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In vitro antimicrobial activity of auxiliary chemical substances and natural extracts on *Candida albicans* and *Enterococcus faecalis* in root canals

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ABSTRACT

Objective: The aim of this study was to evaluate the antimicrobial activity of auxiliary chemical substances and natural extracts on *Candida albicans* and *Enterococcus faecalis* inoculated in root canals. Material and Methods: Seventy-two human tooth roots were contaminated with *C. albicans* and *E. faecalis* for 21 days. The groups were divided according to the auxiliary chemical substance into: G1) 2.5% sodium hypochlorite (NaOCl), G2) 2% chlorhexidine gel (CHX), G3) castor oil, G4) glycolic *Aloe vera* extract, G5) glycolic ginger extract, and G6) sterile saline (control). The samples of the root canal were collected at different intervals: confirmation collection, at 21 days after contamination; 1st collection, after instrumentation; and 2nd collection, seven days after instrumentation. Microbiological samples were grown in culture medium and incubated at 37°C for 48 hours. Results: The results were submitted to the Kruskal-Wallis and Dunn (5%) statistical tests. NaOCl and CHX completely eliminated the microorganisms of the root canals. Castor oil and ginger significantly reduced the number of CFU of the tested bacteria. Reduction of CFU/mL at the 1st and 2nd collections for groups G1, G2, G3 and G4 was greater in comparison to groups G5 and G6. Conclusion: It was concluded that 2.5% sodium hypochlorite and 2% chlorhexidine gel were more effective in eliminating *C. albicans* and *E. faecalis*, followed by the castor oil and glycolic ginger extract. The *Aloe vera* extract showed no antimicrobial activity.

Key words: *Ricinus communis*. *Aloe vera*. *Zingiber officinale*. Sodium hypochlorite. Chlorhexidine.

INTRODUCTION

Micro-organisms activity and their metabolic products have been reported as being part of the etiology of pulp and periapical lesions, which can certainly lead to pulp necrosis and inflammatory reactions. Root canal infections can be caused by a combination of microorganisms[24]. *Enterococcus faecalis* has been frequently isolated from infected pulp and persistent infections in post-endodontic treatment[24]. This type of microorganism has the ability to penetrate into the dentinal tubules and survive in root canals without other bacterial support[16]. Its eradication depends on a high pH value environment (pH=11)[17].

A percentage of yeasts, mainly from the *Candida* genus, ranging from 6 to 55%, can be also found in necrotic pulps[20]. In addition, the presence of *Candida spp*. in refractory periapical granulomas is reported in studies with a polymerase chain reaction (PCR) analysis[21,31]. Yeasts have been particularly

associated with persistent root canal infections that did not respond favorably to conservative root canal therapy[28].

Some properties such as antimicrobial effect, biocompatibility, and ability of tissue dissolving activity are required for irrigating solutions in order to achieve a satisfactory level of cleaning. Sodium hypochlorite has long been recognized as presenting outstanding disinfection properties, and it has been widely used for root canal disinfection[19,28,30]. Amongst its positive properties, sodium hypochlorite is known to be highly irritant to periapical tissues when used at high concentrations[22].

Chlorhexidine, in liquid or gel formats, has great potential to be used as an endodontic auxiliary chemical substance during the biomechanical preparation. It has shown great efficacy against microorganisms found in root canals[7,8,11,29]. On the other hand, it does not present tissue dissolving activity. For that matter, alternative solutions have been proposed, aiming to associate antimicrobial efficacy, tissue dissolving action and biocompatibility[14,26,27].

More recently, there have been an increasing number of studies focused on the use of phytotherapic substances for medical purposes. It is known that plant extracts and several types of teas have been used in popular medicine since remote times. However, their real properties and applications have not been scientifically investigated yet. Several companies, groups and developed countries have shown an increasing interest towards the biodiversity of tropical and subtropical countries such as Brazil.

The aim of this study was to evaluate *in vitro* the antimicrobial activity of auxiliary chemical substances and natural extracts against *Candida albicans* and *Enterococcus faecalis* inoculated in root canals.

MATERIAL AND METHODS

The present study was approved by the Institutional Review Board from Univ. Estadual Paulista – UNESP, São José dos Campos, Brazil (approval n. 093/2005). A total of seventy-two freshly extracted human single-rooted teeth were used in this study. All samples were cleaned and stored in saline prior to use. The crown portion was removed and the length of instrumentation was standardized at 16±0.5 mm.

The root canals were initially over-instrumented to 0.5 mm beyond the apex by means of a #25 K-file (Dentsply Ind. Com. Ltda, Petrópolis, RJ, Brazil), and post-instrumented to 1 mm from the apex with a #30 K-file. The root canals were filled with 17% EDTA solution for 3 minutes and rinsed with 5 mL of saline solution. The apex was sealed using Z-100 composite resin (3M – Saint Paul, USA) and the roots were externally sealed with epoxy adhesive (Araldite, Brascola, São Paulo, SP, Brazil), except for the cervical opening. All samples were included in transparent light-cured acrylic resin (Dencor Artigos Odontológicos Clássico – São Paulo, SP, Brazil). The specimens were distributed on cell plates (24 wells) (Costar, Corning, New York, USA) and further sterilized by Cobalt-60 gamma radiation[6].

The microorganisms strains used were *Candida albicans* (ATCC 18804) and *Enterococcus faecalis* (ATCC 29212). Both microorganisms were seeded on Petri dishes containing Sabouraud Dextrose Agar (SDA) (Himedia Laboratories, Mumbai, India) for *C. albicans*, and Brain Heart Infusion (BHI) (Himedia Laboratories, Mumbai, India) for *E. faecalis*. The SDA dishes were incubated in a bacteriological oven at 37±1°C for 24 hours, while the BHI dishes were incubated for a period of 48 hours.

Standardized saline solution suspensions of *C. albicans* and *E. faecalis* were prepared (10^8 cells/mL) by means of a spectrophotometric technique (λ=530 nm, DO=1.258, and λ=760 nm, DO=1.258, respectively). The root canals were contaminated with 10 µL of each microorganism suspension and 10 µL of BHI broth (Himedia Laboratories, Mumbai, India), resulting in 30 µL of inoculated medium in the root canals. A sterile cotton pellet embedded in BHI broth was placed at the entrance of the canals. The samples were stored in an incubator at 37±1°C in a humid atmosphere for 21 days. During this period, a small amount of BHI broth was placed in the root canals every three days[19].

After the contamination period, samples of all specimens were collected to confirm the contamination of the root canals (confirmation collection). The samples were then divided into 6 experimental groups (n=12), according to the auxiliary chemical substances used.

Group 1 – 2.5% sodium hypochlorite solution (NaOCl) (Byofórmula – Farmácia de Manipulação, São José dos Campos, SP, Brazil);

Group 2 – 2% chlorhexidine gel (Byofórmula – Farmácia de Manipulação, São José dos Campos – SP) and irrigation with saline solution between files Exchange;

Group 3 – Castor oil extract (*Ricinus communis*) (Chemistry Institute of São Carlos – USP, São Carlos, SP, Brazil);

Group 4 – Glycolic ginger extract (*Zingiber officinale*)(Becker – Farmácia de Manipulação, São José dos Campos, SP, Brazil);

Group 5 – Glycolic *Aloe vera* extract (Synthon Especialidades Químicas Ltda.);

Group 6 – Sterile saline solution.

The root canals were biomechanically prepared up to a size #50 K-file and rinsed with 3 mL of irrigating solution after each file.

Microbiological samples were collected immediately post-instrumentation (1st collection) and seven days post-instrumentation (2nd collection).

Root specimens from group 1 were irrigated with 3 mL of 0.6% sodium thiosulphate, previous to the first collection, in order to neutralize the remaining NaOCl, while the root canals from group 2 were irrigated with 3 mL of 0.5% Tween 80+0.07% lecithin to neutralize the remaining chlorhexidine[19].

Each of the three sample collections (confirmation collection, 1st collection, and 2nd collection) was carried out at the same way. A number 30 sterile paper cone (confirmation collection) or a number 50 sterile paper cone (1st and 2nd collection) was placed and left in the root canal for one minute. The paper cone was placed in an Eppendorf test tube containing 0.5 mL sterile saline solution and stirred for 30 seconds. A 0.1 mL aliquot of each content was seeded and duplicated into dishes containing Agar Sabouraud for *C. albicans* and Agar Mitis Salivarius for *E. faecalis*.

Subsequent to the first collection, all the root canals were filled with sterile saline solution, and a sterile cotton pellet was placed at the entrance of the canals. The samples were stored in an incubator at 37±1°C with a humid atmosphere for 7 days prior to the second collection.

Characteristic grown colonies of *E. faecalis* and *C. albicans* were counted and confirmed by the Gram-color staining method. Descriptive statistics,

Table 1- Colony forming units *per* mL (CFU/mL) (log10) for confirmation, first, and second collections

Groups	Confirmation collection		1st collection		2nd collection	
	C. albicans	*E. faecalis*	*C. albicans*	*E. faecalis*	*C. albicans*	*E. faecalis*
G1 2.5% NaOCl	6.09	6.12	0	0	0	0
G2 2% CLX gel	6.17	6.01	0	0	0	0
G3 Castor oil	6.16	6.24	0.50	1.57	2.80	3.49
G4 Ginger	6.29	8.22	0	2.42	3.50	3.49
G5 *Aloe vera*	5.95	7.33	4.90	5.39	5.68	5.73
G6 Saline	6.08	6.17	4.85	5.03	5.53	5.46

Table 2- Percentage reduction values of *C. albicans* and *E. faecalis* CFU/mL, during the first and the second collections in relation to the confirmation collection. The homogeneous groups are also presented

Groups	Confirmation X 1st collection				Confirmation X 2nd collection			
	C. albicans		*E. faecalis*		*C. albicans*		*E. faecalis*	
	Median	HG*	Median	HG*	Median	HG*	Median	HG*
G1 NaOCl 2,5%	100	A	100	A	100	A	100	A
G2 2% CLX gel	100	A	100	A	100	A	100	A
G3 Castor oil	100	A	100	A	99.9	A	99.9	A
G4 Ginger	100	A	99.9	AB	98.8	A	99.9	A
G5 *Aloe vera*	98.5	B	98.66	BC	76.7	B	77	B
G6 Saline	95.1	B	93.21	C	74.8	B	82.6	B

*Homogeneous groups: different letters show statistical significant difference ($p<0.05$)

and the Kruskal-Wallis test and the Dunn's test (5%) were used to evaluate the results. The statistical analysis was based on the percentage of reduction.

RESULTS

The mean values of colony forming units *per* mL (CFU/mL) for each group were determined and are shown in Table 1.

The reduction or complete elimination of *C. albicans* and *E. faecalis* for the first and second collection compared to the confirmation collection, according to the Dunn's test (5%), is shown in Table 2.

DISCUSSION

The root canals within the present study were inoculated with *C. albicans* and *E. faecalis* for 21 days. The literature shows this contamination period is the reference for obtaining mature biofilms in the dentin[2,32]. Wang, et al.[32] (2012) evaluated the antibacterial effect of different disinfecting solutions on young and established *E. faecalis* biofilms in dentin canals using a novel dentin infection model. They verified that within the dentin canals, endodontic medications less easily kill bacteria in established biofilms than bacteria in young biofilms.

The results obtained in the present study showed a range of effects against the microorganisms tested, for both the 1st collection and 2nd collection.

The irrigation of the root canals with 2.5% NaOCl or 2% chlorhexidine gel during the instrumentation process, resulted in negative microbiological collections immediately post-instrumentation (1st collection) and also for the period of seven days post-instrumentation (2nd collection). It shows that those substances are capable of eliminating both *E. faecalis* and *C. albicans*. Valera, et al.[29,30] (2009, 2010) also assessed 1% NaOCl and 2% clorhexidine gel as significantly reducing the quantity of *C. albicans* and *E. faecalis* inoculated into the root canals.

It is evident that both 2.5% NaOCl solution and 2% chlorhexidine gel have antimicrobial activity and great capacity of penetration into the dentinal tubules, but the efficacy of NaOCl seven days post biomechanical preparation has not been established by other studies[19,28]. In the present study, the residual effect of those substances have not been evaluated, due to the neutralization process performed after the biomechanical preparation: roots irrigated with 2.5% NaOCl were neutralized by 3 mL of a 0.6% sodium thiosulphate solution; while roots irrigated with 2% chlorhexidine gel were neutralized by 3 mL of a 0.5% Tween 80+0.07% lecithin[19]. The neutralization process before the microbiological collection was required once some residues from the irrigating solutions might have remained and inhibited the growing process of microorganisms when using culture medium. However, even with the neutralization process it is possible to evaluate whether microorganisms remained in the tubules after the mechanical preparation and, in this study, the biomechanical preparation completely eliminated *C. albicans* and *E. faecalis*.

The efficacy of NaOCl as an irrigating solution has been reported by other studies[3,8,19,30] at different concentrations. Sodium hypochlorite has been capable of promoting biosynthetic cell alterations and phospholipids damage. These properties are related to the formation of chloramines, which interfere with cell metabolism leading to an oxidizing action. As a consequence, irreversible enzymatic inhibition of the sulphidrila present in bacterial enzymes and degradation of fatty acids and lipids are expected[8]. Gomes, et al.[11] (2001) verified a better performance of chlorhexidine on *E. faecalis* in comparison to 2.5% NaOCl.

The efficacy of chlorhexidine has been already reported in previous studies[8,11,19,29]. The antimicrobial activity of chlorhexidine is based on its positively charged molecule that interacts with negatively charged phosphate groups present on the bacterial cell wall, allowing the chlorhexidine molecule to penetrate the bacteria and leading to intracellular toxic effects[7-9]. This substance acts on Gram-positive and Gram-negative microorganisms. Due to its cationic properties, this biguanide is able to connect to hydroxyapatite, dental biofilm, oral mucosa and salivary proteins. When its concentration decreases in the oral environment, this substance is released from those structures. Such a characteristic is called substantivity, and promotes a durable effect to clorhexidine[8,9]. In concentrations ranging from 0.2 to 2%, chlorhexidine presents a wider antimicrobial spectrum, lower toxicity, better diffusion through the dentinal tubules, biocompatibility, and it has been more efficient in removing the smear layer compared to sodium hypochlorite, which makes this substance a good choice for endodontic therapy[7,8,25].

Castor oil detergent (*Ricinus communis*) has shown antimicrobial activity and biocompatibility, non-toxic results, detergent properties, which are important requirements for an irrigant solution[4,5,10,15,27]. The literature has reported that irrigation with castor oil extract is capable of removing debris, showing similar results to 1% NaOCl[18]. Valera, et al.[27] (2012) showed a significant decrease in the number of *Escherichia coli* in the root canals after irrigation with castor oil extract during the biomechanical preparation. These findings suggest that this substance could be utilized in endodontic therapy.

When analyzing the first collection data of the

present study, it could be verified that castor oil was able to completely eliminate *C. albicans* and it was also able to significantly reduce the amount of *E. faecalis*. On the other hand, in the second collection data, the development of two microorganisms (0.1%), especially *E. faecalis*, demonstrated that this solution is promising for endodontic purposes. However, more studies are required to clarify its antimicrobial activity mechanism.

The medical use of *Aloe vera* has been supported by its antimicrobial, anti-inflammatory and regenerative properties[12]. Gontijo, et al.[12] (2013) evaluated *in vivo* dentine-pulp behaviour of rats after direct pulp capping with *Aloe vera*. They observed the presence of acute inflammatory infiltrate (light to moderate) in the first day, while for the calcium hydroxide group (positive control), the presence of acute inflammatory infiltrate was severely related to superficial necrosis. Athiban, et al.[1] (2012) detected the *in vitro* antimicrobial activity of *Aloe vera* over *E. faecalis, E. coli* and *Staphylococcus aureus.* It was concluded *Aloe vera* could be effectively used for decontaminating GP points within a short application time. In the present study, *Aloe vera* did not show antimicrobial efficacy, once it was not able to eliminate all the microorganisms determined in the first sample collection. Moreover, it allowed a growth of microorganisms between the first and second data collection. According to the comparative Dunn's test (5%), the *Aloe vera* group showed similar results compared to the saline group.

The use of ginger (*Zingiber officinale*) has been appreciated since remote times, and it has been widely used in alcoholic drinks, seasonings and in popular medicine. It was verified that this extract was effective in eliminating microorganisms. The antimicrobial activity of the ginger extract on three Gram-negative anaerobes, *Porphyromonas gingivalis*, *Porphyromonas endodontalis* and *Prevotella intermedia* was observed[23]. The effective action of glycolic and alcoholic ginger extract on *S. mutans*, *Staphylococcus aureus*, *E. coli* and *C. albicans*[13] were also reported. This fact might contribute to the treatment of some diseases caused by these types of microorganisms present in the oral cavity. The real mechanism of action of the ginger extract has not yet been elucidated in the literature.

In this study, the irrigation of the root canals with glycolic ginger extract resulted in the negative development of *C. albicans* for the first sample collection (immediately post instrumentation). Although a positive increase of *C. albicans* was observed in the second sample collection, no statistical differences were detected. This result suggests that microorganisms situated deeper in the dentinal tubules were not affected by the irrigating agent, and therefore they were able to recolonize the root canal lumen after seven days. Although *E. faecalis* was not completely eliminated in the first sample collection, the reduction was close to 100%. In the second sample collection, there was an increase in the number of microorganisms in comparison to the first collection, demonstrating no residual effect. In spite of the growth of microorganisms, the results obtained for the ginger group was statistically similar to 2.5% NaOCl and 2% chlorhexidine gel. The present observed antimicrobial effect of the ginger extract may be related to the very low concentration used. Further investigations on higher concentrations of these substances would be necessary to elucidate the action of the ginger over the microorganisms.

The saline solution (control) used in this experiment was the reference for evaluation of the antimicrobial action of the other substances. Due to its absence of antimicrobial effect, it was possible to assume that the physical action of the instrumentation leads to a considerable decrease in the amount of microorganisms in the root canals.

The results obtained in the present study show that phytotherapic substances might be used in the future as alternative irrigating solutions for endodontic treatment, since they are natural products and do not disturb the environment. As previously mentioned, it is important to support further investigations to identify the most suitable concentration of these substances and their effects over other types of microorganisms and their products.

CONCLUSION

According to the methodology used and the results obtained in this experiment, it could be concluded that: 2.5% NaOCl and 2% chlorhexidine gel were the most effective irrigating solutions against *C. albicans* and *E. faecalis*, for both the first and second sample collections. They were able to completely eliminate the microorganisms from the root canals. Castor oil extract and glycolic ginger extracts were able to significantly decrease the amount of microorganisms, not being able to completely eliminate them 7 days post biomechanical preparation. The *Aloe vera* natural extract, did not show antimicrobial efficacy with the methodology used.

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