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Antibiofilm and Antibacterial Activities of Farnesol and Xylitol as Potential Endodontic Irrigants

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This study investigated the antibiofilm and antibacterial effects of farnesol and xylitol in a series of experiments in order to evaluate their potential use as root canal irrigants. The following substances were tested: 0.2% farnesol; 5% and 20% xylitol; 0.2% farnesol plus 20% xylitol; and saline (control). For comparison with an established endodontic irrigant, 2.5% NaOCl was included in each test. Three experiments were conducted: the crystal violet assay, to evaluate the effects on the biofilm biomass; the dentin disinfection test, to evaluate the effects on bacterial viability in biofilms; and the root canal disinfection test, to simulate the use in the root canal environment. Farnesol was the most effective substance in reducing the biofilm biomass, followed by 20% xylitol. All substances affected bacterial viability in biofilms; farnesol showed the best results followed by the farnesol/xylitol combination. Irrigation with all substances significantly reduced the bacterial load ($p<0.001$), but only the farnesol/xylitol combination was significantly more effective than saline ($p=0.02$). NaOCl was more effective than any other substance tested in the three experiments ($p<0.001$). The findings demonstrated that farnesol affected both the biofilm biomass and the viability of cells in the biofilm, while 20% xylitol affected only the biofilm biomass. Although not more effective than NaOCl, the combination of these two antibiofilm substances has potential to be used in endodontics in certain situations.

Key Words: antibiofilm agents, bacterial biofilm, endodontic irrigation, *Enterococcus faecalis*.

Introduction

Apical periodontitis is an infectious disease associated with intraradicular bacterial biofilms (1). The prevalence of bacterial biofilms in the apical part of the root canal system is very high in teeth with either primary or post-treatment apical periodontitis (1). Therefore, endodontic treatment and retreatment in most cases involve management of a biofilm infection. Because biofilms may be very difficult to eliminate and considering that persistent infectious bioburden in the root canal system is the most important barrier to periradicular tissue healing after endodontic treatment (2), special strategies may be required for successful control of endodontic infections.

Mechanical debridement plays an important role in the treatment of biofilms by significantly reducing the bacterial bioburden and removing organic matter that might hamper the antimicrobial effects of irrigants. Debridement is essential to remove endodontic biofilms, but the fact that instrumentation leaves several untouched areas in the root canal indicates that biofilms may remain unaffected in these areas (3). Biofilms have the ability to reconstitute themselves after being partially affected (4), which makes mechanical debridement alone insufficient in endodontic therapy. This reinforces the need of using antimicrobial and/or antibiofilm agents as irrigants.

Sodium hypochlorite (NaOCl) has strong bactericidal and antibiofilm properties (5-6). However, it is highly cytotoxic (7) and its effects are significantly affected by organic matter (8). It was also shown that the clinical performance of NaOCl is inferior to its effects *in vitro* and about 40-60% of the cases of irrigation with NaOCl still harbor bacteria in the main canal (2). Moreover, NaOCl and other nonspecific antimicrobials used in traditional endodontic treatment may cause significant changes in dentin that further interfere with pulp regeneration approaches (9).

Multiple and concurrent strategies may be the most effective way of eliminating biofilms. For instance, attempts to combine different strategies to suppress biofilms in chronic wound infections include mechanical debridement, antimicrobial agents (antibiotics, antiseptics and disinfectants), and antibiofilm agents (4). Examples of antibiofilm agents include those targeting bacterial attachment (lactoferrin), blocking formation or degrading the biofilm matrix (xylitol and farnesol), and disrupting quorum-sensing systems (quorum sensing inhibitors).

Trans-trans farnesol (tt-farnesol) is a sesquiterpene alcohol commonly found in propolis and in essential oils of citrus fruits (e.g., in orange peel and lemon-grass oil). Farnesol has demonstrated to have antibiofilm effects by either preventing biofilm formation or attacking established biofilms. Farnesol inhibits or reduces biofilm

formation by diverse microbial species, including *Candida albicans*, *Streptococcus mutans* and *Staphylococcus aureus* (10-12). This substance inhibits acid production and glucan synthesis by *S. mutans* in biofilms (12). Biofilms formed in the presence of farnesol contain less biomass, and display marked changes in matrix composition (13). Topical applications of farnesol reduce the biofilm matrix content (14). In addition to affecting the biofilm structure by reducing its biomass, farnesol seems to kill bacteria in biofilms, depending on the concentration (13).

Xylitol is a five-carbon alcohol sugar found naturally in small quantities in fruit and vegetables. It is commonly used in chewing gum, with preventive effects on caries, and has been shown to inhibit biofilm formation (15). Biofilms treated with xylitol exhibit weakened overall structure (16). Xylitol can act synergistically with farnesol and the combination with this substance can selectively inhibit the growth of *S. aureus* (15).

This study investigated the antibiofilm and antibacterial effects of farnesol and xylitol in a series of experiments to evaluate their potential to be used as endodontic irrigants.

Material and Methods

Substances

The substances and combinations tested in this study were the following: 0.2% farnesol (Sigma-Aldrich, St. Louis, MO, USA); 5% and 20% xylitol (Sigma-Aldrich); and 0.2% farnesol plus 20% xylitol. For comparison with an established endodontic irrigant, 2.5% NaOCl was included in each test for a separate analysis. Sterile 0.85% saline was used as control for all experiments. As extracted human teeth were used in two experiments, the study protocol was subjected to and approved by the Ethics Committee of the Estácio de Sá University (Process#0248).

Crystal Violet Assay

Biofilm biomass was visualized and quantified by a crystal violet binding assay as previously described (17,18). A 0.5 McFarland standard of an overnight culture of *Enterococcus faecalis* ATCC 29212 was prepared in Tryptic Soy Broth (TSB, Difco, Detroit, MI, USA) supplemented with 1% glucose (Merck, Whitehouse Station, NJ, USA). After agitation in vortex, 200 µL aliquots were distributed in wells of a 96-well microtiter plate (tissue-culture-treated polystyrene, flat bottoms, model 92096 TPP "Techno Plastic Products", Trasadingen, Switzerland), and incubated for 24 h at 35ºC. Next, the content of each well was aspirated, and the wells were rinsed three times with 200 µL of phosphate-buffered saline (PBS, pH 7.2) to remove loosely attached cells. Each test substance was applied (200 µL *per* well) for 1, 3 and 10 min at 37ºC. After washing three times with PBS, adherent bacteria were stained for 20 min with 200 µL of 0.1% violet crystal solution at room temperature. Excess stain was rinsed off by copious washing with distilled water. Plates were overturned and air-dried, and the dye bound to the adherent cells was solubilized with 150 µL of 95% ethanol for 5 min. For quantification of biofilm biomass remaining after exposure to the test solutions, absorbance of the crystal violet solution was measured using an ELISA reader model 680 (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 590 nm. Positive control consisted of saline used instead of the test substance. Negative control used sterile culture broth. All assays were performed with 4 repetitions and on three separate occasions. The cut-off value for optical density (OD) measurements was defined as three standard deviations above the mean OD of the negative control (17). Therefore, final OD values for the test substances were expressed as average OD value reduced by the cutt-off value.

Dentin Disinfection Assay

Root hemicylinders were prepared by cutting off the tooth crown and the apical part of the root of extracted maxillary incisors and canines to obtain 5-mm cylinders. The root canals were enlarged up to a #5 Gates-Glidden bur and then the cylinders were split to generate two hemisections. The root hemicylinders were immersed in fresh TSB, sterilized in an autoclave and contaminated with *E. faecalis* ATCC 29212 for 30 days at 37ºC. Culture medium was replenished every week. Excess culture medium was removed and the specimens were washed by gentle shaking in saline solution to remove nonadhered cells. Four hemicylinders were prepared for scanning electron microscopy (SEM) as described previously (19) to confirm biofilm formation. Five hemicylinders were immersed in each test solution or saline (control) for 3 min at 37ºC. Next, the hemicylinders were transferred to flasks containing saline solution for 1 min. Specimens were placed in 5% sodium thiosulphate for 1 min, then in 1 mL of sterile saline, and subjected to ultrasonic agitation for 2 min in order to loosen the biofilm. After agitation in vortex, aliquots were immediately processed for culture. Samples were tenfold serially diluted in saline, aliquots of 100 µL were plated onto Mitis-Salivarius agar plates (Difco), and then incubated at 37ºC for 48 h. The grown colony forming units (CFU) were counted and then transformed into actual counts based on the known dilution factors.

Root Canal Disinfection Assay

This experiment used 55 single-rooted teeth (maxillary central incisors and canines), extracted for reasons not related to this study. All teeth were sectioned to leave a 10-mm long root segment.

Root canals were prepared as follows: LA Axxess burs

#45 (SybronEndo, Orange, CA, USA) were used along the coronal two-thirds and then the apical root canal was instrumented up to a #50 K-type file 1 mm beyond the apical foramen. All preparation procedures were performed under continuous irrigation with water. The teeth were immersed in TSB (Difco), ultrasonicated for 1 min to release the entrapped air and allow penetration of the culture media into root canal irregularities, and then sterilized in an autoclave for 20 min at 121°C.

E. faecalis strain ATCC 29212 was used for root canal contamination for 30 days at 37°C under gentle shaking. Culture media was replenished every week. Following the contamination period, all teeth had the excess of culture medium dripped off and their external root surface wiped with sterile gauze. The teeth were divided into 5 groups (n=11); 5% xylitol was not tested in this experiment. The apical foramen of each tooth was sealed with a fast setting epoxy resin to prevent apical bacterial leakage and create a closed-end channel that produced a vapor lock effect. Teeth were mounted vertically up to the cervical region in blocks made of a silicone impression material (President Jet; Coltène AG, Cuyahoga Falls, OH, USA). The tooth crown, including the pulp chamber walls, and the silicone surface were disinfected with 2.5% NaOCl, followed by inactivation of this substance with 10% sodium thiosulfate. Next, the working length (WL) was determined by introducing #20 K-file in the canal until it reached the apical foramen. The root canal was rinsed with 1 mL of sterile 0.85% saline solution to remove unattached cells and an initial sample (S1) was taken by the sequential use of 3 to 5 paper points placed to the WL. Each paper point remained in the canal for 1 min. Paper points were transferred to tubes containing 1 mL of sterile saline and immediately processed.

Next, the root canals were irrigated with 2 mL of the test substances. Irrigation was performed with disposable syringes and 30-gauge NaviTip needles (Ultradent, South Jordan, UT, USA) taken up to 3 mm of the WL. The canal was then flooded with the tested irrigant, which was sonically agitated for 1 min by using the EndoActivator blue tip size #35/.04 (Dentsply Tulsa Dental, Tulsa, OK, USA) placed up to 2 mm of the WL, at 10,000 cpm. Then the canals were again irrigated with 3 mL of the same test solution. After leaving the tested solution for 3 min in the canal, 1 mL of sodium thiosulfate was used to rinse the canal and another bacteriological sample (S2) was taken as above. Next, the canals of all groups were irrigated with 5 mL of 2.5% NaOCl for 1 min. NaOCl was inactivated with 10% sodium thiosulfate for 1 min and a third sample (S3) was taken.

Samples were agitated in vortex for 1 min and immediately processed for culture. After tenfold serial dilutions were done in saline, aliquots of 100 µL of each dilution were plated onto Mitis-Salivarius agar plates (Difco), and incubated at 37oC for 48 h. The grown colony forming units (CFU) were counted and then transformed into actual counts based on the known dilution factors.

The Wilcoxon matched pairs test was used for intragroup and the Mann-Whitney U tests for intergroup quantitative analysis. Intragroup evaluation related to the reduction in *E. faecalis* counts from S1 to S2 or S3; and from S2 to S3. Since no significant differences were found between S1 samples from the groups (Mann-Whitney U test), data for intergroup quantitative comparisons consisted of the absolute counts in S2 and S3. Significance level for all analyses was set at $p<0.05$.

Results

Crystal Violet Assay

This test evaluated the effects of the substances on the

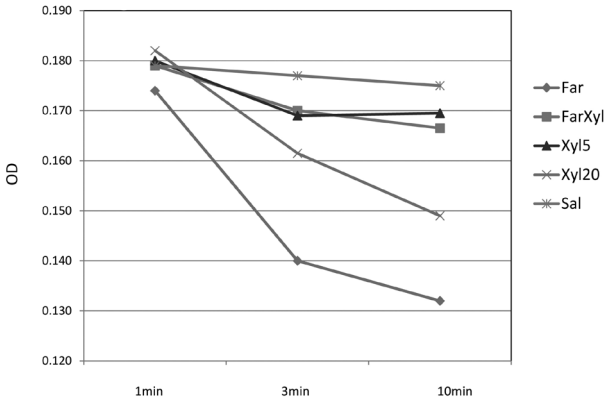

Figure 1. Data from the crystal violet assay, demonstrating the time-dependent effects of the substances on the biomass of *Enterococcus faecalis* biofilms. Xyl5 (5% xylitol); Xyl20 (20% xylitol); Far (0.2% farnesol); FarXyl (0.2% farnesol/20% xylitol); Sal (saline).

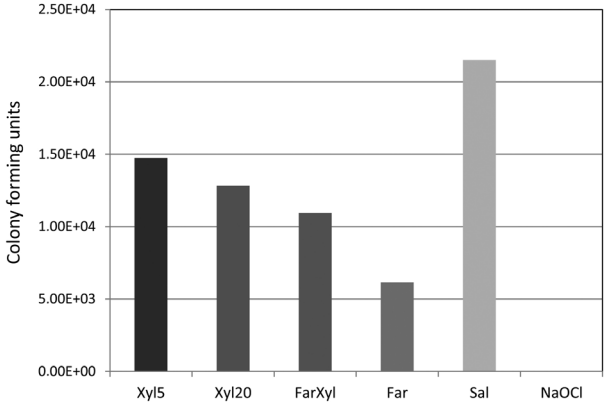

Figure 2. Mean number of colony forming units after exposure of *Enterococcus faecalis* biofilms grown on dentin hemicylinders to the tested substances. Xyl5 (5% xylitol); Xyl20 (20% xylitol); Far (0.2% farnesol); FarXyl (0.2% farnesol/20% xylitol); Sal (saline).

biofilm biomass. The test strain of *E. faecalis* was shown to form biofilms in this assay. All substances managed to reduce the biofilm biomass when compared to controls and the effects were time-dependent (Fig. 1). After 1 min of exposure, the effects of all substances were virtually negligible. After 3 min, all of them reduced the biofilm biomass. A further reduction in biomass was observed after 10 min, except for 5% xylitol. After both 3 and 10 min, the most effective substance was farnesol followed by 20% xylitol. All of the test substances were substantially less effective than 2.5% NaOCl in all time intervals (data not shown).

Dentin Disinfection Assay

This test evaluated the effects of the test substances on the viability of bacteria present in biofilms adhered to dentin. SEM confirmed biofilm formation by *E. faecalis* on dentin from the root hemicylinders (data not shown). In this test, all substances promoted reduction in the number of bacterial cells when compared with saline (Fig. 2). The greatest reduction was obtained with farnesol, followed by the farnesol/xylitol combination. No fragment exposed to NaOCl showed bacterial growth.

Root Canal Disinfection

This test intended to simulate the use of the antibiofilm substances in the root canal environment. Means, medians and ranges for all groups are shown in Table 1. Intragroup analysis revealed that irrigation with all substances, including saline (control), succeeded in significantly reducing the bacterial load ($p<0.001$). However, intergroup analysis of S2 samples demonstrated that only the farnesol/xylitol combination caused a reduction in bacterial counts that was significantly higher than saline ($p=0.02$). When NaOCl entered the analysis, data revealed that this substance was significantly more effective than every other tested substance ($p<0.001$). When a supplementary irrigation with 2.5% NaOCl was performed, intragroup analysis (S2 × S3 comparison) indicated that bacterial levels were still significantly reduced, regardless of the group ($p<0.001$). When comparing S3 samples between groups, both farnesol + NaOCl and farnesol/xylitol + NaOCl were significantly more effective than saline + NaOCl ($p=0.02$). The combination of NaOCl + NaOCl was not more effective than either farnesol + NaOCl or farnesol/xylitol + NaOCl ($p>0.05$).

Discussion

Because biofilms are very frequently observed in the apical root canal system of teeth with apical periodontitis, they should be regarded as targets in endodontic treatment. At present, the most effective treatment of medical biofilms in different body sites is its physical removal by debridement. This is also true for endodontic treatment, as instrumentation may affect most biofilms present in the main root canal. However, biofilms may remain

Table 1. *Enterococcus faecalis* counts before (S1), after irrigation with antibiofilm agents (S2), and after a final rinse with 2.5% NaOCl (S3)

Groups	S1			S2			S3		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
Saline	5.08E+05	5.12E+05	1.00E+03 -1.50E+06	1.45E+04 [a]	1.34E+04	6.20E+02 -2.88E+04	5.51E+02 [d]	4.00E+01	0 -2.56E+03
20% xylitol	2.08E+05	1.20E+05	9.00E+03 -4.96E+05	9.76E+03 [a]	1.09E+04	3.00E+02 -2.50E+04	5.18E+01 [d]	0	0 -3.10E+02
0.2% farnesol	7.04E+04	4.64E+04	1.30E+03 -1.76E+05	1.02E+04 [a]	1.20E+04	3.80E+02 -2.37E+04	8.18E+00 [d,e]	0	0 -8.00E+01
0.2% farnesol/ 20% xylitol	3.66E+05	2.90E+04	2.00E+03 -2.69E+06	6.35E+03 [a,b]	5.28E+03	7.10E+02 -1.60E+04	3.64E+00 [d,e]	0	0 -2.00E+01
2.5% NaOCl	8.01E+05	5.76E+05	2.00E+04 -1.98E+06	5,67E+01 [a,b,c]	0	0 - 5.10E+02	0 [d,e]	0	0

[a] Significant difference when compared to S1 of the respective group. [b] Significant difference when compared to S2 from the saline group. [c] Significant difference when compared to S2 from all the other groups. [d] Significant difference when compared to S2 from the respective group. [e] Significant difference when compared to S3 from the saline group.

unaffected in areas of the main canal that were untouched by instruments and in areas distant from the main canal where instruments cannot reach, such as isthmuses and apical/lateral ramifications (20). Residual biofilms may lead to persistent apical periodontitis (2). Therefore, there is need for chemical substances as irrigants or interappointment medications that have both antibacterial and antibiofilm activities. In the present study it was investigated the antibiofilm and antibacterial effects of farnesol and xylitol against *E. faecalis* biofilms in three experiments. These substances have previously shown to be effective against biofilms and have been suggested for use as potential auxiliary substances in the treatment of biofilms associated with chronic wound infections (21) or caries (22).

The findings in this study demonstrated that farnesol was effective in both reducing the biofilm biomass and killing bacteria in biofilms. Farnesol has previously shown to have antibacterial effects (11). Farnesol is hydrophobic and may accumulate on the bacterial cell membranes, compromising their integrity and leading to a consequent release of the intracellular content (11,13). Even in non-inhibitory concentrations, farnesol induces a decrease in biofilm matrix production and affects biofilm formation over time (13,23). The present findings for farnesol against *E. faecalis* biofilms confirm those from a previous study using *S. epidermidis* (13), showing that in addition to killing bacteria farnesol seems to affect the biofilm structure by disrupting its biomass.

When used alone, xylitol had significant effects on the biofilm biomass only at the 20% concentration. Regardless of the concentration, its effects on bacterial viability were minimal. These findings agree with a previous study that showed that xylitol has a minimal influence on the viability of bacterial cells within biofilms and its antibiofilm effects are probably related to disruption of the biofilm structure (16). When 20% xylitol was combined with farnesol, good results were observed for the test simulating use in the root canal, as this combination was the only one to show significant bacterial reduction when compared with saline. Xylitol has shown to act synergistically with farnesol in terms of antibacterial and antibiofilm activities (15).

The test substances were significantly less effective than NaOCl in either reducing the biofilm biomass or killing bacteria in biofilms. This comes as no surprise, as NaOCl is a potent disinfectant with recognized strong bactericidal and antibiofilm properties (5,6). However, NaOCl has several disadvantages, including its cytotoxicity to vital tissues, foul smell and taste, and the capacity to bleach clothes and corrode metal objects (24). In addition, it may alter the dentin structure and leave residues that may interfere with pulp regeneration procedures and bonding of adhesive materials to dentin (9). Thus, the search for safer (and more effective) substances to be used as root canal irrigants should be encouraged.

In the root canal experiment, an attempt was made to evaluate the effects of using the antibiofilm substances first to attack the biofilm biomass, and then to perform further irrigation with NaOCl, which would supposedly act on a more vulnerable residual biofilm. Although attractive, this protocol did not work as expected, because it was not more effective than the group where NaOCl was used throughout the preparation phase, i.e., without pre-irrigation with the antibiofilm agents. It remains to be determined whether this approach may work out with other irrigants less effective against the biofilm biomass but more biocompatible than NaOCl, such as chlorhexidine.

The present findings suggest that farnesol alone or in combination with xylitol may be a potential endodontic irrigant with antibiofilm properties. Both substances are natural products and have the potential to be more biocompatible than NaOCl. Farnesol has shown to be devoid of toxic and mutagenic effects (25). Further studies are required to assess its biocompatibility when used *in vivo*.

In conclusion, the findings of the present study demonstrated that farnesol affected both the biofilm biomass and the viability of cells in the biofilm, while 20% xylitol affected the biofilm biomass. Combination of these two antibiofilm substances has potential to be used in endodontic treatment to eliminate biofilms. Further studies are required to evaluate other biological properties of these substances before clinical use can be indicated.

Resumo

Este estudo investigou os efeitos antibiofilme e antibacteriano de farnesol e xilitol em uma série de experimentos para avaliar seu uso potencial como irrigante de canais radiculares. As seguintes substâncias foram testadas: farnesol a 0,2%; xilitol a 5% e 20%; farnesol a 0,2% combinado com xilitol a 20%; e solução salina (controle). NaOCl foi testado para comparação. Três experimentos foram conduzidos: o teste do cristal violeta para avaliar os efeitos sobre a biomassa de biofilme, o teste da desinfecção de fragmentos de dentina para avaliar os efeitos na viabilidade bacteriana nos biofilmes e o teste da desinfecção de canal radicular para simular o uso no ambiente do canal radicular. Farnesol foi o mais eficaz, seguido por xylitol a 20%. Todas as substâncias afetaram a viabilidade bacteriana nos biofilmes; farnesol mostrou os melhores resultados, seguido pela combinação farnesol/xilitol. A irrigação com todas as substâncias reduziu significativamente a carga bacteriana ($p<0,001$), mas somente a combinação farnesol/xilitol foi significativamente mais eficaz que a solução salina ($p=0,02$). NaOCl foi mais eficaz que qualquer outra substância testada nos três experimentos ($p<0,001$). Os achados demonstraram que farnesol afetou tanto a biomassa de biofilme quanto a viabilidade das células no biofilme, enquanto que xilitol a 20% afetou a biomassa de biofilme. Embora não mais eficazes que NaOCl, combinações dessas duas substâncias antibiofilmes têm o potencial de ser usadas na Endodontia, em determinadas situações.

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