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Original paper

Stevia vs. triple antibiotic paste: An intracanal battle?

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Abstract

Background. Intracanal medicaments are vital in treating the infections of the deciduous dentition due to the large percentage of accessory canals that hasten the microbial spread to the periradicular region. Though countless medicaments have been produced to reduce the microbial load and aid symptomatic relief, they still do not fulfill every function of an ideal medicament.

Objectives. The aim of the present study was to evaluate and compare the antimicrobial efficacy of *Stevia rebaudiana* (*S. rebaudiana*) and triple antibiotic paste (TAP) against *Enterococcus faecalis* (*E. faecalis*).

Material and methods. The present in vitro, parallel, double-blinded study had an equal allocation ratio. The specimens were prepared, randomly divided into 4 groups and inoculated with *E. faecalis* (ATCC35550). Following incubation, the first 3 groups were treated with *S. rebaudiana*, triple antibiotic therapy or carbopol gel, respectively, with the 4th negative control group left untreated. The microbial samples were collected before and after treatment, and the counts of colony-forming units (CFUs) were compared. The results were analyzed using the Kruskal–Wallis, Dunn's post-hoc and Wilcoxon's signed rank tests.

Results. The first 2 groups displayed a significant decrease in CFUs after drug application, while the carbopol and control groups showed an exponential increase. There was no statistically significant difference between the stevia and TAP groups ($p = 0.630$).

Conclusions. Stevia gel was comparable to TAP in terms of antimicrobial efficacy, and can therefore be considered a new alternative in intracanal treatment.

Keywords: triple antibiotic paste, intracanal medicament, stevia

Introduction

The most important etiologic factors for pulp and periradicular diseases are pathogens, of which *Enterococcus faecalis* (*E. faecalis*) is of paramount importance. It is a facultative gram-positive anaerobe, occurring abundantly in failed endodontic canals.[1] Classical biomechanical preparations can help eliminate microorganisms while extirpating the infected tissue. However, tortuous roots and the complexity of accessory canals, as well as the ability of *E. faecalis* to exist independently as a biofilm without synergistic support, make it difficult to eliminate the possibility of reinfection after obturation. Therefore, better eradication of such microorganisms requires an additional disinfection approach with the use of irrigants and intracanal medications.

Intracanal medications aim to reduce bacterial colonies and prevent reinfection. Various drugs used to disinfect the canal include calcium hydroxide, Formocresol®, triple antibiotic paste (TAP), chlorhexidine digluconate, and Ledermix™, of which calcium hydroxide and TAP are most commonly used. Although their efficacy has been demonstrated in many studies, both have several disadvantages. Calcium hydroxide is less effective against *E. faecalis* and completely ineffective against yeast-like fungi,[2] which are the primary commensals of the infected ducts. In addition, allergic tissue reactions have been reported in numerous cases. Triple antibiotic paste (minocycline, ciprofloxacin, metronidazole), on the other hand, has been very successful in eliminating endodontic pathogens.[3] However, its use has resulted in coronal discoloration[4] and a reduction in dentin hardness.[5] Due to these drawbacks, there is a constant search for better novel materials.

Stevia rebaudiana (*S. rebaudiana*), also called candy leaf, is a flowering plant native to Paraguay that is cultivated for its sweet-tasting leaves. It is used by diabetics in a dried form as a calorie-free natural sweetener, and is rich in terpenes and flavonoids, which have good antibacterial and antifungal properties.[6] In dentistry, stevia was shown to inhibit plaque and gingivitis when used as a mouthwash in an in vivo study.[7] Stevia has been shown to be non-toxic,[8] and have therapeutic effects in treating cancer,[9] inflammation,[10] cysts,[11] and obesity.[12] Also, it can be safely consumed during pregnancy without a fear of teratogenicity.[8] Considering these benefits, the present study evaluated and compared the antimicrobial properties of stevia and TAP against *E. faecalis* when used as intracanal drugs.

Material and methods

Study design

The present study followed an in vitro design, and was planned and performed according to the Checklist for Reporting In-vitro Studies (CRIS) and Consolidated Standards of Reporting Trials (CONSORT) guidelines.

Since the present study was conducted in vitro, it was exempted from the review by the institutional ethics committee.

Study setting

The study was conducted at the Department of Pediatric and Preventive Dentistry of AME's Dental College and Hospital, Raichur, India, in collaboration with the Department of Pharmaceutics of the V.L. College of Pharmacy, Raichur, India, and the Department of Microbiology of Raichur Institute of Medical Sciences.

Sample size

The sample size was estimated using the G*Power software, v. 3.1.9.4 (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower). Considering a power (1-β) of 80% and an α-error of 5%, the total sample size was set at 40 (10 × 4 groups = 40 samples).

Preparation of the teeth

The examined samples included freshly extracted premolars with intact crowns and fully formed root apices.[13] Teeth that were fractured, carious, previously restored, calcified, or with multiple canals or resorbed roots were excluded.[14]

The root length of the specimens was standardized by decoronating the teeth with a 0.1-millimeter-thick double-sided diamond disk (Dentorium Products, Farmingdale, USA). The roots were prepared to a working length of 14 mm, using K files with an apical file size 35–70 (Mani Medical India, New Delhi, India), with an increment of 1 mm to ensure the standardization of measurements. All canals were rinsed with 1 mL of 2% NaOCl (Vishal Dentocare, Ahmedabad, India) for 30 s between the instrumentation stages. A 3-milliliter preparation of 17% ethylenediaminetetraacetic acid (EDTA) (Dental Avenue India, Palghar, India) was administered and the canals were soaked for 5 min, followed by final irrigation with 5 mL of sterile saline. The apical foramen was sealed with light-cured composite resin and the outer surface of the root was made impermeable by coating it with 2 layers of epoxy resin (Fortune Chemie, Bangalore, India).[15] All specimens were autoclaved under standard conditions (121°C for 15 min) and stored in an ultraviolet (UV) chamber to ensure sterility.

Preparation of the drugs

Stevia extraction and formulations

A total of 40 g of dried powdered stevia (Stevia World Agrotech, Bangalore, India) was added to 200 mL

of absolute alcohol and boiled in a Soxhlet apparatus for 23 cycles.[16] The obtained extract was then reduced to 20 mL and formulated at different concentrations (0.1%, 0.2%, 0.6%, 0.7%, and 0.8%) to determine the minimal bactericidal concentration (MBC) (Table 1). The experimental formulations were based on random concentrations, and if positive for microbial inhibition, new formulations with adjacent lower concentrations were used. The MBC of stevia was determined using the spot inoculation technique,[17] in which 1 mL of activated *E. faecalis* suspension was combined with equal parts of different drug concentrations and dropped onto a blood agar plate divided into 5 compartments. The contaminated plate was then incubated at 37°C and 5% CO_2 for 24 h, and changes were noted. The minimum drug concentration at which the complete elimination of the organism occurred was 0.7%. The concentrations for different formulations were chosen randomly, and since 0.6% did not show any inhibition, there was no need for formulations at 0.3%, 0.4% or 0.5% concentrations.

Stevia gel preparation

Carbopol powder (50 mg) was added to 10 mL of distilled water and sonicated for 5 cycles, each lasting 2 min. After achieving a homogeneous solution, 0.7 mL of stevia from a freshly prepared extract was added. Triethanolamine (Qualigens Fine Chemicals, Mumbai, India) was then added dropwise and stirred until a gel-like consistency was obtained. Carbopol served as a thickening agent, while triethanolamine acted as a neutralizing agent.

Triple antibiotic gel preparation

Triple antibiotic gel (TAG) was formulated using Hashimoto's ratio, combining equal parts of minocycline (100 mg), ciprofloxacin (200 mg) and metronidazole (500 mg) (Lifecare Neuro Products, Baddi, India) with 50 mg of Carbopol®-934 (Rolex Pharmaceuticals Limited, Ahmedabad, India) and 10 mL of distilled water. Sonication was performed for 5 cycles. Triethanolamine (Qualigens Fine Chemicals) was then added dropwise and stirred until a gel-like consistency was reached.

Table 1. Different concentrations of the stevia extract formulated for checking the minimal bactericidal concentration (MBC)

No.	Formulation	Concentration [%]
1	0.1 mL of the extract brought up to 10 mL by the addition of ethanol yields 1.007 mg/mL	0.1
2	0.2 mL of the extract brought up to 10 mL by the addition of ethanol yields 2.014 mg/mL	0.2
3	0.6 mL of the extract brought up to 10 mL by the addition of ethanol yields 6.042 mg/mL	0.6
4	0.7 mL of the extract brought up to 10 mL by the addition of ethanol yields 7.049 mg/mL	0.7
5	0.8 mL of the extract brought up to 10 mL by the addition of ethanol yields 8.056 mg/mL	0.8

Inoculation

The samples were fixed in sterile Eppendorf tubes and stabilized on 5-milliliter tubes. A standard strain of *E. faecalis* was reactivated in brain–heart infusion (BHI) broth, and incubated at 37°C and 5% CO_2 for 24 h. After microbial growth, a suspension of the culture was prepared in a tube containing 10 mL of saline (0.9%) at a concentration of 2 on the McFarland scale, with 5 mL of the prepared suspension mixed with 5 mL of BHI broth in a test tube to obtain the final suspension. Then, 20 µL of the final suspension was introduced into the root canal with the use of a 0.3-milliliter insulin syringe, and a cotton pellet dipped in the suspension was inserted into the canal opening. Incubation continued for 21 days in a 5% CO_2 atmosphere at 37°C. The viability and purity of the microorganisms were assessed weekly by Gram staining.[15]

Microbiological examination

After 21 days, the microbial samples were collected by inserting absorbent paper cones into the canal for 1 min, which were then transferred to Eppendorf tubes containing 1 mL of saline and shaken in a tube shaker for 30 s. Serial dilutions of each suspension were prepared to a concentration of 10^{-5} mg/mL, and 0.1 mL of the suspension from each sample was seeded and streaked onto blood agar plates, and incubated at 37°C and 5% CO_2 for 24 h. Colony-forming units (CFUs) were then mechanically counted and the number of viable germs was calculated.[14] Saline irrigation was performed 3 times to remove the free-flowing detached bacteria, and the intracanal medication was then administered: 0.7% stevia gel (group 1); TAP (group 2); or carbopol gel (vehicle) (group 3). Group 4 (control) was left untreated.

Analysis of the root canal samples

After an incubation period of 7 days, the drug was flushed out by rinsing with saline. Absorbent paper cones were inserted and the procedure was repeated.[4] The count of CFUs was noted and compared (Fig. 1).

Statistical analysis

The quantitative data post triplicate experimentation was subjected to statistical analysis and tabulated. The intergroup analysis was performed using the Kruskal–Wallis test, whereas intragroup comparisons used the Wilcoxon test, with a p-value set at 0.05. Pairwise comparisons with Dunn's post-hoc test determined the significant intergroup differences.

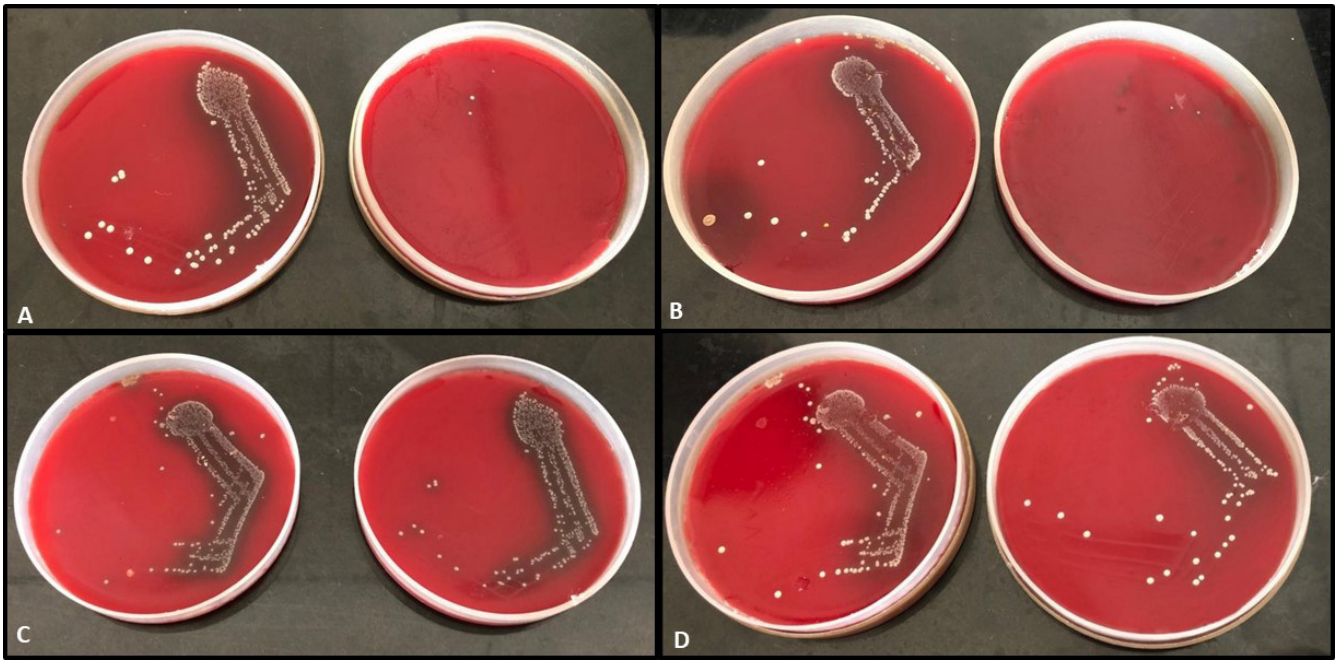

Fig. 1. Microbial colonies formed pre- and post-treatment with different drugs
A – stevia; B – triple antibiotic paste (TAP); C – carbopol; D – control group (untreated).

Results

Minimal bactericidal concentration

The MBC of stevia that totally inhibited the growth of *E. faecalis* was 0.7%.

Intergroup analysis

Pre-treatment

The mean count of CFUs of *E. faecalis* before treatment was 407.60 ±177.37 in the stevia group, 443.50 ±130.04 in the TAP group, 393.90 ±88.34 in the vehicle group, and 444.30 ±52.70 in the control group, with no significant differences between the 4 groups (p = 0.420) (Table 2, Fig. 2).

Post-treatment

The mean count of *E. faecalis* CFUs after treatment was 2.30 ±0.82 in the stevia group, 1.90 ±0.74 in the TAP group, 391.80 ±87.97 in the vehicle group, and 444.30 ±52.37 in the control group. There were significant differences in the *E. faecalis* CFUs between the 4 groups post-intervention (p < 0.001) (Table 2, Fig. 2).

Pairwise analysis

Multiple pairwise comparisons revealed that in groups 1 and 2, the mean count of CFUs was significantly smaller as compared to groups 4 and 5 ($p \leq 0.001$). However, no remarkable differences were observed between groups 1 and 2 (p = 0.630), and groups 3 and 4 (p = 0.400) (Table 3).

Table 2. Comparison of the mean count of colony-forming units (CFUs) of *Enterococcus faecalis* (*E. faecalis*) (×10^8) between the 4 groups pre- and post-treatment (Kruskal–Wallis test)

Group	CFUs of *E. faecalis* (×10^8)			
	pre-treatment	*p*-value	post-treatment	*p*-value
Stevia	407.60 ±177.37	0.420	2.30 ±0.82	<0.001*
TAP	443.50 ±130.04		1.90 ±0.74	
Carbopol	393.90 ±88.34		391.80 ±87.97	
Control	444.30 ±52.70		444.30 ±52.37	

Data presented as mean ± standard deviation (*M* ±*SD*).
* statistically significant.

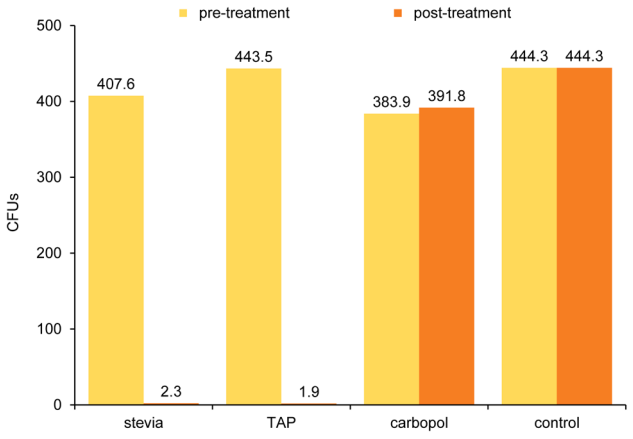

Fig. 2. Mean count of colony-forming units (CFUs) of *Enterococcus faecalis* (*E. faecalis*) before and after drug application

Table 3. Multiple pairwise comparisons of the mean differences in the count of colony-forming units (CFUs) of *Enterococcus faecalis* (*E. faecalis*) ($\times 10^8$) between the 4 groups post-treatment (Dunn's post-hoc test)

Group (A)	Group (B)	*MD* (A – B)	95% *CI* for *MD*		*p*-value
			lower	upper	
Stevia	TAP	0.40	–61.26	62.06	0.630
	carbopol	–389.50	–451.16	–327.84	0.001*
	control	–442.00	–503.66	–380.34	<0.001*
TAP	carbopol	–389.90	–451.56	–328.24	<0.001*
	control	–442.40	–504.06	–380.74	<0.001*
Carbopol	control	–52.50	–114.16	9.16	0.400

MD – mean difference; *CI* – confidence interval; * statistically significant.

Discussion

This study focused on the formulation of a drug that overcomes the disadvantages of commercially available preparations, but also leads the way in terms of antimicrobial properties. Stevia was the preferred alternative, as it has been shown historically to be superior to industrially marketed chlorhexidine mouthwashes in limiting plaque, gingivitis,[16] and the count of caries-forming organisms.[18] The steviol glycoside that the preparation contains also makes it palatable to children because of its sweet taste. The other group in this trial received TAP (in the present study, it was in fact TAG to distinguish the vehicle used), which causes coronal discoloration despite its good canal-disinfecting properties.[19] Also, the minocycline component in TAP has been associated with allergic reactions in many cases.[20] The available literature suggests that a biocompatible herbal drug with minimal/no side effects is preferable.

The overall performance of intracanal drugs depends on the type of carrier used. Gels are generally much less viscous than pastes, which improves penetration deep into the canal and collateral canals. In addition, pastes adversely affect the microhardness of dentin.[5] A study investigating the properties of different carrier materials demonstrated that macrogol and propylene glycol, the traditional carrier materials for TAP, had some antimicrobial effects, leading to some bias.[21] Considering all of the above factors, a carbopol gel-based vehicle was selected as the delivery system, as it has a neutral pH and is efficient in terms of permeability.[22]

Enterococcus faecalis naturally produces reactive oxygen species (ROS), and to combat them, antioxidants are produced simultaneously. This property makes the bacteria tolerant to acidic environments; nevertheless, TAP with its acidic pH (5.5) successfully limits the *E. faecalis* count, suggesting that both acidic and alkaline niches can affect the growth of *E. faecalis*.[23]

The results of the present study indicate that stevia acts very similarly to TAP; its antibacterial properties could result from oxygen radicals (hydroxyl, nitrate and peroxide) causing DNA mutations, base pair modification and intercellular protein cross-linking.[17] Carbopol is acidic in nature, with a pH of 2–2.5, but the pH rises to neutral by adding triethanolamine, which is both a gelling agent and a neutralizer.

Stevia is a novel product with very limited reports in the literature, and this study was conducted to explore the possibility of using stevia as a bactericidal/bacteriostatic agent. The exploratory nature of the study made it justifiable to use a relatively small sample size.

Conclusions

Stevia demonstrated a significant reduction in the count of *E. faecalis*, similar to TAP, when used as an intracanal medicament.

This study concluded that carbopol did not influence the antimicrobial efficacy of the experimental drug used as an intracanal medicament, which was comparable to that of the traditionally used TAP.

However, it should be noted that the oral cavity and the root canal system are inhabited by a variety of microflora, and the lack of substantial data on the antimicrobial efficacy of stevia against this microbiota suggests the need for further studies.

Ethics approval and consent to participate

Not applicable.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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