

In Vitro Antimicrobial Activity of an Experimental Dentifrice Based on *Ricinus Communis*

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This study evaluated the antimicrobial activity of a *Ricinus communis*-based experimental dentifrice for denture hygiene against the following standard strains: *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus mutans*, *Enterococcus faecalis*, *Candida albicans* and *Candida glabrata*. The minimum inhibitory concentration (MIC) assay was performed with *R. communis* in pure oil at 2.5%. Only *E. coli* was not inhibited by *R. communis*, but the MIC (0.0781%) was effective against the other microorganisms. From these results it was determined the *R. communis* concentrations for experimental dentifrices, 1, 2, 5 and 10%, which were evaluated by the test-well diffusion in agar. The commercial dentifrices Colgate, Trihydral and Corega Brite were tested for comparative purposes. The diameter of the zones of bacterial growth inhibition produced around the wells was measured (in mm) with a rule under reflected light. Data were analyzed statistically by analysis of variance and Tukey's post-hoc test ($\alpha=0.05$). Neither the commercial nor the experimental dentifrices were effective against *E. coli*. The experimental dentifrices containing *R. communis* at 2, 5 and 10% presented action against *S. mutans*, *S. aureus* and *E. faecalis*. The experimental dentifrices showed no antimicrobial activity against *Candida* spp. and *E. coli* in any of the tested concentrations. Trihydral was the most effective. Comparing the experimental dentifrices, the product with 10% *R. communis* produced the largest zones of bacterial growth inhibition and had similar antimicrobial activity to the commercial dentifrices, except against *S. aureus*.

Key words: complete denture, hygiene, dentifrice, *Ricinus communis*, microbiology.

Introduction

In contrast with continuous advances in dentistry and the advent of new materials and techniques, the oral health of denture wearers is still deficient. Approximately 11-65% of denture wearers are affected by denture stomatitis, a disease characterized by inflammation of the palatal mucosa. This is a multifactorial disease, however poor denture hygiene is considered to be particularly significant (1).

Inadequate denture hygiene habits lead to biofilm accumulation on the surfaces of removable dentures. Studies have shown that different species of microorganisms are associated with denture plaque. Among the bacteria, the following can be cited: *Staphylococcus aureus*, *Streptococcus mutans*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (2). *Candida albicans* and *Candida glabrata* are commonly found yeast in denture biofilm and are the main etiological factors for atrophic chronic candidiasis (1). The proper cleaning of the prosthesis is vital for biofilm control and for the prevention of inflammation and infection in the mouth of edentulous patients.

Denture cleaning methods can be performed chemically and/or mechanically. Cleaning by chemical method

consists in immersing the denture in solutions with solvent, detergent, antibacterial and antifungal actions (3). A significant feature of the chemical method is the variety of possible active agents. However, these agents present antimicrobial activity against denture biofilm depending on the type of microbial biofilms formed (4) and can cause damages in the acrylic resin denture base (5). The mechanical method is represented by brushing and previous data have shown that brushing with dentifrice is one of the most common methods of denture hygiene (3). This method is inexpensive and simple to use, requiring the adequate combination of brushes and auxiliary agents such as dentifrices (3). The use of adequate brushes and auxiliary agents is essential for the removal of denture biofilm, control of microorganisms and prevent adverse effects to the material of the denture base (6). The combination of mechanical and chemical methods, which consists in brushing with brush plus dentifrice and immersing the denture in chemical cleansers, is recommended for denture cleansing. However, Paranhos et al. (4) showed that combination method provided results similar to the mechanical method and was more effective than the chemical method for the majority of the tested species of microorganisms. Our clinical experience shows

that many denture wearers perceive the combination of methods as a complex procedure. Therefore, combination of the mechanical and chemical methods in one step, i.e. mechanical brushing with a dentifrice that has in its composition an antimicrobial agent, can facilitate the cleaning and sanitizing procedures for denture users. New dentifrices for denture hygiene should be developed in order to obtain an ideal relation between biofilm removal and potential antimicrobial (7,6).

Dentifrices have a complex composition. The main components are water, detergents, thickeners, pigments, flavorings and abrasive agents. Antimicrobial substances or other therapeutic agents can also be included. The development of a dentifrice formulation should consist of adequate antimicrobial and surfactants for a more efficient biofilm removal (8). The literature has increasingly focused its attention on the antimicrobial activity of plants and seeds as raw material for manufacturing dentifrices and soaking solutions. Some of the benefits of their use concern their relative safety when compared with synthetic alternatives, offering significant and more affordable therapeutic benefits (9).

One of these plants is *Ricinus communis*, which is a plant species typically found in tropical climate areas. The oil extracted from the *R. communis* seeds has a strong surfactant with antimicrobial and anti-inflammatory activities, as well as biocompatibility with periapical tissues (10). This study evaluated the antimicrobial activity of a dentifrice formulated with *R. communis* in different concentrations to be employed in denture hygiene.

Material and Methods

This study included the commercial dentifrices Colgate (Colgate-Palmolive Division Kolynos Brazil, Osasco, SP, Brazil) and Trihydral (Perland Pharmacos Ltd., Cornelio Procópio, PR, Brazil) and Corega Brite (GSK - GlaxoSmithKline, Brazil Ltda, Rio de Janeiro, RJ, Brazil). These dentifrices were chosen in order to cover a wide range of clinical indications, i.e., dentate subjects (Colgate), removable denture cleansing (Corega Brite) or both (Trihydral).

The *R. communis* oil was obtained by a reaction of esterification and it consists of triglycerides containing fatty acids, such as ricinoleic acid, linoleic acid, stearic acid, palmitic acid, dihydroxystearic acid, licosanoic acid and linolenic acid (Chemistry Institute of São Carlos, University of São Paulo, São Carlos, SP, Brazil). The Broth Microdilution method was used to determine the minimum inhibitory concentration (MIC) of *R. communis* necessary for formulation of experimental dentifrice.

The microdilution technique in 96-well plates was performed in duplicate against *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *S. mutans* (ATCC 25175), *Enterococcus*

faecalis (ATCC 29212), *C. albicans* (ATCC 10231) and *C. glabrata* (ATCC 2001).

A 5% solution was initially prepared with the dilution of 0.5 g of *R. communis* in 9.5 mL of distilled water. This solution was then diluted in Tryptic Soy Broth (Difco, Sparks, MD, USA) to obtain the initial concentration of 2.5%. Serial two-fold dilutions were added into 100 μ L volumes of Tryptic Soy Broth in the wells. The procedure resulted in 10 concentrations that varied from 2.5 to 0.0048%. An aliquot of 5 μ L of standardized microbial inoculum was added to each well of the microplate.

Microbial suspensions were prepared in saline solution from recent cultures (37 °C for 24 h) containing 10^8 CFU/mL for bacteria and 10^6 CFU/mL for yeast, which correspond to an absorbance of 0.08 and 0.1 at 625 nm wavelength. Next, the suspensions were diluted to 1:10 to order to achieve a bacterial inoculum of approximately 10^7 CFU/mL and 10^5 CFU/mL for yeast. When aliquots of 5 μ L standardized microbial inoculum were inoculated in the culture medium, the final concentration of each microorganism was of about 10^5 CFU/mL for bacteria and 10^3 CFU/mL for the yeasts. Aliquots of 50 μ L of Tryptic Soy Broth added to 50 μ L of saline and 5 μ L standardized microbial inoculum served as a positive control and aliquots of 50 μ L of Tryptic Soy Broth added to 50 μ L of *R. communis* solution at 5% served as negative control.

Figure 1 is a flowchart of the aforementioned technique of the microdilution to obtain the MIC of *R. communis*.

The incubation was performed in a moist chamber at 37 °C for 24 h and the wells with the culture medium *R. communis* and inocula were examined for the turbidity of the medium, which indicated microbial growth. MIC was the lowest concentration of the product able to inhibit visible microbial growth.

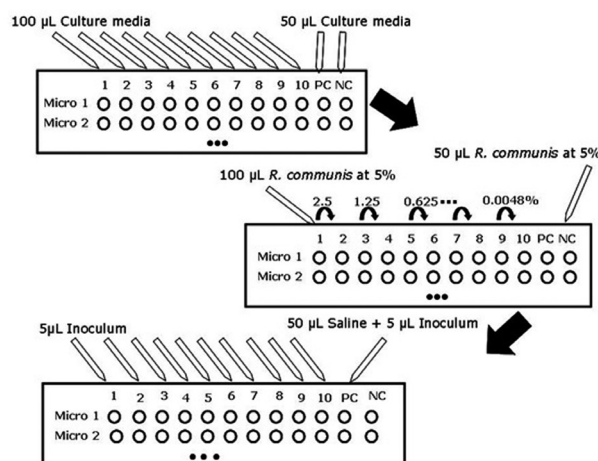


Figure 1. Flowchart of the MIC methodology. PC: Positive Control. NC: Negative Control.

With the results of the MIC test, four formulations of *R. communis* dentifrice were manipulated with concentrations at 1, 2, 5 and 10% (Table 1), according to the Good Practices of Pharmaceutical Manufacturing.

During the preparation of the experimental dentifrice, hydroxyethyl cellulose, glycerin, EDTA, saccharin sodium and water were weighed on a digital balance (Ind. and Com GEHAKA Electrical and Electronics Ltd., São Paulo, Brazil, Mod BG400), manually homogenized and kept in a dark container for 24 h until gel formation. Next, the other components were weighed, gradually added and mixed with the gel in a blender (Model MPDL-6; SEW do Brasil Ltda, São Paulo, Brazil). The *R. communis* was added in sufficient quantity to obtain the desired concentrations of 1, 2, 5 or 10%. After obtaining a homogeneous dentifrice, it was dispensed and stored in opaque colored tubes that have a 60 g capacity.

To evaluate the antimicrobial activity of *R. communis* dentifrices against the microorganisms, the agar well-diffusion double layer technique was used. The experiment was performed in duplicate and the culture mediums used were Sabouraud Dextrose Agar (Difco, Sparks, MD, USA) for *C. albicans* and *C. glabrata* and Tryptic Soy Agar (Difco, Sparks, MD, USA) for *S. aureus*, *S. mutans*, *E. coli* and *E. faecalis*. The culture media were distributed in a Petri dish (20 x 10 mm) to obtain the sterile base layer of 12 mL.

Test tubes (25 x 150 mm) containing 20 mL of sterile culture medium were kept in a water bath at 45 °C after, 200 µL of standardized microbial inoculum were inserted in these tubes to obtain 1% inoculum. The culture medium with the microbial inoculum was homogenized in a tube shaker and transferred to the solidified base layer of the Petri dish, after which the seed layer was formed. Then, 5-mm diameter wells were prepared with sterile straws and

a bacteriological needle sterilized with Bunsen burner. The different dentifrices were added to the well and the Petri plates were pre-incubated at room temperature (25 °C) for 2 h to allow diffusion of the product in the culture medium.

The plates were incubated at 37 °C for 24 h, and the microorganisms *S. mutans* and *E. faecalis* were incubated under microaerobic conditions. To avoid contact of the water condensation with the culture medium, absorbent paper was placed between the bottom and the cover of the plates. The formation of an inhibition zone around the sample indicated the efficacy of the dentifrice. The diameter of the zones of bacterial growth inhibition produced around the wells was measured (in mm) with a rule under reflected light. The measurements were performed three times in each zone to obtain a mean value. Data referring to the antimicrobial activity of the dentifrices were analyzed statistically by analysis of variance and Tukey's post-hoc test ($\alpha=0.05$).

Results

The minimum inhibitory concentration (MIC) of *R. communis* was of 0.0781%. Only *Escherichia coli* was not inhibited with the maximum concentration (Table 2).

The analysis of the antimicrobial activity using the well agar diffusion method revealed statistically significant difference between the dentifrices. Table 3 shows the comparison of the mean diameters (mm) of the zones of bacterial growth inhibition formed around the wells. None of the dentifrices were effective against *E. coli*. Colgate and Trihydral presented similar action against *S. aureus* and *S. mutans*. Corega and the experimental dentifrice at 10% also presented action against *S. mutans*. Trihydral was the most effective against *E. faecallis*, followed by the experimental dentifrice at 10% and Colgate, which showed no significant difference between each other ($p>0.05$). The

Table 1. Experimental dentifrice composition

Components	Manufacturer	Function
Hydroxyethylcellulose	Union Carbide Corp., Houston, TX, USA	Thickening agent
Glycerin	Ely Martins, Ribeirão Preto, SP, Brazil	Humectant
EDTA	Dow Chemical Co., Midland, MI, USA	Chelating agent
Saccharin sodium	Labsynth Produtos para Laboratórios Ltda, Diadema, SP, Brazil	Flavoring
<i>R. communis</i> gel	Institute of Chemistry of São Carlos (IQSC)/ University of São Paulo (USP), São Carlos, SP, Brazil	Preservative, antimicrobial agent, surfactant
Silica (Sident 9)	Evonik Degussa GmbH, Düsseldorf, Germany	Abrasive
Silica (Sident 22S)	Evonik Degussa GmbH, Düsseldorf, Germany	Abrasive
Titanium dioxide	Minérios Ouro Branco, São Paulo, SP, Brazil	Pigment (white)
Menthol - eucalyptol	Givaudan do Brasil Ltda., São Paulo, SP, Brazil	Flavoring
Distilled water	-	Vehicle

experimental dentifrice showed no antimicrobial activity against *Candida spp.* in any of the concentrations tested. Against these yeasts, Colgate, Trihydral and Corega showed greater inhibition capacity; Colgate and Trihydral were similar for *C. albicans*.

Discussion

This study evaluated the antimicrobial activity of the experimental dentifrice of *R. communis* against microorganisms found in the buccal cavity and complete dentures.

The MIC was performed with the unalloyed *R. communis* to select the dentifrice concentrations. Only *E. coli* was not inhibited up to the maximum concentration of 2.5%. This microorganism is a Gram negative bacillus transient of the oral cavity that is responsible for the initial adhesion of yeasts on various surfaces (11). *E. coli* has an outer membrane which covers the thin peptidoglycan layer of the cell wall, which forms a selective barrier to the entry and exit of some substances (12). Additionally, the enzymes in the periplasmic space are capable of destroying foreign molecules (13), which could explain this result. However,

another study showed that the detergent oil of *R. communis* 10% was effective against Gram-negative bacteria (10). This result suggests the need for further tests of MIC with *R. communis* at higher concentrations. Increasing the concentration of *R. communis* is a safe procedure, since it is not toxic to the oral tissues (9).

The other microorganisms were inhibited by the concentration of 0.0781%. These results show that *R. communis* is a potential antimicrobial agent and encourage the development of more studies on its antimicrobial activity against denture biofilm microorganisms. The *S. mutans*, is a Gram positive coccus with a fundamental role in the initial colonization of denture and natural teeth biofilm, considered the main etiological agent of dental caries (14); *S. aureus*, Gram positive coccus, can cause opportunistic infections (15) and intestinal infection in immunocompromised patients; the yeast *C. albicans* and *C. glabrata* are the major etiological factors of denture stomatitis and responsible for its maintenance and exacerbation (1); *E. faecalis* is a Gram-positive coccus involved in various diseases of the oral cavity, such as apical periodontitis, recurrent caries and endodontic infections and also the colonization of other sites in the human body, favoring the occurrence of bacterial endocarditis (16).

The action of *R. communis* against these microorganisms can be attributed to its action as "protractors", water catalyzing the hydrolytic reaction by breaking the glycosidic bonds, which are present in the cell wall of the bacteria (17). Although this possibility exists, there are no other studies evaluating the mechanism of antimicrobial action of *R. communis*. Thus, studies need to be conducted since the knowledge of the mechanism of action of antimicrobials is of great importance for adequate and conscious rational use of the product as well as for the elucidation of biochemical phenomena at various levels of the structure of the cells.

The MIC test results enabled choosing to formulate the experimental dentifrices at concentrations of 1 and 2% with a margin of safety for the most resistant bacteria and, at concentrations of 5 and 10% to achieve an antimicrobial activity against *E. coli*.

Comparing the antimicrobial activity of the experimental dentifrices and the other commercial dentifrices, it was found that they have no antimicrobial activity against *E. coli*. For other microorganisms, the commercial formulations Colgate and Trihydral, considered with positive controls in this

Table 2. Minimum inhibitory concentration (MIC) of *R. communis* against the standardized microorganisms

Microorganism	MIC (µg/L)	MIC (%)
<i>S. aureus</i>	195	0.0195
<i>E. coli</i>	0*	0*
<i>S. mutans</i>	< 48	< 0.0048
<i>E. faecalis</i>	195	0.0195
<i>C. albicans</i>	781	0.0781
<i>C. glabrata</i>	781	0.0781

*Initial concentration of *R. communis* analyzed 25.000 µg/L or 2.5%.

Table 3. Diameters (in mm) of the zones of bacterial growth inhibition against of the bacterial strains

Dentifrice	<i>S. aureus</i>	<i>E. coli</i>	<i>S. mutans</i>	<i>E. faecalis</i>	<i>C. albicans</i>	<i>C. glabrata</i>
Colgate	16.5 ^a	0	17 ^a	15 ^a	20.5 ^a	21.5 ^a
Trihydral	16.5 ^a	0	22 ^a	20 ^b	19.5 ^a	20 ^b
Corega	15 ^b	0	13 ^b	11 ^c	17 ^b	15.5 ^c
Experimental 10%	12 ^c	0	13.5 ^b	16 ^a	0	0
Experimental 2%	9.5 ^d	0	10.5 ^c	8.5 ^d	0	0
Experimental 5%	9 ^d	0	12 ^c	10 ^c	0	0
Experimental 1%	6 ^e	0	0	0	0	0

Different letters in rows indicate statistically significant difference.

study, promote inhibition zone. The antimicrobial action of Trihydral can be attributed to the presence of Chloramine T. This component destroys the cellular material because it reacts with the organic material of microorganisms, causing oxidative reaction and protein hydrolysis, breaking down the cell walls of gram positive bacteria, gram negative bacteria, fungi, viruses, mycobacteria, yeasts, in vegetative form (spores) or not. Panzeri et al. (6) found that a dentifrice containing Chloramine-T was effective against *S. mutans*, but the *Candida* spp. counts were not affected by the treatment. The antimicrobial action of Colgate dentifrice can be attributed to the presence of sodium monofluorophosphate in their composition. The fluoride inhibits the enzymatic metabolism of microorganisms in the biofilm, reducing the ability of the microorganisms to decompose carbohydrates (18). The Corega brite, although considered with negative control, also showed action against all microorganisms, exception of *E. coli*. This dentifrice contains sodium bicarbonate in their composition which has an antimicrobial effect over several microorganisms isolated from the oral cavity, including *C. albicans* (19). In addition, in the presence of water the sodium bicarbonate provides an alkaline pH to solubilize the fatty acids on the surface, which might hinder bacterial adhesion to the surface (20). However, the effect of sodium bicarbonate on the adherence of these microorganisms should be evaluated since this adhesion can interfere with the biofilm development mechanisms.

The antimicrobial activity results of the experimental dentifrice at 5 and 10 % showed inhibitory activity against *S. aureus*, *S. mutans* and *E. faecalis*. None of the experimental dentifrices inhibit the growth of *Candida* spp. However, a decrease was observed in the concentration of the yeast colonies around the wells corresponding to the experimental dentifrice, suggesting that they presented light antimicrobial activity. Regarding the antimicrobial activity of *R. communis* solutions, the irrigating solution for root canal treatment did not appear to be effective against Gram-negative bacteria (21), while it was effective against Gram-positive bacteria and yeast (9). It is difficult to make comparisons with other studies because there is no available data on the use of *R. communis* associated with dentifrices.

In this study, the *R. communis* gel in its pure form inhibited *Candida* spp., but it was not effective when used as a component in the experimental dentifrice formulation. Many substances, such as thickening agents, abrasives, humectants, antimicrobial, surfactants, sweeteners and flavorings are present in the composition of dentifrices (22). According to Moran et al. (22), some additives in the formulation of dentifrices can reduce or inhibit the activity of some of their components, but may also increase the

substantivity and clinical activity (23). The interaction between different components must be characterized to not impair the action of the formulated product. A complex mixture of the components of a dentifrice should be compatible with the antimicrobial agent, both physically and chemically, so that it is a stable product during storage as well as effective in its application (24). Another factor that could have influenced on the results related to the methodology and techniques used (25). For an antimicrobial agent performs its action by this method (well-agar diffusion), it is necessary that the active ingredient to diffuse through for mean. *Ricinus communis* presents as the hydrophobic characteristics and the culture medium used for the growth of yeasts, hydrophilic characteristics, their action may have been prejudiced against yeasts.

Further studies with *R. communis* and studies evaluating the interaction between the components are necessary. The components of experimental dentifrices must be fractionated and tested separately.

In the present study, the *R. communis*-based dentifrices presented higher antimicrobial activity against microorganisms commonly found in the oral biofilm of dentate patients, such as *Streptococci*. These preliminary results suggest that the experimental dentifrices could be better indicated for dentate individuals. However, clinical studies are also necessary to prove the efficacy of *R. communis*. The use of these dentifrice by denture wearers seems to be limited, based on the tested concentrations and methodology used, since they showed no antimicrobial activity against *Candida* sp., the most common microorganism on denture biofilm. The limitation of this study was the lack of comparison between the antimicrobial agent, *R. communis*, and the antimicrobial agents of commercial dentifrices, due to the different formulation of each dentifrice. However, as the objective was to assess whether the experimental dentifrices presented antimicrobial activity similar to the commercial ones, this study was not impaired. Further research aimed at comparing *R. communis* with antimicrobial agents of different dentifrices and their antimicrobial action on biofilms, will be our next step.

In conclusion, the experimental dentifrices based on *R. communis* at concentrations of 2, 5 and 10% presented antimicrobial activity against all microorganisms studied, except for *E. coli* and *Candida* spp. The dentifrice at 10% showed the best antimicrobial activity and presented a similar antimicrobial activity to commercial dentifrices, except against *S. aureus*.

Resumo

Este estudo avaliou a atividade antimicrobiana de um dentifrício experimental a base de *Ricinus communis* para higiene de prótese contra

as seguintes cepas padrão: *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus mutans*, *Enterococcus faecalis*, *Candida albicans* e *Candida glabrata*. O ensaio de concentração inibitória mínima foi realizado com *R. communis* em óleo puro a 2,5%. Apenas a *E. coli* não foi inibida por *R. communis*, no entanto, a concentração mínima (0,0781%) foi eficaz contra os outros microrganismos. A partir destes resultados foram determinadas as concentrações dos dentifrícios experimentais: 1, 2, 5 e 10%, as quais foram avaliadas pelo teste de difusão em Agar. Os dentifrícios comerciais Colgate, Trihydral e Corega Brite foram testados para fins comparativos. O diâmetro dos halos de inibição do crescimento bacteriano, em torno dos poços, foi medido (em mm) com uma régua sob uma luz refletida. Os dados foram analisados estatisticamente por meio de análise de variância e teste post-hoc de Tukey ($\alpha=0,05$). Nem os dentifrícios comerciais nem os experimentais foram eficazes contra *E. coli*. Os dentifrícios experimentais contendo *R. communis* a 2, 5 e 10% apresentaram ação contra *S. mutans*, *S. aureus* e *E. faecalis*. Os dentifrícios experimentais não mostraram atividade antimicrobiana contra *Candida spp* e *E. coli* em nenhuma das concentrações testadas. O Trihydral foi o mais eficaz. Comparando os dentifrícios experimentais, o produto com 10% de *R. communis* produziu os maiores halos de inibição do crescimento microbiano e apresentou atividade antimicrobiana.

Acknowledgements

The authors are grateful to the funding agency FAPESP for the research grant (#2010/50819-8), to Prof. Denise de Andrade from the Laboratory of Infection Control and Prevention of the Nursing School of Ribeirão Preto, University of São Paulo, to Professors Heitor Panzeri (*In memoriam*) and Elza Helena Guimarães Lara, from the Clinical Analysis Laboratory HELP and to Professor Gilberto Orivaldo Chierice, from the Institute of Chemistry of São Carlos, University of São Paulo.

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Received December 19, 2013

Accepted April 17, 2014