




## Depth of Penetration and Antimicrobial Activity of 5% and 10% Bamboo Salt, 2% Chlorhexidine Gel and Calcium Hydroxide Against *Enterococcus faecalis* – An *In Vitro* Study

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### ABSTRACT

**Objective:** *E. faecalis* is one of the most commonly found species in persistent and secondary infections associated with endodontic failure. This *in vitro* study aimed to evaluate the depth of penetration and antimicrobial efficacy of 5% and 10% bamboo salt (BS), 2% chlorhexidine gel (CHX) and calcium hydroxide (CH) against *E. faecalis*.

**Methods:** *E. faecalis* was inoculated in dentine blocks for 21 days following which the antibacterial efficacy of the experimental medicaments were quantitatively assessed by harvesting the dentinal debris from depths of 200 µm and 400 µm from the block lumen on the 2<sup>nd</sup> and 7<sup>th</sup> day. The depth of penetration of the medicaments was measured using LIVE/DEAD BacLight stain under CLSM.

**Results:** Results showed that the medicaments had varying degrees of antimicrobial efficacy and depth of penetration. Among the medicaments, CHX showed the highest antimicrobial activity on both the time intervals ( $P < 0.05$ ), followed by 10% BS, 5% BS and the least efficacy was observed in CH group. CHX and 10% BS exhibited the highest depth of penetration, which was proximate to the penetration of *E. faecalis*.

**Conclusion:** It can be concluded that 10% bamboo salt can serve as a viable natural antimicrobial in endodontic therapy.

**Keywords:** Antimicrobial, bamboo salt, chlorhexidine, calcium hydroxide, *E. faecalis*

### HIGHLIGHTS

- This is the first study to use bamboo salt, a Korean folk medicine as an intracanal medicament against *E. faecalis* in an endodontic study model.
- 10% bamboo salt exerted significantly superior antimicrobial efficacy against *E. faecalis*, compared to the gold standard medicament, calcium hydroxide.
- The depth of penetration of conventional intracanal medicaments was found to be lesser compared to the depth of penetration of *E. faecalis*.
- The depth of penetration of 10% bamboo salt was 968.82 µm, which was proximate to that of *E. faecalis*.

### INTRODUCTION

The main goal of endodontic therapy is to eliminate microbes and their by-products from the root canal system prior to obturation (1). This can be achieved by chemomechanical preparation and disinfection of the canal with an antimicrobial agent. Studies have proven that microorganisms persist even after thorough cleaning and shaping, within the anatomical complexities of the root canals, due to limited access to the instruments and irrigants used during the procedure (2, 3). It is evidenced that teeth with negative cultures before obturation had

significantly higher success rates compared to teeth with positive cultures at the time of obturation (4). Placement of an intracanal dressing between successive treatment sessions has been shown to be effective in preventing repopulation of the canal by residual microorganisms thereby reducing the microbial load, especially in cases with large periapical lesions (5).

Endodontic infection involves many bacterial species, with *E. faecalis* being the most frequently associated strain in about 24%-77% of persistent endodontic infections and failed endodontic treatments (6). Commonly used intra canal medicaments (ICMs) are calcium hydroxide (CH) and

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2% chlorhexidine (CHX). The hydroxyl ions released from CH make the local environment highly alkaline (pH-12.5), which causes protein denaturation and damage to bacterial cytoplasmic membrane and DNA (7). Nevertheless, studies have shown that *E. faecalis* has become resistant to CH (7,8,9). CH is known to exert antimicrobial efficacy only up to a depth of 200  $\mu\text{m}$  from the canal lumen into the dentinal tubules, which is far remote compared to the depth of penetration of *E. faecalis*, which ranges from 244  $\mu\text{m}$  to 1175  $\mu\text{m}$  (10,11). Moreover, it is challenging to remove CH in its entirety from the root canal, which may in turn have a negative effect on the bonding of resin-based endodontic sealers to root dentine (12).

CHX is capable of achieving thorough disinfection of dentinal tubules. But, it has been reported to cause allergic and cytotoxic reactions (13,14). Thus, there is a constant quest for an ideal ICM that would overcome the deficiencies of the medications used currently.

Various natural products with a wide range of therapeutic properties including *Curcuma longa*, *Morinda citrifolia*, aloe vera and propolis have been experimented as irrigants and ICMs against *E. faecalis* (14-16). Bamboo salt (BS) is one such natural product, which shows promising antimicrobial, antioxidant and anti-inflammatory properties (17-20). It is a form of salt processed by repeatedly roasting sun-dried salt within a bamboo trunk, which is sealed by yellow soil. The roasting process is performed within a furnace and is fuelled by pinewood and pine resin (18). Shin et al. (17), proved that bamboo salt had antibacterial effect against *Streptococcus mutans*. Moon et al. (18), observed that bamboo salt was effective against *Salmonella enteritidis* and suggested that it could be used as a natural antimicrobial. A recent agar diffusion study showed that bamboo salt was effective against *E. faecalis* and this effect was concentration dependent (19). Whether it is effective in penetrating into the dentinal tubules and exerting an antimicrobial effect when used as an ICM is not yet known. Hence, the aim of this *in vitro* study was to comparatively evaluate the depth of penetration and antimicrobial efficacy of 5% and

10% bamboo salt, calcium hydroxide and 2% chlorhexidine gel against *E. faecalis*.

## MATERIALS AND METHODS

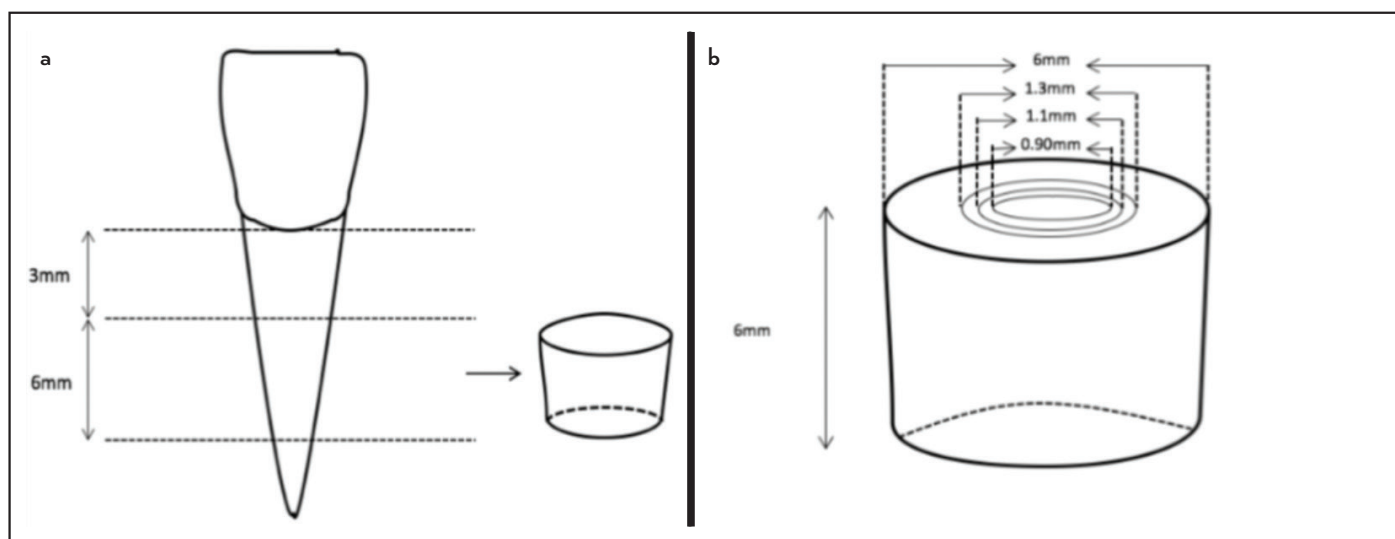
The research protocol was duly submitted to the university Institutional Review Board (IRB) and Ethical Committee approval was obtained (IRB/2015/MDS/No.303).

### Preparation of dentine blocks

Sample collection was in conformation with the provisions of declaration of Helsinki. 185 extracted single rooted human premolar teeth were collected after obtaining informed consent from patients, who had teeth scheduled for orthodontic or periodontal extractions. These extracted teeth were examined radiographically to confirm the presence of single canal, cleaned, stored in 0.1% thymol (Chenchems, Chennai, India) and were used within one month post extraction. A rotary diamond disk was used to section the tooth so as to obtain 6 mm of the middle third of the root for the experimental procedure. Figure 1 shows the schematic representation of the prepared sample. Cementum was removed from the root surface and the external diameter was standardized to 6 mm using a rotating diamond abrasive. The internal diameter of the dentine block was standardized to a size of 0.9 mm using Gates Glidden drill no.3 (Mani Inc, Tochigi, Japan) in a slow speed hand piece. Smear layer was removed by immersing the blocks in an ultrasonic bath containing 17% EDTA (Anabond Stedman Pharma Research Ltd, Kanchipuram, India) for 5 min followed by consecutive immersion in 5% NaOCl (Nice chemicals, Kochi, India) for 5 min. The residual chemicals were removed by rinsing the blocks in an ultrasonic bath containing distilled water (Chenchems, Chennai, India) for 5 mins.

### Sterilization of dentine blocks

The dentine blocks were subjected to two cycles of sterilization in an autoclave. In the first cycle, the blocks alone were autoclaved at 121°C for 15 min at 15 psi pressure. In the second cycle, the blocks were suspended in individual microcentrifuge tubes with 1 mL of tryptone soya (TS) broth (Himedia, Mumbai, India) prior to autoclaving.



**Figure 1.** Schematic representation of the dentine block model. The dentine block was secured from an intact tooth (a) and its internal and external diameters were standardized (b)

### Inoculation of dentinal tubules with *E. faecalis*

*E. faecalis* (ATCC 29212) was grown in TS agar (Himedia, Mumbai, India) for 24 h at 37°C. The culture of *E. faecalis* was suspended in 5 mL of TS broth and incubated for 4 hrs at 37°C. The *E. faecalis* suspension was standardized by matching its turbidity equivalent to 0.5 McFarland standard. 50 µL of the inoculum was transferred into each of the microcentrifuge tubes containing the dentine blocks. The TS broth was changed every 3 days up to 21 days. All the procedures were carried out in a biosafety cabinet. The purity of the culture was checked by sub culturing 5 µL of the broth from the incubated blocks, on agar plates. After the incubation period, the blocks were irrigated with 5 mL of sterile saline to remove the broth. The penetration of *E. faecalis* into the dentinal tubules was confirmed using 5 random samples, splitting them longitudinally and viewing them under a field emission scanning electron microscope (FESEM, JSM-5410, JOEL, Tokyo, Germany).

### Grouping, preparation and placement of medicaments

The remaining 180 dentine blocks were randomly assigned to 5 groups (n=36). 5% bamboo salt (Korea salt, Seoul, South Korea) (BS 5, group 1) and 10% bamboo salt (BS 10, group 2) were prepared by dissolving 5 g and 10 g of bamboo salt in 100 mL of distilled water respectively. 2% CHX (Anabond Stedman Pharma Research Ltd, Kanchipuram, India) and CH (Azure Laboratories Pvt Ltd, Kochi, India) mixed with distilled water served as groups 3 and 4 respectively. 25 g of methylcellulose (Chenchems, Chennai, India) was added as a thickening agent in groups 1-4. Dentine blocks filled with TS broth served as controls (TS, group 5). The respective ICMs were placed into the respective dentine block lumens using a plastic instrument. After placement of the medicament, the blocks were sealed with paraffin wax at both the ends, externally coated with paraffin wax and incubated in an aerobic environment at 37°C. These groups were further sub-divided into two subgroups (n=18 each), based on the time period of the medicament placement, namely 2 and 7 days (subgroups A and B). The medicaments were removed from the blocks after the prescribed time periods by placing the latter in an ultrasonic bath containing distilled water.

### Quantitative assessment of antimicrobial activity of medicaments

Collection of dentinal debris was done according to an established protocol (21). Dentine debris from nine blocks from each subgroup was individually harvested at depths of approximately 200 µm and 400 µm from the block lumen using

Gates Glidden drills (Mani, Tochigi, Japan.) no. 4 and 5 respectively in a single stroke downward movement. The debris was collected in 1 ml of sterile broth and incubated in an anaerobic environment at 37°C for 24 hrs, after which the content of each micro-centrifuge tube was serially diluted, 100 µL of broth in 100 µL of normal saline for five times. Five µL of this diluted sample was plated on TS agar plate and incubated at 37°C for 24 hrs. The colony forming units (CFU) were counted and tabulated.

### Depth of penetration of *E. faecalis* and experimental medicaments into the dentinal tubules

One mm thick slice was obtained by horizontally sectioning each of the remaining nine blocks from both the subgroup. The obtained slices were stained using LIVE/DEAD BaCLight fluorescent stain (Thermo Fisher Scientific, Massachusetts, United States) that was diluted in phosphate buffer solution (PBS) in a ratio of 1.5:1000. The specimens were placed in the prepared stain at room temperature in the dark for 30 minutes and later washed with PBS. The stained specimens were mounted on a slide and viewed using confocal laser scanning microscope (CLSM, LSM 700, Carl Zeiss, Germany) under oil immersion at 10X magnification. An inbuilt measuring tool was used to determine the farthest distance from the lumen to the viable cells (green fluorescence) in the control group, which was taken as the depth of penetration of *E. faecalis*. The farthest distance from the lumen to the dead bacteria (red fluorescence) in groups 1 to 4 was taken as the depth of penetration of the medicament. An average of three such measurements obtained from each slice was taken as the depth of penetration value for that slice. All the measurements were made by a single investigator.

### Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS, version 17) for Microsoft windows. The data was normally distributed. Therefore, parametric tests were performed. Independent sample student t test was used to compare continuous variables between two groups. Paired sample test was used to compare within groups. A one-way analysis of variance with a post-hoc Tukey HSD was used for continuous data. A two-sided p value < 0.05 was considered statistically significant.

### RESULTS

FESEM confirmed the penetration of *E. faecalis* into the dentinal tubules. The mean CFU of all the groups at 200 µm and

**TABLE 1.** Mean±SD of CFU (log 105) of all the groups at both the depths on days 2 and 7

Groups	200 µm		400 µm	
	Day 2	Day 7	Day 2	Day 7
Group 1 (BS 5)	2.63±0.23 <sup>c</sup>	5.14±0.69 <sup>c</sup>	4.30±0.23 <sup>c</sup>	5.20±0.41 <sup>d</sup>
Group 2 (BS 10)	1.33±0.19 <sup>b</sup>	2.04±0.07 <sup>b</sup>	1.40±0.19 <sup>b</sup>	2.28±0.12 <sup>b</sup>
Group 3 (2% CHX)	0.86±0.01 <sup>a</sup>	0.92±0.03 <sup>a</sup>	0.80±0.01 <sup>a</sup>	0.89±0.02 <sup>a</sup>
Group 4 (CH)	6.55±0.19 <sup>d</sup>	4.79±0.08 <sup>c*</sup>	4.49±0.60 <sup>c</sup>	3.81±0.23 <sup>c*</sup>
Group 5 (TS broth)	7.55±0.28 <sup>e</sup>	6.63±0.28 <sup>d</sup>	9.18±0.34 <sup>d</sup>	8.11±0.25 <sup>e</sup>

SD: Standard deviation, <sup>a</sup>For each column different superscript letters indicate significant difference (P<0.05) between the groups.

\*Among the experimental groups, CH showed a significant reduction in CFU (P<0.05) on day 7 compared to day 2 at both the depths



**TABLE 2.** Mean±SD of depth of penetration of all the medicaments on days 2 and 7 (in  $\mu\text{m}$ )

Groups	Day 2	Day 7
Group 1 (BS 5)	611.77±59.79 <sup>c</sup>	543.48±74.24 <sup>c</sup>
Group 2 (BS 10)	758.07±69.79 <sup>b</sup>	968±137.34 <sup>a</sup>
Group 3 (2% CHX)	861.91±84.92 <sup>a</sup>	812.18±96.77 <sup>b</sup>
Group 4 (CH)	517.66±73.57 <sup>d</sup>	454.73±78.57 <sup>c</sup>

SD: Standard deviation, \*Under each time period, different alphabets in superscript denote significant difference between the groups ( $P<0.05$ )

400  $\mu\text{m}$  on days 2 and 7 is given in Table 1. The highest and the lowest mean CFU were recorded for control (group 5, TS Broth) and CHX (group 3) respectively, at both the depths and on both the days ( $P<0.05$ ). CHX was followed by BS 10 (group 2), which showed significantly lower mean CFU when compared to BS 5 (group 1) and CH (group 4) ( $P<0.05$ ). BS 5 was superior or equivalent to CH except at 400  $\mu\text{m}$  on day 7. At both the depths, a significant increase in CFU was seen on day 7 when compared to day 2 in BS 5, BS 10 and CHX, whereas a significant decrease was observed in CH and control ( $P<0.05$ ).

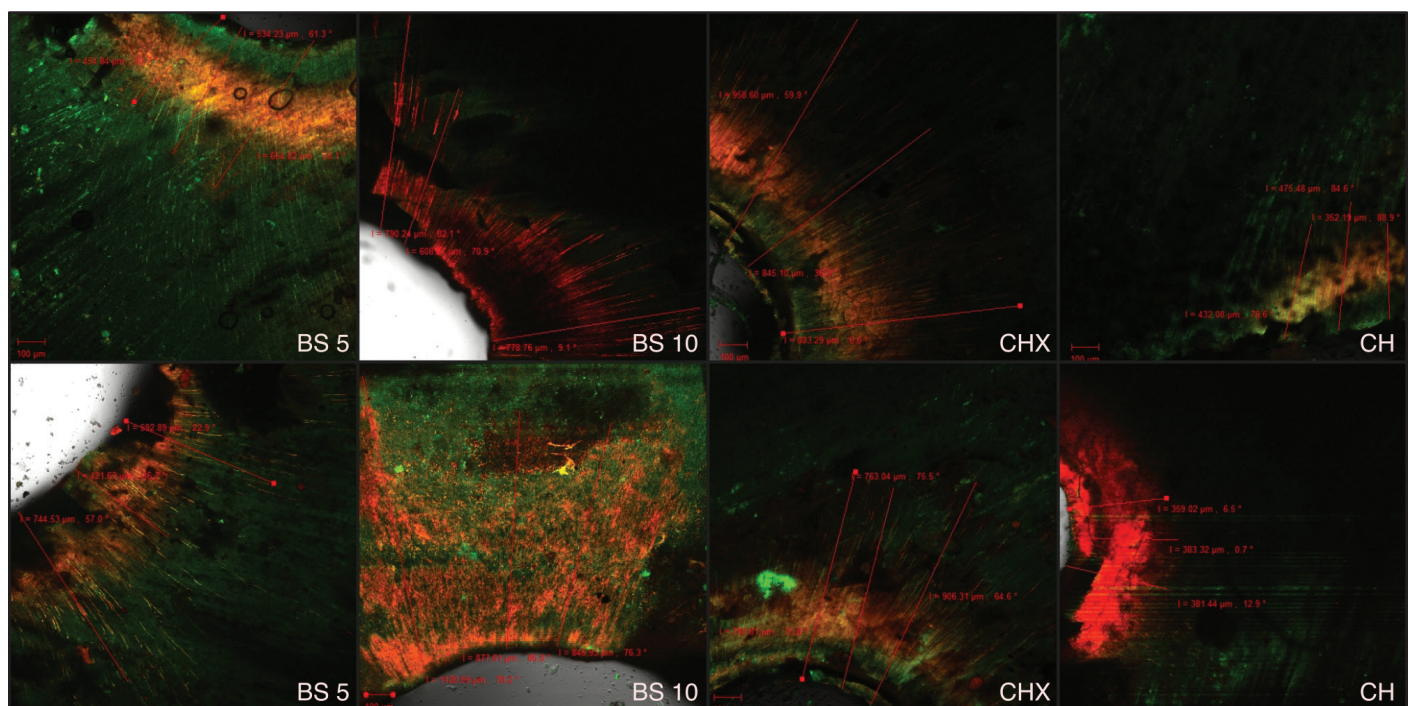
CLSM images showed a significant increase in the mean depth of penetration (DOP) of *E. faecalis* on day 7 (1061.79  $\mu\text{m}$ ) compared to day 2 (821.92  $\mu\text{m}$ ) ( $P<0.05$ ). The mean DOP of all the medicaments on both the days and the representative CLSM images showing their penetration are given in Table 2 and Figure 2 respectively. On day 2, CHX (861.91  $\mu\text{m}$ ) showed the highest mean DOP, while on day 7, BS 10 (968.82  $\mu\text{m}$ ) showed the highest ( $P<0.05$ ). CH showed the least penetration on both the days. While a significant increase in DOP was found in BS 10 from day 2 to day 7, a decrease was found in the other groups, which was significant only for BS 5 ( $P<0.05$ ).

## DISCUSSION

The antimicrobial efficacy of the ICM was evaluated using the modified Haapasalo and Ørstavik dentine block model (21). Blocks with definite internal and external diameters enabled standardized quantification of depth of penetration of the medicaments into the dentinal tubules. Dentine debris was harvested from two specific depths in accordance with previous literature (9, 16). This also correlates with the established depth of penetration of CH (22). LIVE/DEAD BacLight fluorescent solution (SYTO 9 and propidium iodide) works by using the integrity of the bacterial cell membrane as a parameter. The red-fluorescent nucleic acid stain, propidium iodide (PI) penetrates only those cells with disrupted membranes, thus identifying dead bacteria. On the contrary, the green-fluorescent nucleic acid stain SYTO 9 enters live and dead bacterial cells. When both dyes are present, PI exhibits a stronger affinity for nucleic acids than SYTO 9, and hence, PI displaces SYTO 9 (23). The use of CLSM enabled visualization of stained microbes and quantification of their penetration depth using an in-built measuring tool.

## Antibacterial efficacy

According to the results of the present study, all the experimental medicaments showed antibacterial activity, but to varying degrees. CHX exhibited the highest antibacterial activity at both the depths and on both the days. These results are in accordance with previous studies (14-16). The superior antimicrobial activity of CHX can be explained by the fact that CHX being a cationic bis-guanide interacts with the negatively charged phosphate groups of microbial cell wall altering their osmotic equilibrium (24). This results in increased cell wall permeability, facilitating the entry of CHX molecule into the microbial cell (25). CHX causes coagulation or precipitation of cytoplasm by protein cross-linking at higher concentrations, like the one used in this study (2%), making it bactericidal in nature (26). However, complete elimination of *E. faecalis* was not



**Figure 2.** CLSM images showing depth of penetration of the medicaments on day 2 (upper panel) and day 7 (lower panel)

achieved, in contrast to previous studies where CHX showed 100% elimination of *E. faecalis* (15, 16). This could be due to the decreased sensitivity of *E. faecalis* to CHX. Long-term use of a medicament could result in decreased sensitivity of the microbes towards it, as evidenced in the literature (27).

CH demonstrated significantly lesser antimicrobial activity compared to all the other experimental groups on both the days and at both the depths, except on day 7 at 400  $\mu\text{m}$ , where CH was significantly better than BS 5. CH can create alkalinity corresponding to a pH of 10.3 within the root dentine, whereas *E. faecalis* can resist an alkaline environment with a pH of up to 11.5, owing to its proton pump mechanism and pH homeostasis (18). By maintaining pH homeostasis in acidic or alkaline environment, the bacterial cell maintains an internal pH, which is kept within a narrow range such that enzymes and proteins maintain a normal function (28). Also, the buffering capacity of dentine prevents CH from reaching and maintaining a high alkaline pH, thus diminishing the potency of the latter (29). When comparing the antimicrobial activity at both the depths, CH showed consistently superior antimicrobial activity at 400  $\mu\text{m}$  compared to 200  $\mu\text{m}$  on both days. In addition, an increase in antimicrobial activity was observed on day 7 compared to day 2 at both the depths in CH treated specimens, unlike the other experimental groups. This unique pattern could be attributed to the diffusion of hydroxyl ions from CH to the outer root dentine, which takes one to seven days and thus their ensuing antimicrobial activity (30). Nevertheless, Camargo et al. (31), observed that it takes at least 14 days for ions from CH to reach the outer root dentine. Therefore, further studies using longer residence time of ICM are warranted in this regard (31).

In the case of CHX, while no significant difference was detected in its activity at both the depths on day 2, a significantly superior activity was seen at 400  $\mu\text{m}$  compared to 200  $\mu\text{m}$  on day 7. Komorowski et al. (2000), observed that CHX is capable of exerting satisfactory antimicrobial action if it is applied for a period of at least 7 days (25). This property of CHX helps in preventing reinfection of the canal, not only during the treatment period but also following obturation. Hence, based on these sound observations from previous studies, a seven-day protocol was adopted in this study.

Among the experimental groups, 10% bamboo salt (BS 10) showed better antibacterial activity at both the depths and on both the days. Bamboo salt exerts its antibacterial property by diminishing the water content in the bacterial cell, thereby causing hypersomatic shock to the bacteria. It also contains ions of Zn, Mn, Ca, P, K, Mg and Fe (18). These metal ions, along with its high alkaline pH (11.4) cause oxidative stress, protein dysfunction and membrane damage to the bacterial cell (32). The present study also implies that the antimicrobial activity of BS was concentration dependent with BS 10 showing superior activity than BS 5 in all tested conditions.

In the present study, a decrease in the efficacy of CHX, BS 10 and BS 5 was found on day 7 compared to day 2. This can be explained by the fact that during interaction of medicament with the microbes in the root canal, in addition to the death of microbes, there is also a gradual weakening and/or inactivation of the disinfecting agent. Rinsing the specimen with che-

lating agents such as ethylenediaminetetraacetic acid prior to treatment with disinfecting agents might also weaken the effect of the latter (33).

In the current study, methylcellulose was added as a thickening agent in order to adjust the consistency of the medicaments to a paste form. Studies have shown that methylcellulose possesses an inherent antimicrobial activity. This could also have influenced the antibacterial activity of the medicaments.

### Depth of penetration

A magnification of 40X is preferred to observe the microbial load on the tooth specimens under CLSM. But, in the present study a 10X magnification has been used to get a panoramic view from the lumen of the root canal to the outer surface of root dentine. In the present study, *E. faecalis* was shown to penetrate to a depth of 821.91  $\mu\text{m}$  to 1061.79  $\mu\text{m}$  into the dentinal tubules. This result can be compared to a previous study by Vatkar et al. (11), who observed that *E. faecalis* penetrated up to a depth of 1175.78  $\mu\text{m}$ . CHX and BS 10 showed significantly higher penetration depths than BS 5 and CH. These results correlate well with their antimicrobial efficacy. Therefore, it can be inferred that the medicaments, which have penetrated deeper into the dentinal tubules, have successfully eradicated *E. faecalis* compared to the other experimental medicaments. Fewer studies are available in the literature evaluating the depth of penetration of ICMs. Vadhana et al. (34), observed that CHX could penetrate up to 138  $\mu\text{m}$  into root dentine. But direct comparison of the results of the present study to the above-mentioned study is difficult owing to the varying time of contact of the disinfectant with dentine when used as an irrigant and ICM. Among the medicaments tested in this study, CH showed the least depth of penetration into the dentinal tubules, which correlated well with its poor antimicrobial activity. Similar observations were made by Sireesha et al. (22), who found that CH could penetrate only to a depth ranging from 111.99  $\mu\text{m}$  to 459.45  $\mu\text{m}$ , which was significantly lesser than the other medicaments tested in that study.

Any *in vitro* study testing the antimicrobial efficacy of a substance does not completely simulate the *in vivo* environment of an infected canal. Further, the inorganic components of dentine, the inflammatory mediators and the serum present may adversely affect the action and efficacy of the medicaments (33). Hence, caution must be exercised when drawing conclusions to *in vivo* conditions by using the results of this *in vitro* study.

Although bamboo salt has been extensively used in food industry, its biocompatibility when used as an ICM needs to be studied. Bamboo salt also possesses anti-inflammatory activity. Whether this property would further augment its use as an ICM has to be evaluated. Further *in vivo* studies are also required to confirm the findings of the present study. Since the antimicrobial action of bamboo salt was found to be concentration dependent, the results of this study open avenues for further research using higher concentrations of the salt.

### CONCLUSION

Within the limitations of this *in vitro* study, it can be concluded that *E. faecalis* penetrated up to a depth of 1061.79  $\mu\text{m}$  into the



dentinal tubules. All the experimental medicaments showed antibacterial efficacy to varying degrees. 2% CHX and CH showed the highest and the least antimicrobial efficacy and depth of penetration respectively. The performance of bamboo salt was concentration dependent, with BS 10 showing superior antimicrobial efficacy and penetration than BS 5 in all tested conditions.

#### Disclosures

**Conflict of interest:** The authors deny any conflict of interest associated with the study.

**Ethics Committee Approval:** The research protocol was duly submitted to the university Institutional Review Board (IRB) and Ethical Committee approval was obtained (IRB/2015/MDS/No.303).

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