups at different intervals were presented in table (1).

After 24, 48, and 72 hrs., group 1 was significantly the lowest, group 2 and 3 were significantly the highest with insignificant difference between them (Figure 1).

After 1 week, there was a significant difference between all groups as, group 1 was significantly the lowest, followed by group 2 and 3 were significantly the highest.

TABLE (1) Minimum, maximum, mean and standard deviation of antibacterial activity of all groups, and comparison between different groups (effect of material):

		N	Min	Max	M	SD	P value
24 hours	Group 1	6	14	15	14.33 ^{a}	0.52	0.002*
	Group 2	6	16	17	16.33 ^{b}	0.52	
	Group 3	6	15	17	16.00 ^{b}	0.89	
48 hours	Group 1	6	14	16	15.00 ^{a}	0.89	<0.0001*
	Group 2	6	19	21	20.00 ^{b}	0.89	
	Group 3	6	18	20	19.33 ^{b}	1.03	
72 hours	Group 1	6	14	16	15.00 ^{a}	0.89	0.007*
	Group 2	6	19	21	20.00 ^{b}	0.89	
	Group 3	6	18	25	20.33 ^{b}	3.61	
After one week	Group 1	6	12	14	13.33 ^{a}	1.03	<0.0001*
	Group 2	6	19	20	19.67 ^{c}	0.52	
	Group 3	6	15	18	16.33 ^{b}	1.37	

N: count Min: minimum Max: maximum
M: mean SD: standard deviation*Significant difference as P < 0.05.
Means with different superscript letters were significantly different as P<0.05.
Means with the same superscript letters were insignificantly different as P>0.05.

Effect of time (comparison between different intervals):

- In group 1, the antibacterial activity was significantly the lowest after 1 week (13.33±1.03) , while after 48 and 72 hrs. it were significantly the highest (15.00±0.89). After 24hrs. it revealed insignificant difference with other intervals (14.33±0.52).
- In group 2, the antibacterial activity after 24 hrs. was significantly the lowest (16.33±0.52), while after 48 hrs. (20.00 ± 0.89), 72 hrs. (20.00±0.89) and 1 week (19.67±0.52) were significantly the highest with insignificant difference between them.
- Ingroup 3, after 24 hrs. (16.00±0.89) and 1 week (16.33±1.37) were significantly the lowest, while after 48 hrs. (19.33±1.03) and 72hrs. (20.33±3.61) were significantly the highest with insignificant difference between them.

TABLE (2) Mean and standard deviation of antibacterial activity all groups, and comparison between different intervals (effect of time):

Group	24 hours		48 hours		72 hours		After one week		P value
	M	SD	M	SD	M	SD	M	SD	
Group 1	14.33 ^{ab}	0.52	15.00 ^{a}	0.89	15.00 ^{a}	0.89	13.33 ^{b}	1.03	0.009*
Group 2	16.33 ^{a}	0.52	20.00 ^{b}	0.89	20.00 ^{b}	0.89	19.67 ^{b}	0.52	<0.0001*
Group 3	16.00 ^{a}	0.89	19.33 ^{b}	1.03	20.33 ^{b}	3.61	16.33 ^{a}	1.37	0.003*

N: count Min: minimum Max: maximum
M: mean SD: standard deviation*Significant difference as P < 0.05.
Means with different superscript letters were significantly different as P<0.05.
Means with the same superscript letters were insignificantly different as P>0.05.

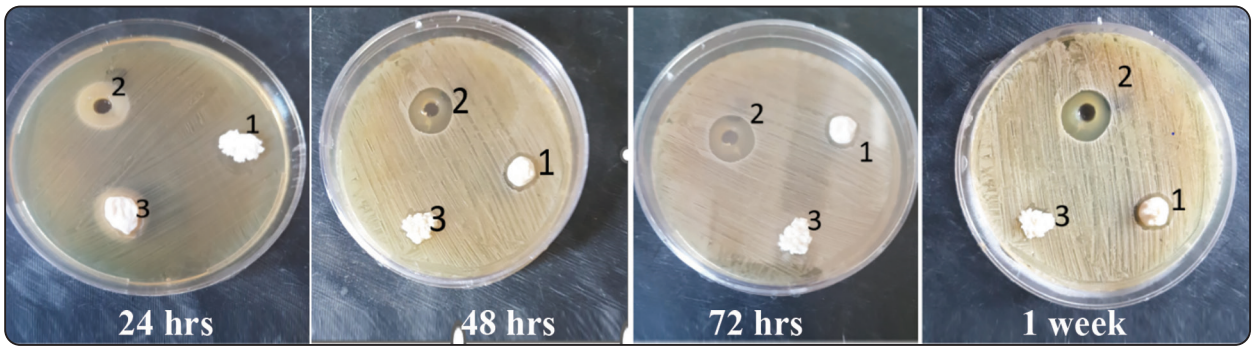

Fig. (1) Zone of inhibition of antibacterial activity of the three groups at 24, 48,72 and 1 week. [(1) Stevia gel by Carbopol, (2) Chitosan solution (3) Stevia gel by Chitosan].

DISCUSSION

The efficacy of root canal therapy depends on the removal of bacteria from root canal systems [29]. Despite significant advancements in recent years, the endodontic chemo-mechanical disinfection techniques now in use are unable to reliably maintain the sterility of root canal complexes. No endodontic therapy component, including the host defence system, instruments and irrigation, intracanal medications, permanent root fillings, and coronal restoration, can provide total sterility [30].

The bacteria that are typically connected to the aetiology of persistent peri radicular lesions is E. faecalis[31]. It has a number of virulence characteristics that let it withstand the effects of traditional root canal therapy [32]. Additionally, this Gram-positive facultative anaerobe can attach to collagen and enter dentine tubules [33]. Due to these factors, E. faecalis was chosen as the microbe in our study to assess the antimicrobial capabilities of the endodontic materials put to the test.

The agar well diffusion assay was extensively applied to assess the antimicrobial activity of natural products [34,35] .It was chosen in the current study to evaluate the experimental materials' antimicrobial activity because it is a straightforward method that can be used to determine the antimicrobial activity of endodontic material before doing more complex experiments[29 ,36] .it had 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[36].

Following chitosan and stevia, the antibacterial activity of stevia gel by chitosan in this investigation revealed significantly the highest antibacterial effect after 24, 48, and 72 hours. This has to do with the combination of chitosan and stevia, both of which have antibacterial properties on their own[37]. After 1 week, the antibacterial activity of all groups decreased. Stevia gel is the lowest, followed by chitosan, and stevia gel is followed by chitosan significantly.

Stevia extract used in this study had the ability to prevent the growth of E. faecalis bacteria because it includes several bioactive substances (phytochemicals) like phenols, tannins, saponins, glycosides, terpenoids and flavonoids which had an antibacterial action [38,39].

Tannic phytochemicalshinder the production of the bacterial cell wall by creating irreversible compounds with proline-rich proteins, which is the mechanism they exert their antibacterial effect against E. faecalis. The capacity of saponin phytochemicals to digest bacterial cell proteins and enzymes was the basis for their antibacterial effect. The breakdown of bacterial cell walls is how terpenoid phytochemicals exert their antibacterial effect[38,40]. Furthermore, complex formation with soluble proteins and bacterial cell walls is facilitated by flavonoid phytochemicals. Bacterial activity is capable of being induced by steroidal phytochemicals [39,40,41] .

A naturally occurring polysaccharide, chitosan is made up of copolymers of glucosamine and N-acetylglucosamine. Because it is biodegradable and harmless, chitosan is utilised to produce nanoparticles for a variety of purposes. Because the free amino groups on its polymeric chain protonate and contribute to its beneficial modifications, it is insoluble in acidic environments. It is believed that the mechanism of action of chitosan is that cationically charged amino groups may combine with anionic components such as N-acetyl muramic acid, sialic acid, and neuramic acid on the cell surface and inhibit bacterial growth by obstructing exchanges with the medium, chelating transition metal ions, and inhibiting enzymes [42] .

CONCLUSION

The results of the current study suggest that, mixture of stevia gel by chitosan may improve the antimicrobial activity against E. faecalis rather than using stevia gel and Chitosan solution alone.

RECOMMENDATIONS

Further studies are required to employ these mixtures with chitosan as an intracanal medicaments in vivo investigations. Local intracanal medication administration requires extreme caution because the patient may be chemically sensitive. It is necessary to analyse and compare the extent to which the medication combinations penetrate the dentinal tubules, as well as their duration of action, concentrations, and dosage requirements. However, in order to suggest these intracanal medication combinations for therapeutic use, biocompatibility and safety must be assessed through preclinical and clinical research.

REFERENCES

1. Samaranayake L. Essential microbiology for dentistry-E-Book. Elsevier Health Sciences; 2018 Mar 28.
2. Deviyanti S, Abraham S, Pasiga BD. Antibacterial Potential of Stevia rebaudiana Bertoni Leaf Extract on The Growth of Enterococcus faecalis Bacteries.
3. Bjørndal L, Kirkevang LL, Whitworth J, editors. Textbook of endodontology. John Wiley & Sons; 2018 Aug 6.
4. Borzini L, Condò R, De Dominicis P, Casaglia A, Cerroni L. Root canal irrigation: Chemical agents and plant extracts against Enterococcus faecalis. The open dentistry journals. 2016; 10:692.
5. Nakornchai S, Banditsing P, Visetratana N. Clinical evaluation of 3Mix and Vitapex® as treatment options for pulpally involved primary molars. International Journal of Paediatric Dentistry. 2010 May; 20:214-21.
6. Reddy GA, Sridevi E, Sankar AS, Pranitha K, Gowd MP, Vinay C. Endodontic treatment of chronically infected primary teeth using triple antibiotic paste: An in vivo study. Journal of Conservative Dentistry: JCD. 2017 Nov;20 :405.
7. Dutta B, Dhull KS, Das D, Samir PV, Verma RK, Singh N. Evaluation of antimicrobial efficacy of various intracanal medicaments in primary teeth: an in vivo study. International Journal of Clinical Pediatric Dentistry. 2017 Jul;10 :267.
8. Manzur A, González AM, Pozos A, Silva-Herzog D, Friedman S. Bacterial quantification in teeth with apical periodontitis related to instrumentation and different intracanal medications: a randomized clinical trial. Journal of Endodontics. 2007 Feb 1; 33:114-8.
9. Lana PE, Scelza MF, Silva LE, Mattos-Guaraldi AL, Hirata Júnior R. Antimicrobial activity of calcium hydroxide pastes on Enterococcus faecalis cultivated in root canal systems. Brazilian dental journal. 2009; 20:32-6.
10. Mohammadi Z, Shalavi S, Yazdizadeh M. Antimicrobial activity of calcium hydroxide in endodontics: a review. Chonnam medical journal. 2012 Dec 1; 48:133-40.
11. Cogulu D, Uzel A, Oncag O, Eronat C. PCR-based identification of selected pathogens associated with endodontic infections in deciduous and permanent teeth. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2008 Sep 1; 106:443-9.
12. Forghani, M., Afshari, E., Parisay, I. and Garajian, R., 2017. Effect of a passive sonic irrigation system on elimination of Enterococcus faecalis from root canal systems of primary teeth, using different concentrations of sodium hypochlorite: An in vitro evaluation. Journal of Dental Research, Dental Clinics, Dental Prospects, 11, 177.
13. Moradi F, Haghgoo R. Evaluation of antimicrobial efficacy of nanosilver solution, sodium hypochlorite and normal saline in root canal irrigation of primary teeth. Contemporary clinical dentistry. 2018 Sep; 9:227.
14. Akıncıbay H, Şenel S, Yetkin Ay Z. Application of chitosan gel in the treatment of chronic periodontitis. Journal of Biomedical Materials Research Part B: Applied Biomaterials: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials. 2007 Feb;80(2):290-6.
15. Shrestha A, Zhilong S, Gee NK, Kishen A. Nanoparticulates for antibiofilm treatment and effect of aging on its antibacterial activity. Journal of endodontics. 2010 Jun 1; 36:1030-5.
16. Silva PV, Guedes DF, Nakadi FV, Pécora JD, Cruz-Filho AM. Chitosan: a new solution for removal of smear layer after root canal instrumentation. International endodontic journal. 2013 Apr; 46:332-8.
17. Chi J, Jiang Z, Qiao J, Peng Y, Liu W, Han B. Synthesis and anti-metastasis activities of nor cantharidin-conjugated carboxymethyl chitosan as a novel drug delivery system. Carbohydrate polymers. 2019 Jun 15; 214:80-9.
18. Saravanan S, Vimalraj S, Thanikaivelan P, Banudevi S, Manivasagam G. A review on injectable chitosan/beta glycerophosphate hydrogels for bone tissue regeneration.

International journal of biological macromolecules. 2019 Jan 1; 121:38-54.

19. Palla-Rubio B, Araujo-Gomes N, Fernandez-Gutierrez M, Rojo L, Suay J, Gurruchaga M and Goni I: Synthesis and characterization of silica-chitosan hybrid materials as antibacterial coatings for titanium implants. Carbohydr Polym. 203:331–341. 2019.
20. Kwak SY, Lew TT, Sweeney CJ, Koman VB, Wong MH, Bohmert-Tatarev K, Snell KD, Seo JS, Chua NH, Strano MS. Chloroplast-selective gene delivery and expression in planta using chitosan-complexed single-walled carbon nanotube carriers. Nature nanotechnology. 2019 May; 14:447-55.
21. Carroll EC, Jin L, Mori A, Munoz-Wolf N, Oleszycka E, Moran HB, Mansouri S, McEntee CP, Lambe E, Agger EM, Andersen P. The vaccine adjuvant chitosan promotes cellular immunity via DNA sensor cGAS-STING-dependent induction of type I interferons. Immunity. 2016 Mar 15;44:597-608.
22. Chen C, Liu Y, Wang H, Chen G, Wu X, Ren J, Zhang H, Zhao Y. Multifunctional chitosan inverse opal particles for wound healing. ACS nano. 2018 Sep 26;12:10493-500.
23. Borgo J, Laurella LC, Martini F, Catalán CA, Sülsen VP. Stevia genus: phytochemistry and biological activities update. Molecules. 2021 May 6;26:2733.
24. Gunasena MD, Senarath RM, Senarath WT. A review on chemical composition, biosynthesis of steviol glycosides, application, cultivation, and phytochemical screening of Stevia rebaudiana (Bert.) bertoni.
25. Ballal NV, Kundabala M, Bhat KS, Acharya S, Ballal M, Kumar R, Prakash PY. Susceptibility of Candida albicans and Enterococcus faecalis to Chitosan, Chlorhexidine gluconate and their combination in vitro. Australian Endodontic Journal. 2009 Apr;35:29-33.
26. Gamboa F, Chaves M. Antimicrobial potential of extracts from Stevia rebaudiana leaves against bacteria of importance in dental caries. Acta odontológica latinoamericana. 2012 Oct; 25:171-5.
27. Sharma S, Gupta S, Kumari D, Kothari SL, Jain R, Kachhwaha S. Exploring Plant Tissue Culture and Steviol Glycosides Production in Stevia rebaudiana (Bert.) Bertoni: A Review. Agriculture. 2023 Feb 16; 13:475.
28. Demiryürek EÖ, Özyürek T, Gülhan T, Keskin C. Evaluation of antibacterial and antifungal activity of calcium silicate based retrograde filling materials. Int J Applied Dent Sci. 2016; 2:85-.
29. 29.Sjögren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. International endodontic journal. 1997 Sep;30:297-306.
30. Haapasalo M, Endal U, Zandi H, Coil JM. Eradication of endodontic infection by instrumentation and irrigation solutions. Endodontic topics. 2005 Mar; 10:77-102
31. Gomes BP, Pinheiro ET, Sousa EL, Jacinto RC, Zaia AA, Ferraz CC, de Souza-Filho FJ. Enterococcus faecalis in dental root canals detected by culture and by polymerase chain reaction analysis. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2006 Aug 1; 102:247-53.
32. 32.Kayaoglu G, Erten H, Alaçam T, Ørstavik D. Short-term antibacterial activity of root canal sealers towards Enterococcus faecalis. International Endodontic Journal. 2005 Jul;38:483-8.
33. Love RM. Enterococcus faecalis–a mechanism for its role in endodontic failure. International endodontic journal. 2001 Jul;34(5):399-405.
34. Magaldi, S.; Mata-Essayag, S.; de Capriles, C.H.; Perez, C.; Colella, M.T.; Olaizola, C.; Ontiveros, Y. Well diffusion for antifungal susceptibility testing. Int. J. Infect. Dis. 2004, 8, 39–45.
35. Valgas, C.; de Souza, S.M.; Smânia, E.F.A.; Smânia, A., Jr. Screening methods to determine antibacterial activity of natural products. Braz. J. Microbiol. 2007, 38, 369–380.
36. Reller LB, Weinstein M, Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clinical infectious diseases. 2009 Dec 1;49:1749-55.
37. Jahan N, Khatoon R, Ahmad S. In vitro evaluation of antibacterial potential of Stevia rebaudiana Bertoni against various bacterial pathogens including resistant isolates with bla genes. Medicinal Plants-International Journal of Phytomedicines and Related Industries. 2014;6:114-9.
38. Gupta E, Vajpayee G, Purwar S, Shakyawar S, Alok S, Sundaram S. Phytochemical screening and in-vitro studies of antioxidant and antimicrobial activity of extracts of dried Stevia rebaudiana leaves. International Journal of Pharmaceutical Sciences and Research. 2017;8:3354-60.
39. Mujeeb F, Bajpai P, Pathak N. Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of Aegle marmelos. BioMed research international. 2014 May 11;2014.

40. 40.Azzam C.R., Al-Taweel S.K., Abdel-Aziz R.M., Rabea K.M., Abou-Sreea A., Rady M.M., Ali E.F. Salinity effects on gene expression, morphological, and physio-biochemical responses of *Stevia rebaudiana* Bertoni in vitro. Plants (Basel, Switzerland) 2021;10 :820

41. Kahrizi D., Ghari S.M., Ghaheri M., Fallah F., Ghorbani T., Beheshti Ale Agha A., Kazemi E., Ansarypour Z. Effect of KH2PO4 on gene expression, morphological and biochemical characteristics of stevia rebaudiana Bertoni under in vitro conditions. Cell. Mol. Biol. (Noisy-le-Grand, France) 2017;63 :107–111.

42. Raafat D, Sahl HG. Chitosan and its antimicrobial potential-a critical literature survey. Microb Biotechnol. 2009; 2:186–201