# Inhibitory activity of root canal irrigants against *Candida albicans*, *Enterococcus faecalis* and *Staphylococcus aureus*

**Tatiana Kelly da Silva Fidalgo**[a]
**Roberta Barcelos**[b]
**Maristela Barbosa Portela**[c]
**Rosangela Maria de Araújo Soares**[d]
**Rogério Gleiser**[c]
**Fernando Costa e Silva-Filho**[e]

[a] School of Dentistry of Rio de Janeiro, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

[b] Department of Specific Formation, School of Dentistry, Fluminense Federal University, Nova Friburgo, RJ, Brazil.

[c] Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

[d] Professor Paulo de Goes Institute of Microbiology, University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

[e] Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

**Abstract:** The present study evaluated the antimicrobial activity of three root canal irrigants against *Enterococcus faecalis*, *Candida albicans*, and *Staphylococcus aureus*. These microorganisms were incubated in the presence of citric acid (6 and 10%), EDTA (17%), and NaOCl (0.5, 1.0, 2.5, and 5.25%). Agar diffusion tests were performed and redox indicator resazurin was used to evaluate the inhibitory effect of the irrigants on the metabolic activity of these microorganisms. The mean diameters of the inhibition zones for the *C. albicans* cultures were 11.6 mm (17% EDTA), 5.5 mm (0.5% NaOCl), 12.9 mm (1% NaOCl), 22.1 mm (2.5% NaOCl), and 28.5 mm (5.25% NaOCl). The mean diameters of the inhibition zones for *E. faecalis* were 2.8 mm (1% NaOCl), 5.4 mm (2.5% NaOCl), and 8.3 mm (5.25% NaOCl). For *S. aureus*, the mean values were 8.0 mm (17% EDTA), 3.0 mm (1% NaOCl), 8.8 mm (2.5% NaOCl), and 10.0 mm (5.25% NaOCl). Most of the irrigant solutions presented effective antimicrobial activity against *C. albicans*. A high inhibitory effect on the metabolic activity of *E. faecalis* was detected when the microorganisms were incubated with 17% EDTA. The same result was reached when *S. aureus* was incubated in the presence of ≥ 2.5% NaOCl. Altogether, these results indicate that 2.5% and 5.25% NaOCl are microbicides against *S. aureus* while 0.5% and 1% NaOCl are only microbiostatic against the tested bacteria. The 6% and 10% citric acid as well as 17% EDTA did not affect the viability of any of the assayed microorganisms.

**Descriptors:** *Candida albicans; Enterococcus faecalis; Staphylococcus aureus*; Root Canal Irrigants.

## Introduction

Microorganisms are the major causative factor associated with endodontic treatment failure.[1,2] The success of endodontic treatment depends on the reduction or elimination of bacteria present in the root canal system. Residual pulpal tissue, bacteria, and dentine debris may persist in the irregularities of root canal systems, even after meticulous mechanical preparation.[3] Therefore, irrigant solutions should be used in combination with canal preparation.[4-6]

Root canal irrigants are used during chemomechanical procedures not only as antimicrobial agents but also to flush out loose debris, to lubricate the dentinal walls, and to dissolve organic compounds in the

**Corresponding author:**
Tatiana Kelly da Silva Fidalgo
Rua Joaquim Távora 244/202 - Icarai
Niterói - RJ - Brazil
CEP: 24230-541
E-mail: *tatiana_kelly@yahoo.com.br*

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canal.[7,8] Chemomechanical preparation is one of the most important phases of endodontic treatment and irrigants such as sodium hypochlorite (NaOCl), citric acid, and ethylene diamine tetra-acetic acid (EDTA) are commonly used. The efficacy of these procedures also depends upon the vulnerability of the species involved.[9]

Many *in vitro* studies relate the antimicrobial activity of root canal irrigants against microorganisms, but studies on the metabolic activity of microorganisms after contact with these irrigants was not found in the literature.[5,6] Therefore, this study first evaluated the effectiveness of NaOCl (0.5, 1, 2.5, 4, and 5.25%), EDTA (17%), and citric acid (6 and 10%) against *Enterococcus faecalis, Candida albicans*, and *Staphylococcus aureus*, and then assessed their metabolic activities under the same conditions.

## Material and Methods

### Microorganisms

The following microorganisms and their related ATCC strains were used throughout: *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212) and *Candida albicans* (ATCC 10231). All microorganisms were grown in BHI-agar medium (Difco, Rio de Janeiro, RJ, Brazil) supplemented with 10% sheep blood (24 h, 37°C, 5% $CO_2$ atmosphere or in anaerobiosis). The microorganisms were then collected with a Drigalsky spatula for standardization of quantities collected and resuspended in sterile 0.01 M phosphate buffer pH 7.2, containing 0.15 M NaCl (PBS pH 7.2). The quantity of all microorganisms was adjusted using McFarland's scale (McFarland Standard no. 0.5) and spectrophotometrically adjusted to $10^5$ CFU.$ml^{-1}$ through optical density measurement at 600 nm ($OD_{600}$).

### Root canal irrigants

The root canal irrigants used were: citric acid (6 and 10% - VETEC, Rio de Janeiro, RJ, Brazil), EDTA (17% - Biodinâmica, Rio de Janeiro, RJ, Brazil), and NaOCl (0.50, 1.00, 2.50, and 5.25% - Fórmula & Ação, São Paulo, SP, Brazil). All of these compounds were diluted in sterile PBS pH 7.2.

### Agar Diffusion Test (ADT)

For this test, nitrocellulose membranes (13.0 mm - Catalogue Nº GSWP04700; Millipore Co., Billerica, MA, USA) impregnated with the root canal irrigants (30 µl each), described above, were placed on the top of *S. aureus*, *E. faecalis*, and *C. albicans* (0.1 ml, $10^5$ CFU.$ml^{-1}$, resuspended in PBS pH 7.2) homogeneously spread onto separated Petri dishes (10 cm diameter) containing BHI agar medium. The microorganism cultures were kept in an incubator (24 h, 37°C, 5% $CO_2$ atmosphere or in anaerobiosis) before measuring the growth inhibition zones. Cell viability (negative control) was determined by incubating the bacteria with Bactrin (Sulfametoxazol-trimetoprima - RJ 0401, Roche, Rio de Janeiro, RJ, Brazil) and the fungus with Fluconazole (Ache, Rio de Janeiro, RJ, Brazil); and the positive controls consisted in incubation of the same microorganisms in PBS pH 7.2. The inhibition zone limit was measured from the edge of the membrane disk to the end of the inhibition zone. A millimeter marking rule was used for this purpose.[9] All assays were done in triplicate.

### Metabolic activity

In order to collect a standard quantity of samples submitted to ADT, the authors developed the following method. Briefly, micropipette tips (S1111-0006; TipOne; Blakelands, MK, UK) with 15 mm cut off from the thinner end were used to collect a fixed (standard) quantity of sample from the inhibition zone. For each sample collection, the tip was inserted 1 mm from the nitrocellulose membrane, and then the collected sample was resuspended in PBS (300 µl). Equivalent selection points to collect the microorganism are essential to maintain the same parameters for all microorganisms studied. To adjust the quantity of microorganisms to be tested, $OD_{530}$ of the samples was taken using PBS as a blank solution. The $OD_{530}$ of all samples was ~0.5.

Microorganisms (100 µl), in the above conditions, were reacted (3 h, 37°C) with resazurin[10] (30 µl - R7017, Sigma-Aldrich Corp. St. Louis, MO, USA), a redox potential indicator, in 96 plastic well plates (Millipore, Bedford, MA, USA). The reactions were carried out in triplicate and read spectro-

metrically at 530 nm ($OD_{530}$).

### Statistical analysis

The data were analyzed by the SPSS 16.0 (SPSS Inc, Chicago, IL, USA) software using the analysis of variance (ANOVA) and the Tukey ($p < 0.05$) test.

## Results

The data summarized in Table 1 show that incubation of the microorganisms with citric acid (6 or 10%) did not inhibit their growth, since inhibition zones were not apparent (data not shown). Neither 17% EDTA nor 0.5% NaOCl inhibited the growth of *Enterococcus fecalis*, and the latter did not have any effect on *Staphylococcus aureus* either. In contrast, 17% EDTA presented activity against both *Candida albicans* and *Staphylococcus aureus*. NaOCl at higher concentrations (1, 2.5, and 5.25%) presented inhibitory activity against all microorganisms tested. Furthermore, the antimicrobial effect of 5.25% NaOCl was comparable to the results presented by the negative controls (Bactrim and Fluconazole). EDTA (17%) presented higher antimicrobial activity than 0.5% NaOCl, when tested against *Candida albicans* and *Staphylococcus aureus*. The effect of EDTA (17%) on *Candida albicans* was similar to that exhibited by 1% NaOCl, but more effective on *Staphylococcus aureus*. Higher concentrations of NaOCl (2.5 and 5.25%) were more effective on *Candida albicans* than 17% EDTA, however their activities on *Staphylococcus aureus* were similar.

EDTA (17%) presented more effective antimicrobial activity than citric acid (6 and 10%) against all assayed microorganisms. It can also be noted that the effectiveness of NaOCl on *E. faecalis* was dose-dependent. Furthermore, 5.25% NaOCl was lethal to all assayed microorganisms (Table 2).

To evaluate the microbicidal or microbiostatic activity of the root canal irrigants studied here, redox analyses of samples taken from the inhibition zones were carried out. *Candida albicans* and *Enterococcus faecalis* metabolic activities were detected in all tested root canal irrigants (that formed inhibition zones). However, no metabolic activity of *Staphylococcus aureus* was detected for 2.5% and 5.25% NaOCl (Table 3 and Graph 1).

## Discussion

Chemomechanical procedure plays an important role in reducing microorganisms in the root canal.[11] Previous studies have shown that irrigation with 0.5% NaOCl eliminated bacteria in 50% to 75% of infected root canals at the end of the first treatment.[12] In the present study, only *Candida albicans* was sensitive to 0.5% NaOCl. However, all microbial species tested were sensitive to 5.25% NaOCl. EDTA is an auxiliary substance that has a chelating action, biocompatibility with the periapical tissues[13] and optimal cleansing abilities.[14] In the current study, 17% EDTA showed a superior antimicrobial effect in the inhibition zone test against *Candida albicans* when compared to 0.5% NaOCl.

*Enterococcus faecalis* is considered one of the most resistant species in the oral cavity and a possible cause of failure in root canal treatments. It can survive after instrumentation and irrigation with NaOCl up to 2.5%.[15] The findings of the present study showed that 5.25% NaOCl had a significantly better performance than 2.5% NaOCl against this

**Table 1 -** Inhibition zones (mm) detected when microorganisms were cultured in the presence of different concentrations (%) of citric acid, EDTA or NaOCl.

| Microorganism | Irrigant solution | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 6% Citric acid | 10% Citric acid | 17% EDTA | 0.5% NaOCl | 1.0% NaOCl | 2.5% NaOCl | 5.25% NaOCl | Positive Control | Negative Control* |
| *C. albicans* | - | - | 11.6 | 5.5 | 12.9 | 22.1 | 28.4 | - | 29.5 |
| *Enterococcus faecalis* | - | - | - | - | 2.8 | 5.4 | 8.3 | - | 6.3 |
| *Staphylococcus aureus* | - | - | 8.0 | - | 3.0 | 8.8 | 10.0 | - | 12.2 |

*For negative control *E. faecalis* and *S. aureus* microorganisms were treated with Bactrin, and *C. albicans* was treated with Fluconazole.

**Table 2 -** A comparative statistical analysis of the data obtained from assays carried out to generate inhibition zones by incubating the microorganisms *Staphylococcus aureus* (SA), *Enterococcus faecalis* (EF), and *Candida albicans* (CA) with different concentrations (%) of canal irrigant solutions

| Microorganism | | Irrigant solution | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C (–) Bac/ Flu | C (+) PBS | 6% citric acid | 10% citric acid | 17% EDTA | 0.5% NaOCl | 1.0% NaOCl | 2.5% NaOCl | 5.25% NaOCl |
| C (–) Bac/ Flu | CA | - | < 0.001* | < 0.001* | < 0.001* | 0.003* | < 0.001* | 0.005* | 0.356 | 1.000 |
| | EF | - | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | 0.012* | 0.001* |
| | SA | - | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* |
| C (+) PBS | CA | < 0.001* | - | 1.000 | 1.000 | 0.051 | 0.693 | 0.028* | 0.001* | < 0.001* |
| | EF | < 0.001* | - | 1.000 | 1.000 | 1.000 | 1.000 | < 0.001* | < 0.001* | < 0.001* |
| | SA | < 0.001* | - | 1.000 | 1.000 | 0.055 | 1.000 | 0.266 | 0.157 | 0.581 |
| 6% Citric acid | CA | 1.000 | 1.000 | - | 1.000 | 0.051 | 0.693 | 0.028* | 0.001* | 0.001* |
| | EF | 1.000 | 1.000 | - | 1.000 | 1.000 | 1.000 | < 0.001* | < 0.001* | < 0.001* |
| | SA | 1.000 | 1.000 | - | 1.000 | 0.001* | 1.000 | 0.266 | < 0.001* | < 0.001* |
| 10% Citric acid | CA | 1.000 | 1.000 | 1.000 | - | 0.051 | 0.693 | 0.028* | 0.001* | < 0.001* |
| | EF | 1.000 | 1.000 | 1.000 | - | 1.000 | 1.000 | < 0.001* | < 0.001* | < 0.001* |
| | SA | 1.000 | 1.000 | 1.000 | - | < 0.001* | 1.000 | 0.266 | < 0.001* | < 0.001* |
| 17% EDTA | CA | 0.051 | 0.051 | 0.051 | 0.051 | - | 0.570 | 1.000 | 0.091 | 0.004* |
| | EF | 1.000 | 1.000 | 1.000 | 1.000 | - | 1.000 | < 0.001* | < 0.001* | < 0.001* |
| | SA | 0.001* | 0.055 | 0.001* | < 0.001* | - | 0.001* | 0.020* | 0.998 | 0.708 |
| 0.5% NaOCl | CA | 0.693 | 0.693 | 0.693 | 0.693 | 0.570 | - | 0.361 | 0.005* | < 0.001* |
| | EF | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | < 0.001* | < 0.001* | < 0.001* |
| | SA | 1.000 | 1.000 | 1.000 | 1.000 | 0.001* | - | 0.266 | < 0.001* | < 0.001* |
| 1.0% NaOCl | CA | 0.028* | 0.028* | 0.028* | 0.028* | 1.000 | 0.361 | - | 0.165 | 0.008* |
| | EF | < 0.001* | < 0.001* | < 0.001* | < 0.001* | 0.001* | < 0.001* | - | < 0.001* | < 0.001* |
| | SA | 0.266 | 0.266 | 0.266 | 0.266 | 0.020* | 0.266 | - | 0.007* | 0.002* |
| 2.5% NaOCl | CA | 0.001* | 0.001* | 0.001* | 0.001* | 0.091 | 0.005* | 0.165 | - | 0.528 |
| | EF | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | - | < 0.001* |
| | SA | < 0.001* | 0.157 | < 0.001* | < 0.001* | 0.998 | < 0.001* | 0.007* | - | 0.975 |
| 5.25% NaOCl | CA | < 0.001* | < 0.001* | 0.001* | < 0.001* | 0.004* | < 0.001* | 0.008* | 0.528 | - |
| | EF | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | < 0.001* | - |
| | SA | < 0.001* | 0.581 | < 0.001* | < 0.001* | 0.708 | < 0.001* | 0.002* | 0.975 | - |

*Values of statistical significance ($p < 0.05$). C (–) and C (+) are respectively related to microorganisms which were incubated in root canal irrigant-depleted medium (–) or PBS (+) before cultivation in BHI medium.

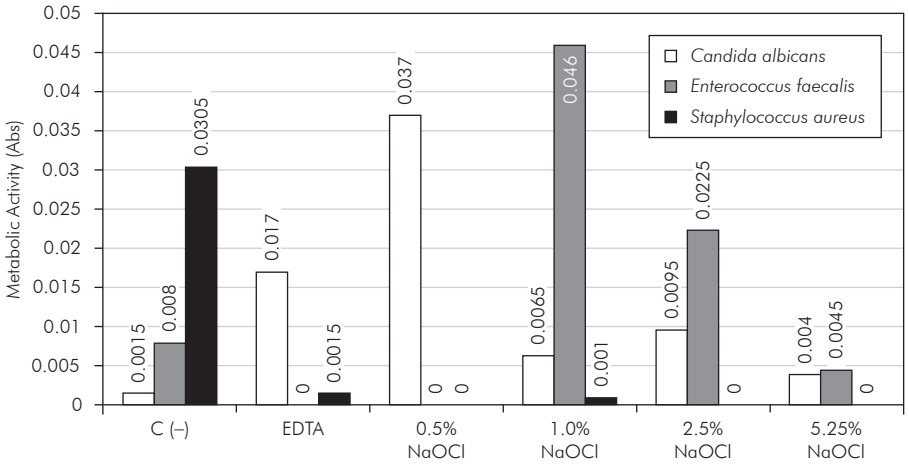
**Table 3 -** Metabolic activity of the microorganisms before [C (–)] and after their incubation with different concentrations (%) of EDTA or NaOCl

| Microorganisms | Irrigant solution | | | | | |
|---|---|---|---|---|---|---|
| | C (–) | 17% EDTA | 0.5% NaOCl | 1.0% NaOCl | 2.5% NaOCl | 5.25% NaOCl |
| *Candida albicans* | 0.0015 | 0.0170 | 0.0370 | 0.0065 | 0.0095 | 0.0040 |
| *Enterococcus faecalis* | 0.0080 | NA | NA | 0.0460 | 0.0225 | 0.0045 |
| *Staphylococcus aureus* | 0.0305 | 0.0015 | NA | 0.0010 | 0 | 0 |

No significant ($p < 0.05$) differences could be seen between groups. Numbers represent the diameters of the inhibition zones. NA = not applicable (In these cases, inhibition zones were not formed in the diffusion agar test, so the metabolic activity test was not applicable). C (–) = Negative control

microorganism.

Chelate and acidic solutions, including EDTA and citric acid, have been recommended for removing the smear layer from instrumented root canals.[16] In the present study, the EDTA solution presented lack of microbicidal activity against *Enterococcus faecalis*, corroborating the findings obtained by Arias-Miliz and Ferrer Luque.[16] By contrast, the




**Graph 1 -** Metabolic activity of *Candida albicans*, *Enterococcus faecalis* and *Staphylococcus aureus* after their incubation for 24 h with EDTA and NaOCl.

same authors showed an inhibition zone using 10% citric acid against *Enterococcus faecalis*. Although citric acid presents low cytotoxity as an advantage,[17] in the present study, 6% and 10% citric acid did not form any inhibition zone against any of the microorganisms tested. In agreement with Grawehr *et al.*,[18] the current study demonstrated that 17% EDTA was more effective than 0.5% NaOCl against *Candida albicans* and *Staphylococcus aureus*. The maintenance of microorganisms in contact with EDTA over a prolonged period is as lethal as short periods of contact, increases the permeability of the outer membrane to hydrophobic molecules, and improves the action of antibacterial agents.[19] Branin at al,[20] reported that the metal chelator EDTA is known to have activity against biofilms of gram-positive bacteria such as *Staphylococcus aureus*.

The agar diffusion test does not distinguish microbiostatic and microbicidal properties of dental materials neither does it provide any information about the microorganisms viability after the test.[21] The bacteria around the inhibition zone might grow back after some days. In clinical practice, it may be possible that after contact with root canal irrigants, microorganisms could still remain viable; this would depend on the irrigant and its concentration. For that reason it is important to associate the metabolic activity to the agar diffusion test, when evaluating the inhibitory activity of root canal irrigants.

Even after irrigation of the root canal with an antimicrobial solution, it may not be possible to eliminate all microorganisms from the root canal.[22] The microorganisms may multiply rapidly in 2-4 days, almost returning to their original numbers, if the canal is not filled with an antimicrobial substance between visits.[12] In the present study, a low metabolic activity that was not statistically significant was noted. Perhaps the low activity could be explained by the short period of incubation, since the metabolic activity was evaluated after only 24 h. More studies are necessary to evaluate metabolic activity after longer periods. The high pH of NaOCl interferes in cytoplasmatic membrane integrity with irreversible enzymatic inhibition, biosynthetic alterations in cell metabolism and phospholipids destruction,[9] and probably 24 h was not sufficient to allow bacteria recuperation, the same occurred with 17% EDTA against *Candida albicans* and *Staphylococcus aureus*.

In the inhibition zone test, *Candida albicans*, *Enterecoccus faecalis* and *Staphylococcus aureus* were sensitive to 1% NaOCl and higher concentrations. However, only 2.5 and 5.25% NaOCl were confirmed as microbicidal irrigants for *Staphylococcus aureus* after the metabolic activity test. The association of the two tests demonstrated that the other irrigants tested had a microbiostatic effect against *Candida albicans* and *Enterecoccus faecalis*. These results suggest that microorganism eradication in an endodontic infection may be obtained in association with other measures such as intracanal medication between sessions. Moreover, the results obtained by association of the two tests demonstrated the importance of the metabolic activity test as a complemen-

tary test to assess antimicrobial properties of root canal irrigants.

The present results support an initial hypothesis that there is a metabolic activity around the inhibition zone after the inhibition test, although without statistical significance. The short period of incubation may contribute to this result. Further studies are necessary to evaluate metabolic activity over a prolonged period.

## Conclusion

Citric acid did not present antimicrobial activity. Only 2.5% and 5.25% NaOCl presented antimicrobial activity against *Staphylococcus aureus*. In addition, 17% EDTA, 0.5% and 1% NaOCl presented only microbiostatic activity against some of the microorganisms tested. The highest concentration of NaOCl (5.25%) presented superior antimicrobial activity when compared with other irrigants used.

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