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Effectiveness of curcumin against Enterococcus faecalis biofilm

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Abstract

Objectives. To evaluate the antimicrobial efficacy of curcumin against Enterococcus faecalis biofilm formed on tooth substrate in vitro. Sodium hypochlorite (NaOCl) and chlorhexidine (CHX) served as standards for comparison. ***Materials and methods.*** Biofilms of E.faecalis were formed on instrumented, extracted human teeth (n = 96). At the end of the 2nd day, 2nd and 8th weeks, specimens were treated for 30 min with one of the test solutions or saline (control) and the surviving colony-forming units (CFU/mL) was recorded. Results were analyzed by Kruskal-Wallis test and Dunnet test for pair-wise comparison with Bonferroni correction ($p = 0.05$). ***Results.*** Only NaOCl showed complete eradication of bacteria at all time periods. In the 2-day and 2nd week biofilms, curcumin and NaOCl showed complete inhibition, which was significantly lower than the CFU recovered in the CHX and saline groups ($p < 0.05$). In 8 week biofilms, samples treated with curcumin showed 553 ± 137.6 CFU/mL, which was significantly higher than NaOCl (0 CFU/mL), but significantly lower than CHX (2551 ± 129.8) and saline control ($1.42 \times 1011 \pm 2.12 \times 1010$; $p < 0.05$). ***Conclusions.*** Sodium hypochlorite (3%) showed maximum antibacterial activity against E.faecalis biofilm formed on the tooth substrate, followed by curcumin and CHX. Considering the potential for undesirable properties of NaOCl, the use of herbal alternatives in endodontics might prove to be advantageous.

Key Words: *biofilm, chlorhexidine, curcumin, Enterococcus faecalis, irrigant, microbiology, phytotherapy, sodium hypochlorite*

Introduction

Chemomechanical preparation is a pivotal step for infection control during root canal treatment [1]. During the stages of cleaning and shaping, instrumentation and irrigation promote significant microbial reduction. Despite this, the complete eradication of intra-canal infection is unachievable by the current available methods. Thus, remaining bacteria might lead to reinfection of the root canal space [2–4]. *Enterococcus faecalis*, a facultative anaerobic gram-positive coccus, is the most common *Enterococcus* sp. cultured from non-healing endodontic cases [5,6]. *E. faecalis* can adhere to the root canal walls, accumulate and form communities organized in biofilm, which enables the bacteria to become more highly resistant to phagocytosis, antibodies and antimicrobials than non-biofilm-producing organisms [7,8]. *E. faecalis* that invades the dentinal tubules may survive chemomechanical instrumentation and intra-canal medication; it can colonize the tubules and reinfect the obturated root canal [9].

Sodium hypochlorite (NaOCl) has been documented as the most effective irrigant in terms of antimicrobial activity [10]. Recently, there has been a growing trend to seek natural remedies as part of dental treatment [11]. Murray et al. [12] reported that *Morinda citrofolia* (Indian Noni) can be used as an alternative to NaOCl, in conjunction with Ethylene diamone tetraacetic acid (EDTA) [12], whereas Prabhakar et al. [13] concluded that the Indian ayurvedic triphala presents significant antibacterial activity, although it was less effective than 5% NaOCl.

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Turmeric (*Curcuma longa*) is extensively used as a spice, food preservative and coloring material in India, China and South East Asia. It has been used in traditional medicine for the treatment of numerous diseases. Curcumin (diferuloylmethane), the main yellow bioactive component of turmeric, has been shown to have a wide spectrum of biological actions, including antimicrobial, anti-inflammatory and antioxidant activities [14–16]. Many studies have attributed a wide spectrum of activities to this compound [17,18] and this provides a suitable basis for exploring its endodontic applications.

Within this background, the present study examined the antimicrobial effect of curcumin against *E. faecalis* biofilm, considering 2% chlorhexidine and 3% NaOCl as references solutions for comparison. The null hypothesis tested was that there is no difference in the antimicrobial activity between the solutions tested.

Materials and methods

Test materials

The antibacterial activity of the test solutions (Curcumin, Biopurify Phytochemicals Ltd., Sichuan, PR China; 2% Chlorhexidine (CHX), Asep RC, Stedman Pharmaceuticals, Chennai, India; 3% sodium hypochlorite, Prime Dental Products, Mumbai, India) were tested on *E. faecalis* biofilm formed on tooth substrate. The absolute purity of curcumin (100%) was assessed by High Performance Liquid Chromatography (HPLC) prior to testing.

Bacteriological culture

Cultures of *E.fecalis* [ATCC 29212] were maintained at 37°C on nutrient agar (Hi Media Labs, Bangalore, India) under aerobic conditions. Individual colonies of the bacteria were seeded into 10 mL of brain heart infusion (BHI) broth (Hi Media Labs) and grown overnight at 37°C with agitation. Following incubation, the cultures were centrifuged and the cell pellet was washed in Phosphate Buffered Saline (PBS) before being re-suspended in fresh BHI. This washing step minimized the carry-over effect of exhausted culture medium and waste metabolic products into the biofilm model. The final optical density of the culture was adjusted to 0.1 units (OD 650 nm) with additional BHI; this was subsequently determined as containing 5×10^7 CFU/mL.

Tube dilution method. Double dilution was made from a higher dilution of 100 mg/mL to a lower dilution in a series of test tubes. For curcumin, the solvent was dimethyl sulfoxide (DMSO). Pilot studies established that DMSO does not have any antibacterial activity. Each tube was inoculated with bacterial suspensions and incubated at 37°C overnight. The Minimum Inhibitory Concentration (MIC) was regarded as the lowest concentration in the series of dilutions, which did not permit the growth of the susceptible bacteria. The sub-cultures were made from the tubes which did not yield any visible turbidity in the MIC assay on freshly prepared Mueller-Hinton agar plates. After 24 h of incubation at 37°C, the Minimum Bactericidal Concentration (MBC) was regarded as the lowest concentration of the test solution that allowed less than 0.1% of the original inoculum to grow on the surface of the medium. Test solutions were tested in triplicate (biological repeats) for each experiment.

Analysis of time to kill. The bacteria (*E. faecalis* - ATCC 29212) were exposed to the bactericidal concentration of the test solutions for 30 min. This was done by inoculating a loop full of the sample at regular intervals (2 min) on a Mueller-Hinton agar plate which was incubated at 37°C for 24 h and observed for growth.

Biofilm formation and susceptibility assay

Single-rooted mandibular premolars (n = 96), extracted for orthodontic reasons, were prepared using a crown-down method. Following access cavity preparation, the coronal two-thirds of each canal was prepared using Gates Glidden drills size 1, 2 and 3. The apical third was prepared to a master apical file size of 40. Smear layer was removed using 17% EDTA followed by 5.25% NaOCl. A final flush of phosphate buffered saline (PBS) was done for all the specimens. To ensure no leakage of microbial media, the roots were planed, to remove any soft tissue and sealed using an acid-resistant nail varnish. The apex was then sealed using composite resin (Filtek Z350, 3M ESPE, St. Paul, MN, USA). Each tooth was located in a piece of addition silicone impression material (Virtual, Ivoclar vivadent, Liechtenstein) to maintain it vertically in a glass jar before being autoclaved at 121°C for 15 min.

The specimens were inoculated with 2 mL of *E. faecalis* cultured overnight at 37°C in All Culture medium (Sigma Aldrich, St. Louis, MO, USA), adjusted to an optical density of 1 at 600 nm having 10^8 cells/mL (UV–VISIBLE Spectrophotometer, Shimadzu, Japan). To ensure a constant supply of medium and to remove excess bacterial cells, the culture medium was replaced every alternative day for a period of 2 weeks or 8 weeks. Hence, three time periods were considered for evaluation—2 days, 2 weeks and 8 weeks. A separate cohort of teeth (n = 3 2) was used for each of the time periods. A pilot study done by staining 5 μm longitudinal sections of teeth with Taylor's modified Brown and Brenn technique [19,20] and observed at 1000× magnification

Table I. Susceptibility of planktonic *E.Faecalis* cells to the test solutions.

Group	Zone of inhibition (mm)	Minimum inhibitory concentration	Minimum bactericidal concentration
Curcumin	32 ± 4	625 μg/mL	2.5 mg/mL
CHX	30 ± 3	0.5%	1.5%
NaOCl	34 ± 5	0.4%	1.1%

confirmed and validated the formation of a uniform endodontic biofilm on the surface of the root canal. This validation was done for all time periods considered in this study.

The clinical irrigation procedure was mimicked in the laboratory by using an electronic dispensing pipette (Pro-line 50–1000 μl, Biohit, Torquay, Devon, UK) to supply the irrigant in a reproducible manner. The pipette tip was located into the root canal to 3 mm short of the working length. With this particular unit set at output speed setting #3, the flow rate was 0.5 mL/s, which corresponded to a flow velocity at the tip of ~ 1 m/s. The pipette tip was loaded with 1 ml of irrigant ($n = 8$): group 1, curcumin (2.5 mg/mL of DMSO); group 2, 2% Chlorhexidine (CHX, Asep RC, Stedman Pharmaceuticals); group 3, 3% sodium hypochlorite (Prime Dental Products) or PBS control.

The irrigant was dispensed into the tooth as a series of five consecutive 200 μL aliquots. The final aliquot of irrigant was allowed to remain for 30 min. The root canals of samples of groups 1 and 3 were flushed with neutralizing broth and sodium thiosulfate (neutralizing agent for NaOCl), respectively, for 5 min, to flush the residual irrigant and also to minimize its carry-over into the subsequent sampling and dilution phases. Similarly, samples of group 2 were flushed with L-α-lecithin in 3% Tween 80 (neutralizing agent for chlorhexidine). Finally, all canals were flushed with sterile saline. Bacteria were recovered from the root canal using a series of five sterile paper points (ISO size 40, Dentsply), which were in turn rubbed against the walls of the root canals and allowed to draw up their full capacity of liquid. The paper points were transferred to BHI broth, vortex-mixed for 30 s and incubated at 37°C for 6 h to enrich the number of bacteria. *E.faecalis* colony forming units were enumerated by performing serial dilutions and growth on nutrient agar after 16 h aerobic incubation at 37°C. The same procedure was followed for the samples with 2 week and 8 week biofilms (immature and mature biofilms, respectively).

Data presentation and analysis.. The main outcome variable in this study was microbial counts (in log CFU/mL). The distribution of microbial counts was skewed and, consequently, non-parametric statistical tests (Kruskal-Wallis test and Dunnet test for pairwise comparison with Bonferroni correction) were applied. The alpha-type error was set at 0.05.

Results

The MIC and MBC of test solutions are summarized (Table I). Analysis of time to kill showed that NaOCl, curcumin and CHX achieved 100% killing of *E. faecalis* at 2 min, 5 min and 10 min, respectively.

The data for bacterial counts in the different biofilms after treatment with the test solutions is presented (Table II). Both NaOCl and curcumin showed complete inhibition of bacteria in the 2 days and 2 week biofilms, while the samples treated with CHX and control showed a significantly higher bacterial growth ($p < 0.05$). In the 8 week biofilms, NaOCl demonstrated 100% eradication of bacteria, while the surviving CFU in the samples treated with curcumin was significantly lesser than the groups treated with CHX or PBS control ($p < 0.05$).

Discussion

The present study compared the antibacterial activity of the predominant active component of turmeric, i.e. curcumin, against *E.faecalis* biofilm. This appears to be the first report on the endodontic applications of curcumin. This material has shown antibacterial, antifungal and antiviral activity in previous studies [21–23]. The MIC of curcumin against *E. faecalis* in this study was ~ 625 μg/mL, which is in accordance with an earlier report [24].

Components of turmeric are named curcuminoids (curcumin or diferuloyl methane, demethoxycurcumin and bisdemethoxycurcumin). These components are

Table II. Colony forming units/mL (mean ± standard deviation) in the 2 days, 2 and 8 week biofilms for different test solutions.

Group	2 days	2 weeks	8 weeks
Curcumin	$0^{A,a}$	$0^{A,a}$	$553 \pm 137.6^{A,b}$
CHX	$268 \pm 75.3^{B,a}$	$765 \pm 126.5^{B,b}$	$2551 \pm 129.8^{B,c}$
NaOCl	$0^{A,a}$	$0^{A,a}$	$0^{C,a}$
Saline (control)	$1 \times 10^8 \pm 1.23 \times 10^{7\ C}$	$1.18 \times 10^{11} \pm 1.56 \times 10^{10\ D}$	$1.42 \times 10^{11} \pm 2.12 \times 10^{10\ D}$

Mean values labeled with different superscript upper case letter and lower case letters were significantly different from the saline control within the same time point (Kruskal-Wallis test) and the same irrigant at different time points (Dunnet test) at the 5% level, respectively.

polyphenols with a strong antioxidant function [25]. Curcumin, the most important fraction, is responsible for the biological activities of turmeric. It has been hypothesized that curcumin inhibits the assembly of a protein-filamenting temperature-sensitive mutant Z (FtsZ) protofilaments and also increases the GTPase activity of FtsZ. The perturbation of the GTPase activity of FtsZ assembly is lethal to bacteria [26].

A pilot study carried out prior to this experimental protocol showed that 3% NaOCl was able to completely eradicate *E. faecalis* in the 2 days, 1 week and 6 weeks biofilms, while curcumin was able to demonstrate complete eradication of *E. faecalis* in the 2 day and 1 week biofilms (data not shown). The results of the present study showed that both 3% NaOCl and curcumin were able to eradicate 2 week biofilms of *E.faecalis*, but curcumin could not eradicate the 8 week biofilms. The ability of 3% NaOCl to eliminate *E. faecalis* biofilms is in accordance with other reports [13,27,28]. It is possible that curcumin is able to penetrate and disrupt *E. faecalis* biofilms, as is NaOCl. For an irrigant to be effective against biofilms, the action on biofilms should involve the elimination of the extracellular polysaccharide matrix (EPS) as well as the bacteria, because this matrix could act as an additional source of nutrients and/or as a suitable surface for further cell growth [28,29]. From the results of the present study, it may be hypothesized that turmeric (curcumin) is able to eliminate the EPS matrix and the bacteria. However, this hypothesis warrants further investigation. On the other hand, this was not true for the 8 week biofilms, probably due to the thickness and more complex dynamic nature of the biofilm. Studies show that chlorhexidine has significant antibacterial activity against most of the bacterial species isolated from infected root canals [30,31]. However, the results of this study showed that chlorhexidine was unable to completely eradicate *E. faecalis* in biofilms, which is in agreement with previous reports [32–34]. This variation may be explained by the difference in study design in the aforementioned studies.

Root canal sampling was done using paper points in this study. This may be a problem in oval root canals where oval extensions are not touched by the paper point during sampling collections and induce false negative results. However, in the present work, root canals were prepared with gates glidden drills to assume a circular shape, which ensures uniform contact of paper points with the root canal walls, thereby offsetting the possibility of false results.

Microbial communities *in vivo* are quite resistant to and difficult to eradicate with antimicrobials, owing to the fact that the microorganisms to be targeted are organized in structures attached to each other and/or the root canal walls often involving a multitude of species known as microbial biofilms [35,36]. The testing of antimicrobial agents against bacterial biofilms is yet to be standardized and no *in vitro* method accurately reflects the conditions under which microorganisms grow *in vivo*. Therefore, caution should be exercised while extrapolating results of biofilm-based studies to the clinical scenario. Although this study was not performed *in vivo*, the model used herein provides a well-standardized method which allowed comparison of different irrigants.

A recent report suggested that curcumin in aqueous preparations exhibits a phototoxic effect against gram positive and gram negative bacteria [37,38]. This opens up avenues for further research on the use of turmeric in photodynamic therapy of root canal systems. In conclusion, curcumin was able to eradicate 2 day and 2 week biofilms of *E. faecalis* and this action was similar to 3% NaOCl. The major advantages of using herbal alternatives are easy availability, cost-effectiveness, increased shelf-life, low toxicity and lack of microbial resistance [39]. Considering the documented disadvantages of NaOCl including unpleasant taste, toxicity, inability to remove the smear layer, limited *in vivo* antibacterial activity, detrimental effect on dentin microhardness, structural integrity, elasticity and flexural strength [40] and the aforementioned advantages of turmeric/curcumin, further research is warranted on this substance prior to clinical application as an irrigant and intra-canal medicament.

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