Original Article

Antibacterial efficacy of carnosic acid as an intracanal medicament against *Enterococcus faecalis*: Quantitative polymerase chain reaction study

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Abstract

Aim: The aim of this study was to quantitatively assess the antibacterial effectiveness of carnosic acid, propolis, and calcium hydroxide when used as intracanal medications against *Enterococcus faecalis*.

Methodology: Forty-six human mandibular single-rooted premolar root lengths were standardized to 12 mm after decoronation. Cleaning and shaping were performed. The specimens were placed in Eppendorf Tubes and autoclaved. *E. faecalis* was inoculated into the canals and incubated for 21 days. The teeth were categorized into four groups: I - carnosic acid, II - propolis, III - calcium hydroxide, and IV - nonmedicated group. The medicaments were applied to the root canals and incubated for 14 days. The DNA extraction of *E. faecalis* was obtained from dentinal shavings harvested at 400-μm depth and a real-time quantitative polymerase chain reaction was performed.

Statistical Analysis: Data were analyzed using the Kruskal–Wallis test and Dunn's intergroup comparison test in SPSS software.

Results: *E. faecalis* present were 4.14, 6.98, 3.80, and 56.84 mean copies/μl in groups I, III, III, and IV, respectively. A significant difference in antibacterial efficacy was observed between medicated and untreated control groups. However, no statistically significant differences were observed among the groups treated with different medicaments.

Conclusion: Carnosic acid has promising antibacterial activity against *E. faecalis* when used as an intracanal medicament.

Keywords: Carnosic acid; *Enterococcus faecalis*; intracanal medicament; propolis; quantitative polymerase chain reaction

INTRODUCTION

The intricate anatomy and limited instrument access challenge the effective disinfection of root canals. Utilizing intracanal medicaments holds significant importance in eliminating remaining microbes after cleaning and shaping to establish an optimal environment for repairing peri-radicular tissues. A systematic review by Pak *et al*. comprising 33 cross-sectional studies across multiple countries, indicates that apical periodontitis was found in 36% of the 28,881 teeth that underwent endodontic treatment.[1,2]

Anaerobic bacterial microflora, particularly facultative anaerobes such as *Enterococcus faecalis*, are present in 22%–77% of endodontically failed teeth with peri-radicular infection cases. *E. faecalis* demonstrates resilience in adverse conditions through adaptable physicochemical properties and biofilm formation that shields bacteria from

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Date of submission : 26.06.2024
Review completed : 30.07.2024
Date of acceptance : 12.08.2024
Published : 05.10.2024

Access this article online

Quick Response Code:

Website: https://journals.lww.com/jcde

DOI: 10.4103/JCDE.JCDE_405_24

How to cite this article: Arun V, Govindaraju L, Paranthaman RV, Priyadharshini R, Kalaiselvam R, Rajan RM. Antibacterial efficacy of carnosic acid as an intracanal medicament against *Enterococcus faecalis*: Quantitative polymerase chain reaction study. J Conserv Dent Endod 2024;27:1010-4.

destruction by phagocytosis, antibodies, and antimicrobial agents. *E. faecalis*, besides antibiotic resistance, persists in the root canal system due to various virulence factors such as quorum sensing, capsular polysaccharide, adhesins, secretory factors, hemolysin, collagen-binding protein, gelatinase, and enterococcal aggregation substance.[3] Hence, a potent antibacterial agent postcanal preparation is crucial, especially in persistent or secondary endodontic infections.

Calcium hydroxide, first identified by Hermann in 1920, finds extensive application in dentistry because of its antimicrobial effect, primarily achieved through direct interaction with bacteria through pH modulation. On *E. faecalis*, calcium hydroxide as an intracanal medicament has limited effect.[4] This is due to its ability to persist in alkaline environments as *E. faecalis* uses a functioning proton pump in its cell membrane to maintain a cytoplasmic homeostasis.[5]

The increase in antimicrobial-resistant bacteria in traditional medicine has spurred research into novel microbial control methods. Herbal products such as essential oils, extracts, and phytochemicals are promising alternatives due to their potential antimicrobial properties and less adverse effects on the mechanical properties of dentin.[6]

The major extracts: carnosic acid, carnosol, rosmarinic acid, oleanolic acid, genkwanin, apigenin, ursolic acid, and luteolin are obtained from an edible shrub *Rosmarinus officinalis L. Lamiaceae,* possess antibacterial, anti-inflammatory, anti-tumor, and antioxidant properties. Bernardes *et al*. stated that carnosic acid and carnosol are the chief chemicals from commercially available rosemary extract that were efficient against oral pathogens such as *Streptococcus* species and *E. faecalis*.[7]

Research on the application of carnosic acid as an intracanal medication was done qualitatively, whereas research on quantitative analysis is scarce. Thus, this study aims to quantitatively assess the antibacterial effectiveness of carnosic acid, propolis, and calcium hydroxide against *E. faecalis* using quantitative polymerase chain reaction (qPCR). The null hypothesis proposed in this study states there is no difference in the antibacterial efficacy among the various experimental intracanal medicaments.

METHODOLOGY

Ethical approval

The protocol for this *in vitro* study was approved by the committee for the Student's proposal, Institutional Ethical Committee of Sri Ramachandra Institute of Higher Education and Research (DU), Porur, Chennai, under CSP/23/AUG/135/762.

Sample size estimation

G*Power (version 3.1.9.2, Heinrich-Heine-Universitat Dusseldorf, Germany) with power of 90%, alpha error of 5% was used to calculate the sample size. A sample size of $n = 11$ per group and in total 44 was determined. Statistical significance is considered to be at $P < 0.05$ level.

Selection of teeth

Forty-six freshly extracted human single-rooted mandibular premolar teeth with mature apices extracted for orthodontic and periodontal purposes were obtained from the Department of Oral and Maxillofacial Surgery, Sri Ramachandra Dental College and Hospital, Porur, and stored in distilled water to prevent dehydration.[8] Teeth with fracture lines, dental caries, resorption, cracks, abnormal root canal morphology, and previous endodontic or restorative treatment were excluded.

Preparation of tooth samples

Soft surface tissues and organic debris from the extracted teeth were removed. Below the cementoenamel junction, tooth samples were decoronated with a rotating diamond disc, and the length was standardized to 12 mm. Following the verification of apical patency using a #10 K-file (Mani, Japan), standardized to a 12 mm working length, the root canals were enlarged using a #20 K-file (Mani, Japan). Using ProTaper Gold rotary instrument (Dentsply Tulsa Dental, Johnson City, TN), each canal was prepared up to size F5 in accordance to the manufacturer's instructions,[9] 1 ml of 3% NaOCl was used between each instrumentation. The canals were irrigated with 5 ml of 3% NaOCl and 5 ml of 17% ethylenediaminetetraacetic acid followed by a final rinse with 5 ml of distilled water. Acrylic was used to seal the root apices. Each specimen was autoclaved for 15 min at 121°C to sterilize it, and it was then stored aseptically at 30°C with 100% humidity.[8]

Contamination of blocks

ATCC 29212 *E. faecalis* suspension at the concentration of 1.5×10^9 CFU/ml was delivered into the sterilized root canals using sterile 1-ml syringes in a laminar air-flow chamber and incubated for 21 days at 37°C. To verify the sterility of the samples, two samples that had not been incubated with organisms were employed. The medium was refreshed every day. Following incubation, the samples underwent a gentle saline wash to eliminate any remaining excess culture from their surface. The infected samples were then divided into the following groups with 11 samples per group:

- Group 1: Carnosic acid-treated tooth samples
- Group 2: Propolis-treated tooth samples
- Group 3: Calcium hydroxide-treated tooth samples
- Group 4: Tooth samples without any treatment (control).

Preparation and placement of intracanal medicaments

A readily available mixture of calcium hydroxide (Ultracal

Table 1: Kruskal–Wallis test for comparative analysis of mean copies/μL in different study groups

Group	*n*	Mean copies/μL	SD	Mean rank	Median	IQR	*P*
Group 1: Carnosic acid	11	4.144	2.594	15.45	3.753	5.000	<0.001
Group 2: Propolis	11	6.980	3.875	22.55	5.638	6.946	
Group 3: Calcium hydroxide	11	3.807	3.159	13.41	3.221	3.626	
Group 4: Control	11	56.84	99.512	38.59	28.068	30.500	

SD: Standard deviation, IQR: Interquartile range

Table 2: Dunn's *post hoc* test for pair-wise comparative analysis of mean rank copies/μL in different study groups

Sample 1–Sample 2	Test statistic	SE	Standard test statistic	Significant	Adjusted significant	Test statistic
Group 3–Group 1	2.045	5.477	0.373	0.709	1.000	2.045
Group 3–Group 2	9.136	5.477	1.668	0.095	0.572	9.136
Group 1–Group 2	−25.182	5.477	−4.598	0.000	<0.001	−25.182
Group 1–Group 4	7.091	5.477	1.295	0.195	1.000	7.091
Group 2–Group 4	−23.136	5.477	−4.225	0.000	<0.001	−23.136
Group 3–Group 4	−16.045	5.477	−2.930	0.003	0.020	−16.045

SE: Standard error

XS) was injected into the canal. 20 mg carnosic acid (Tokyo Chemical Industry Pvt. Ltd., India) with 97% purity was mixed with 0.2 ml of saline[3] and propolis powder (Hi-Tech Natural products, India) was mixed with saline in a ratio of 2:1 to obtain a paste, packed into the root canal using pluggers, and incubated at 37°C for 14 days. After 14 days of incubation, the samples were gently washed with saline and dentinal chips were harvested at 400 μm depth using a size 3 peso reamer.[10]

Real-time quantitative polymerase chain reaction

DNA extraction was done according to the manufacturer's instructions for the QIAamp DNA mini kit (QIAGEN). The presence of DNA was confirmed using nanodrop. Real-time qPCR assessed the *E. faecalis* bacterial load in dentinal chips. ddl gene target was used to determine the copy number of *E. faecalis*. The forward primer (5' - CCACAAGTACCATTCGTGC - 3') and reverse primer (5' – GCGACATCTTTCACCACTTC - 3') sequences were used.[11] The qPCR was performed in QuantStudio 5 Real-time PCR instrument. The assay was performed using SYBR green (TB Green® Premix Ex TaqTMII (Tli RNaseH Plus) PCR Kit (Takara, Japan) Cat No. RR820A. The software system in QuantStudio 5 Real-time PCR instrument, automatically calculated the number of copies in the sample. The reactions were performed in duplicate.[12]

RESULTS

SPSS software (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY, USA: IBM Corp. Released 2019) was used for data analysis. Statistical analysis by the Kruskal–Wallis test [Tables 1 and 2] showed that there is a significant difference in colonies between the groups treated with intracanal medicament (Groups 1, 2, and 3) compared to the group treated with normal saline (Group 4) ($P < 0.001$). The null hypothesis was accepted it was observed that the herbal intracanal medicaments (propolis and carnosic acid) were as efficacious as the gold standard intracanal medicament calcium hydroxide against *E. faecalis*.

DISCUSSION

An ideal intracanal medication should demonstrate high efficacy against microbes in planktonic, biofilm states, and their toxins. In addition, intracanal medications must be biocompatible when interacting with the surrounding periodontal tissues.

E. faecalis, a facultative anaerobe, was selected for the study due to its high prevalence in secondary endodontic infections. The eradication of *E. faecalis* is particularly challenging due to its inherent ability to withstand starvation, high pH levels, high salt concentrations, biofilm formation, spore formation, and antibiotic resistance. In addition, under *in vivo* conditions, the pathogenesis of enterococcal infection at the molecular level is oxidative stress due to the production of free radicals such as superoxide and hydrogen peroxide.[13]

PCR can detect even low levels of bacteria compared to traditional methods, often yielding positive results when viable bacteria are present despite negative cultures. PCR relies on DNA polymerase enzyme to duplicate DNA or genes, enabling rapid identification of challenging-to-culture microbial strains.[14] Studies in literature have assessed the presence of *E. faecalis* qualitatively. qPCR has high sensitivity and specificity and helps in bacterial quantification.

Calcium hydroxide is considered gold standard intracanal medicament. Its antimicrobial activity is attributed to its high alkaline pH (11–12.5) and the release of hydroxyl ions in an aqueous environment. Their mortal effects on bacterial cells are probably because of mechanisms such as damage to the DNA and bacterial cytoplasmic membrane,[15] protein denaturation by inducing the disruption of ionic bonds that preserve the tertiary structure of proteins,[15]

and its physical presence in the root canal prevents reinfection by preventing the ingress, nourishment to the residual bacteria.

In vitro studies have demonstrated that prolonged exposure to calcium hydroxide leads to decrease in the mechanical properties of radicular dentin. Herbal intracanal medicaments do not adversely affect dentin's mechanical properties and also demonstrate comparable antimicrobial properties.[16]

Carnosic acid, a herbal medicament, stands out due to its antioxidative qualities essential for reducing oxidative stress and cellular damage caused by free radicals[17] and antimicrobial activity. In addition, it has anti-inflammatory properties and a relatively low toxicity profile.[18]

In this study, the antimicrobial efficacy of carnosic acid was comparable to the gold standard calcium hydroxide, as evidenced by the low proportion of *E. faecalis* growth, 4.14 and 3.8 mean copies/µl, respectively. This suggests that The antibacterial efficacy of carnosic acid could be attributed to the inherent lipophilic nature that modulates the membrane permeability and penetrate the bacterial membrane.[19,20] Also functions as an efflux pump modulator as the ethidium bromide efflux mechanism extrudes the antimicrobial agents and produces resistance,[20] inactivates genes that cause virulence and biofilm development, making it a potential quorum-sensing inhibitor against *E. faecalis*,[21] modifies the dentinal serum by reducing microorganism's adhesion which prevents and limits the growth of *E faecalis*.[22] In addition, its antioxidant activity could have acted as scavengers of free radicals produced by *E. faecalis* and curbed its proliferative ability.[13,23]

Propolis, a well-studied herbal medicament, is a brownish resinous substance that bees primarily collect from plants, which has potent antibacterial, antifungal, antioxidant, and anti-inflammatory action. The antimicrobial properties of propolis have been hypothesized to be due to its high flavonoid content, particularly galangin and pinocembrin with high antioxidant activity that impacts membrane permeability of *E. faecalis,* thereby reducing the resistance of these cells[24] and propolis is not influenced by dentine's buffering action.[25]

It also contains caffeic acid and phenethyl ester, which possess anti-inflammatory properties and exhibit cytotoxicity that is ten times lower than that of calcium hydroxide. Furthermore, the ethanol extract of propolis is shown to promote bone regeneration.[26]

In contrast to our study results, evidence has shown that carnosic acid and propolis had significantly better antimicrobial efficacy against *E. faecalis* than calcium hydroxide.[3,15,27] It was stated that calcium hydroxide with rosemary essential oil had an additive effect against *E. faecalis* than using only calcium hydroxide.[28]

Although there was no statistically significant difference among the groups receiving different medicaments, calcium hydroxide demonstrated relatively better results when compared to carnosic acid followed by propolis. This could be attributed to variations in the concentration of the test medicaments. Furthermore, proprietary, standardized, premixed calcium hydroxide with propylene glycol vehicle is better due to the slow diffusion and sustained release of calcium and hydroxyl ions, enhancing its properties compared to the herbal intracanal medicament paste prepared with saline.[29]

The limitations of this study include multispecies biofilm and clinical isolate *E. faecalis* were not employed.

CONCLUSION

Within the parameters of this study, it can be inferred that carnosic acid has promising antibacterial activity as an intracanal medicament against *E. faecalis.*

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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