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ORIGINAL ARTICLE

Antimicrobial activity of tt-farnesol associated with an endodontic sealer against *Enterococcus faecalis*

ABSTRACT

Aim: *This study aimed to evaluate* in vitro *the antibacterial activity of trans-trans farnesol (tt-farnesol) associated with Sealapex sealer against* Enterococcus faecalis.

Methodology: *Initially, the minimum bactericidal concentration (MBC) of tt-farnesol was determined by microdilution technique. The sealer was associated with 350 µg/g tt-farnesol (GS+0.35f); 1,750 µg/g tt-farnesol (GS+0.175f); or only Sealapex (GS). For antimicrobial activity test,* E faecalis *suspension was added in tubes containing the sealer samples and incubated for 24, 48, 72, 96, 120 and 144 h. After each time point, two blinded and calibrated evaluators performed the CFU count. Data were analyzed statistically by* one-way *ANOVA and Tukey* post-hoc *tests (significance level P<0.05).*

Results: *It was observed difference in the CFU count between G_S, GS+0.35f and GS+0.175 after 48 and 72 h, and between GS for the other groups in 96, 120 and 144 h (P<0.05). The CFU count was lower in GS+0.35f than in GS+0.175f after 48 and 72 h (P<0.05). In GS+0.35f, there was a decrease in CFU count after 48 h and in GS+0.175f after 72 h (P<0.01).*

Conclusions: *The association of tt-farnesol with Sealapex decreased* E faecalis *growth* in vitro *after 48 h of incubation. The MBC of 0.35 and 0.175 mg/mL of tt-farnesol reduced the CFU count after 48 and 72 h, respectively.*

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Introduction

The presence of microorganisms is one of the main conditions for the maintenance of periapical pathologies, after endodontic treatment (1). Therefore, the effectiveness of disinfection and preparation of root canals are crucial, to avoid secondary endodontic infections (1, 2). In addition to the chemical and mechanical preparation, the use of an intracanal dressing and an adequate filling of the root canal are necessary to eliminate pathogenic microorganisms and achieve more success (2).
The root canal filling is the final stage of endodontic treatment and requires a three-dimensional filling for the success of the treatment (3). It was performed using a material, usually of thermoplastic origin, in combination with an endodontic sealer (3). The filling materials should promote a great sealing of the pulp cavity, avoiding recontamination or proliferation of microorganisms that eventually survive the endodontic preparation (3, 4). Besides, it is interesting that endodontic sealers have an antibacterial effect against microorganisms that remain in isthmuses, dentinal tubules and apical deltas (3, 5). There are endodontic sealers of different bases, such as zinc oxide-eugenols, calcium hydroxide, epoxy resins and bioceramics (6-8).
Enterococcus faecalis is a Gram+ bacteria commonly found in cases of secondary endodontic infections (9-12), with a prevalence of 24% to 80% (10, 11), corresponding to 9-99.8% of the total bacterial count (12). It can invade and survive inside dentinal tubules, where it forms a resistant biofilm, which is difficult to eliminate during the root canal preparation (13, 14). The persistence of this bacteria in hostile environments and its resistance to endodontic dressings that raise the pH of the dentin (such as calcium hydroxide) can contribute to damage the periapical tissues, which can be considered a possible cause of post-treatment apical periodontitis (14-16). Also, *E. faecalis* produces several virulence factors, such as surface adhesins and gelatinase, which contribute to bacterial adhesion, colonization, biofilm formation and tissue damage (17-19). Therefore, the elimination of this resistant microorganism is essential to prevent root canal reinfection.
Propolis has been used in Dentistry since the 1990s and consists of an association of wax, oils and bioactive compounds known as bioflavonoid or terpenoid, such as trans-trans farnesol (tt-farnesol), which has antimicrobial activity (20-22). The association between different therapies and bioactive compounds of propolis has been studied. One example is the tt-farnesol that has been associated with fluoride, dental adhesives and glass ionomer cement in anti-caries therapy, and recently used in primary teeth endodontic therapy (21, 23, 24). Combination therapy negatively influenced the virulence of *Streptococcus mutans* biofilms, being effective in controlling the growth of cariogenic bacteria (25-29). However, few studies have attempted to evaluate tt-farnesol action against *E faecalis*. Therefore, this study aimed to evaluate the antibacterial activity of tt-farnesol associated with an endodontic sealer against *E faecalis*.

Material and Methods

Minimum bactericidal concentration determination
Initially, the minimum bactericidal concentration (MBC) of tt-farnesol ($C_{15}H_{26}O$ 96% Aldrich Chemistry INC; St. Louis, USA) was determined through the microdilution technique (30). The diluent was made of 20% 92.8° alcohol (Alcool Santa Cruz; São Paulo, Brazil), 0.75% dimethyl sulfoxide (DMSO, Rio de Janeiro, Brazil) and 79.25% distilled water, totaling 100 mL.
For dilution, 10 ml of diluent was added to tt-farnesol (28 mg), which resulted in a final concentration of 2.800 µg/mL. The weight of tt-farnesol solution was determined using an analytical balance. Then, tt-farnesol was diluted successively, obtaining final concentrations of 1.4, 0.7, 0.35, 0.175, 0.0875, 0.043, 0.021, 0.010 and 0.0054 mg/mL.

For the inoculum preparation, 100 µL of *E faecalis* (ATCC 10542) was transferred to a tube containing 5 mL of brain heart infusion (BHI) broth (DIFCO, São Paulo, Brazil) and incubated at 37 ºC in 5% CO_2 for 18 h. An aliquot was grown on BHI agar plates and incubated at 37 ºC in 5% CO_2 for 24 h, to obtain isolated colonies and confirm the uniform growth of *E faecalis*. The purity of the inoculum was confirmed by optical microscopy (x1000). The BHI agar plates provided five isolated colonies of *E faecalis* that were replicated, in a tube containing 5 mL of BHI, which was further incubated at 37 ºC in 5% CO_2 for 18 h.
After bacterial growth, the test tubes (n=18) were filled with 100 µL of the diluted solutions. Subsequently, it was filled with the inoculum and standardized using a spectrophotometer (330 model Metrolab, Buenos Aires, Argentina) with a wavelength of 625 nm and absorbance of 0.09. The cultures were adjusted to 0.5 Mc Farland standard (an approximation of $1.5x10^8$ colony forming units - CFU/mL).

Table 1
CFU count (mean) in different concentrations of tt-farnesol and controls in the incubation times

Solution concentration	E faecalis ($CFUx10^6$)
tt-farnesol (mg/mL)	
1.4	0
0.7	0
0.35	0
0.175	9
0.0875	50
0.043	Uncountable
0.021	Uncountable
0.010	Uncountable
0.0054	Uncountable
Positive control	Uncountable
Negative control	0
Buffer solution control	0
Culture medium control	0

To test the antimicrobial activity of tt-farnesol, the wells of a 96 ELISA tray were filled with 100 µL of BHI. The wells (n=7) received 100 µL of each diluted solution and added with the inoculum. Then it was incubated at 37 ºC in 5% CO_2 for 24 h. Positive control (BHI + inoculum), negative control (BHI + inoculum + 0.12% chlorhexidine), buffer solution control (DMSO 100 µL + BHI 100 µL) and culture medium control (BHI) were also performed in the wells (n=7).
An aliquot from each well was grown on BHI agar plates. Two blindly calibrated evaluators (*Kappa* test=1.0) performed the CFU count and the mean values are shown in Table 1. The solution was considered effective when 99.9% growth of the inoculum was inhibited. The concentrations of 0.35 and 0.175 mg/mL of tt-farnesol were those chosen, as they were the borderline results between inhibiting or not bacterial growth.

Sealer preparation
The sealer used is this study was Sealapex (Kerr, Washington, EUA). This sealer is an endodontic sealer calcium hydroxide-based and its mechanism of action is obtained through ionic dissociation of Ca^{2+} and OH^- ions (31). The addition of calcium hydroxide in root canal sealers improves physico-chemical properties, mainly due to a decrease in the flow rate of the sealer (31).
The sealer and tt-farnesol were weighed with a precision scale (Ohaus Corporation Pine Brook, New Jersey, USA). The sealer was prepared by mixing 0.025 g of Sealapex paste and 0.025 g of Sealapex catalyst associated with different proportions of tt-farnesol: only Sealapex (GS); Sealapex + 350 µg/g tt-farnesol (GS+0.35f); Sealapex + 1750 µg/g tt-farnesol (GS+0.175f). The sealer was mixed following the manufacturer's instructions. Then, it was dispensed in 2 mm-diameter and 6 mm-long polyethilene tubes (n=108) with a Centrix syringe (DFL, Rio de Janeiro, Brazil), to avoid bubbles inside the sealer. The end of the tubes was sealed with plastic tape to prevent the material from overflowing. Once the initial hardening time was reached, the polyethilene tubes were cut with a

scalpel blade (15C model Swann-Morton, Sheffield, United Kingdom) and the sample size was confirmed with the help of a calliper.
Each group was placed in a previously identified 3 mL Eppendorf tubes and incubated at 37 °C for 7 days. Thus, the setting could be attained and, as a result, by-products could be released during the setting reaction.

Inoculum preparation
E faecalis culture was prepared in Mueller-Hinton broth (BD, New Jersey, USA) and adjusted to a concentration of 10^6 CFU/mL simulating body fluid (SBF). The adjustment was performed in a spectrophotometer. The absorbance was measured at 660 nm.

Antimicrobial activity test of sealer
The bacterial suspension was then added, in a 1:10 ratio (weight/volume), into the Eppendorf tubes containing the samples. Therefore, at the initial time, all tubes had the same concentration of bacteria. In specific times, 100 µL of each group were removed and twofold serial dilution (decimal dilution) were prepared in saline solution, to obtain a concentration of 10^6 CFU/mL. Subsequently, the aliquots were immediately seeded in BHI agar (Isofar Indústrias Comércio de Produtos Químicos, Rio de Janeiro, Brazil) and incubated at 35°C for 24, 48, 72, 96, 120 and 144 h. After each time, they were removed and two blindly calibrated evaluators (*Kappa* test=1.0) performed the CFU count by group (n=10), under an x25 magnification with the aid of stereomicroscopic analysis. The mean values obtained were considered for each group at different times.
All manipulations were carried out in aseptic conditions and a laminar flow cabinet, to minimize the risks of contamination.

Statistical Analysis
Statistical difference between the groups was tested by the Analysis of Variance (*one-way* ANOVA). Tukey HSD multiple comparison *post-hoc* test was used to complement the analysis. The level of significance was set at 5%. The analysis was performed with the aid of SPSS 20 Software for Windows (IBM SPSS Statistics, Chicago, Il, USA).

Results

The mean of CFU count by the group at incubation times are shown in Table 2. It was observed a statistically significant difference in the CFU count between the groups after 48 h (*one-way* ANOVA, *P*0.01). The *post-hoc* test revealed differences between GS, GS+0.35f and GS+0.175 after 48 and 72 h, and between GS and all other groups in 96, 120 and 144 h of incubation (Tukey HSD, $P<0.05$). The CFU count was lower in GS+0.35f than in GS+0.175f after 48 and 72 h (Tukey HSD, $P<0.05$). In GS+0,35f, there was a decrease in CFU count after 48 h, in GS+0.175f after 72 h and in GS only after 120 h of incubation (*one-way* ANOVA $P<0.01$, Tukey HSD $P<0.05$).

Discussion

Secondary endodontic infection occurs due to the presence of microorganisms that resist to chemical and mechanical preparation of root canal (1, 2). *E faecalis* is a microorganism associated with the appearance or maintenance of periapical pathologies after endodontic treatment (9-12). The use of irrigation solution and intracanal dressing contributes to the root canal disinfection, but it is not always able to eliminate *E faecalis* (16). The use of an endodontic sealer with antibacterial activity can favour the elimination of microorganism that can survive in isthmuses, dentinal tubules and apical deltas, prolonging this effect even after root canal filling and preventing the recontamination (13, 14).
An ideal endodontic sealer must have antimicrobial activity, good sealing ability, be highly penetrating, have good fluidity and be able to stimulate the repair of periapical tissues (3-5). In this study, Sealapex was the endodontic sealer chosen for presenting good sealing capacity and flow (32, 33). Also, it is calcium hydroxide-based, which is favorable for repairing the periapical tissues (32-34). However, previous

Table 2

CFU count by groups in the incubation times

Incubation time	CFU count – mean (±SD)			
	GS	**GS+0.35f**	**GS+0.175f**	***P***
0 h	$1.36x10^{7}$ $(\pm3.2x10^{6})^{Aa}$	$1.38x10^{7}$ $(\pm1.6x10^{6})^{Aa}$	$1.7x10^{7}$ $(\pm4x10^{6})^{Aa}$	0.069
24 h	$5.4x10^{7}$ $(\pm8x10^{6})^{Aa}$	$2.8x10^{7}$ $(\pm1.6x10^{7})^{Aa}$	$5.2x10^{7}$ $(\pm1.4x10^{7})^{Aa}$	0.055
48 h	$4x10^{7}$ $(\pm1x10^{7})^{Aa}$	$1.7x10^{7}$ $(\pm4x10^{6})^{Bb}$	$8x10^{6}$ $(\pm3.7x10^{6})^{Ca}$	<0.01
72 h	$4.3x10^{7}$ $(\pm1.8x10^{7})^{Aa}$	$7x10^{5}$ $(\pm4x10^{5})^{Bb}$	$3.2x10^{6}$ $(\pm1.4x10^{6})^{Cb}$	<0.01
96 h	$2.6x10^{6}$ $(\pm1.2x10^{6})^{Aa}$	$1.96x10^{4}$ $(\pm1.5x10^{4})^{Bb}$	0 $(\pm0)^{Bb}$	<0.01
120 h	$3.5x10^{5}$ $(\pm1x10^{5})^{Aa}$	0 $(\pm0)^{Bb}$	0 $(\pm0)^{Bb}$	<0.01
144 h	$4.3x10^{5}$ $(\pm1.4x10^{5})^{Aa}$	0 $(\pm0)^{Bb}$	0 $(\pm0)^{Bb}$	<0.01
P	<0.01	<0.01	<0.01	

SD Standard Deviation
Same lower letter indicate statistically similarity between groups in lines and same capital letter represents statistically similarity between groups in columns (Tukey's test, P>0.05)

studies have shown that this endodontic sealer was not able to eliminate *E faecalis*, because its antibacterial activity occurs through ionic dissociation of calcium hydroxide, which increases the pH but is unable to reach deeper into the dentinal tubules, where microorganisms may be located (35-37). Therefore, to improve Sealapex antibacterial activity, this study proposed an association with the bioactive compound known for tt-farnesol.

Tt-farnesol is a terpenoid responsible for the antibacterial activity of propolis. Its use in Dentistry is safe, because of its pharmacological characteristics such as low cytotoxicity and genotoxicity, which allows it to be used as an active or adjuvant medication (38-40). Rezende et al. (24) evaluated two pastes containing propolis extract associated with calcium hydroxide for root canal filling of primary molars. The agar-well diffusion technique showed that the association between propolis and calcium hydroxide promoted greater inhibition zones of bacterial growth, being effective in the control of dental infections *in vitro*. These findings corroborate with this study, which showed that the association of the Sealapex sealer with tt-farnesol, a component of propolis, promoted inhibition of *E faecalis* growth in vitro, which could contribute to prolonging the disinfection of the root canals after filling. Studies show that tt-farnesol was effective in dental caries control and inhibition of *S mutans* growth (21, 23, 25, 37). Thus, the application Sealapex sealer associated with tt-farnesol in endodontic therapy has great importance, mainly because it is an original combination. A methodological advantage of this study was the use of simulated body fluid (SBF) as the medium for sealer and bacterial inoculum interaction (25, 26). SBF is a medium that favors the dissolution of the sealer and has similarity to the human body fluid, which resembles the real conditions of the root canal. It is different from studies that use only the agar culture medium (25, 26).

In this study, there was a decrease in CFU count in $GS+0.175_f$ after 72 h and GS+0.35f after 48 h of incubation. In the GS group, which has no association with tt-farnesol, CFU count was higher after 48 h, compared to the other groups. The association of tt-farnesol with Sealapex sealer seemed to inhibit bacterial growth. This result can be explained by the study by Schäfer and Zandbiglari (32) which demonstrated that Sealapex sealer presents low solubility in

the first hours, but increase its solubility over time, reaching 10% after 28 days.
In this study, CFU count decreased in G_S only after 120 h of incubation. This can be explained by the ability of *E faecalis* to remain viable, even after raising the medium pH (14-16). The association between Sealapex and tt-farnesol seems to improve the action of the sealer against *E faecalis*. The action mechanism of tt-farnesol (as well as other terpenoids) is in its ability to act on the bacterial membrane, promoting the lysis of the microorganism (22, 26). Koo et al. (26) demonstrated that tt-farnesol promoted a visible rupture of the membrane of *Streptococcus* in phase-contrast microscopy.
The anti-inflammatory activity of propolis was associated with its ability to remove free radicals through its bioactive compounds, which favors tissue regeneration and repair, stimulating the formation of hard tissue (22). Based on the results of the present study and considering tt-farnesol as an anti-inflammatory agent that favors histological repair, it seems essential to develop further research to understand the mechanism of action of this substance, particularly to provide fundamentals for combinatorial therapy. This would provide this therapy with stronger biological support, thereby allowing it to be clinically tested in the future.

Conclusions

From this study, we can conclude that the association of tt-farnesol with Sealapex sealer decreased *E. faecalis* growth in vitro after 48 h of incubation in comparison to the group that used only the sealer. The MBC of 0.35 and 0.175 mg/mL of tt-farnesol reduced the CFU count after 48 and 72 h, respectively.

Clinical Relevance

An adequate endodontic treatment with a substance that promote microbial control mainly against *E. faecalis* strains can results in the greatest success of the treatmen.

Conflict of Interest

None.

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None.

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