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Effectiveness of experimental dentifrices based on essential oils on biofilm on complete dentures: an *in vitro* study

Andrezza Cristina Moura dos SANTOS[1]
Filipe Santos Ferreira MENDES[1]
Fernanda Thaís POMPEO[1]
Evandro WATANABE[2]
Ana Paula MACEDO[3]
Valéria Oliveira Pagnano de SOUZA[1]
Helena de Freitas Oliveira PARANHOS[1]
Cláudia Helena SILVA-LOVATO[1]

Abstract

Specific products containing natural resources can contribute to the innovation of complete denture hygiene. Objective: To conduct an *in vitro* evaluation of experimental dentifrices containing essential oils of *Bowdichia virgilioides Kunth* (BvK), *Copaifera officinalis* (Co), *Eucalyptus citriodora* (Ec), *Melaleuca alternifolia* (Ma) and *Pinus strobus* (Ps) at 1%. Methodology: The variables evaluated were organoleptic and physicochemical characteristics, abrasiveness (mechanical brushing machine) simulating 2.5 years, and microbial load (Colony Forming Units - CFU/mL), metabolic activity (XTT assay) and cell viability (Live/Dead® BacLight™ kit) of the multispecies biofilm (*Streptococcus mutans*: Sm, *Staphylococcus aureus*: Sa, *Candida albicans*: Ca and *Candida glabrata*: Cg). Specimens of heat-polymerized acrylic resins (n=256) (n=96 specimens for abrasiveness, n=72 for microbial load count, n=72 for biofilm metabolic activity, n=16 for cell viability and total biofilm quantification) with formed biofilm were divided into eight groups for manual brushing (20 seconds) with a dental brush and distilled water (NC: negative control), Trihydral (PC: positive control), placebo (Pl), BvK, Co, Ec, Ma or Ps. After brushing, the specimens were washed with PBS and immersed in Letheen Broth medium, and the suspension was sown in solid specific medium. The organoleptic characteristics were presented by descriptive analysis. The values of density, pH, consistency and viscosity were presented in a table. The data were analyzed with the Wald test in a generalized linear model, followed by the Kruskal-Wallis test, Dunn's test (mass change) and the Bonferroni test (UFC and XTT). The Wald test in Generalized Estimating Equations and the Bonferroni test were used to analyze cell viability. Results: All dentifrices showed stable organoleptic characteristics and adequate physicochemical properties. CN, Ec, Ps, Pl and PC showed low abrasiveness. There was a significant difference between the groups ($p<0.001$) for microbial load, metabolic activity and biofilm viability. Conclusions: It was concluded that the BvK, Ec and Ps dentifrices are useful for cleaning complete dentures, as they have antimicrobial activity against biofilm. The dentifrices containing *Bowdichia virgilioides Kunth* showed medium abrasiveness and should be used with caution.

Keywords: Complete denture. Denture base resins. Dentifrices. Biofilms. Denture cleaners. Essential oils.

Corresponding address:
Andrezza Cristina Moura dos Santos
Universidade de São Paulo - Faculdade de Odontologia de Ribeirão Preto - Avenida do Café, s/n - Campus da USP - Ribeirão Preto - 14040-904 - Brasil. Phone: (+55 16) 99614-4218
e-mail: andrezzamoura@usp.br

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[1]Universidade de São Paulo, Faculdade de Odontologia de Ribeirão Preto, Departamento de Materiais Dentários e Prótese, Ribeirão Preto, São Paulo, Brasil.
[2]Universidade de São Paulo, Faculdade de Odontologia de Ribeirão Preto, Departamento de Odontologia Restauradora, Ribeirão Preto, São Paulo, Brasil.
[3]Universidade de São Paulo, Faculdade de Odontologia de Ribeirão Preto, Departamento de Materiais Dentários e Prótese, Ribeirão Preto, São Paulo, Brasil.

Introduction

The biofilm on complete dentures is composed of bacterial and fungal species[1] that are capable of promoting infections in the oral cavity and compromising the general health of individuals. Brushing is a hygiene method commonly proposed for complete dentures, as it is a simple, low-cost method that can promote biofilm disorganization and clean the prosthetic surface.[2,3,4]

Complete dentures are made of heath-polymerized acrylic resin (polymethylmethacrylate-PMMA) and have the advantages of ease of processing, biocompatibility and aesthetics.[5] However, it has been reported in the literature that over time and due to inadequate use of hygiene methods, this type of resin changes color and undergoes alterations in mechanical resistance, porosity and surface abrasion, which favors the accumulation of biofilm[6] Therefore, to improve the effectiveness of brushing, it is necessary to use toothpastes that have low abrasiveness and promote biofilm removal, antimicrobial action and surface polishing. Conventional toothpastes typically do not meet all of these requirements.[7,8,9,10]

To find alternatives to commercial dentifrices, research has been carried out to obtain specific formulations for complete dentures with promising results in terms of abrasiveness classification and antimicrobial activity against all microorganisms.[7-12,13,14,15] Specific dentifrices must have effective antimicrobial activity against multispecies biofilms, since denture biofilm is complex,[16] and must show stability over time.[17,18,19]

Natural products have been used in different medical fields.[20,21,22] In Dentistry, *Copaiba officinalis*, *Melaleuca alternifólia*, *Eucalyptus citriodora*, *Ricinus communis*, *Bowdichia virgilioides Kunth and Pinus strobus* have been used in the composition of dentifrices with anti-inflammatory, antioxidant activity[10,23,24] and have shown antimicrobial activity against *Streptococcus* spp.,[23] *Candida* spp.[24] and multispecies biofilm formed by *Streptococcus mutans*, *Staphylococcus aureus* and *Candida albicans.*[10]

Despite the promising results obtained with dentifrices for complete dentures based on natural products,[2,9,10,20] there is a need to improve such formulations[10] by modifying the concentrations of essential oils and components, with the aim of expanding the spectrum of action against relevant microorganisms from denture biofilm and adapting their organoleptic and physicochemical properties, which can contribute to innovation and the prevention of infections in the field of complete denture hygiene. Therefore, in this study, dentifrices based on five essential oils (*Bowdichia virgilioides Kunth, Copaifera officinalis, Eucalyptus citriodora, Melaleuca alternifolia* and *Pinus strobus*) were formulated and evaluated for organoleptic properties (appearance, color, odor, flavor), physicochemical properties (pH, density, consistency, rheology, abrasiveness [mass loss]) and activity against multispecies biofilms of *Streptococcus mutans*, *Staphylococcus aureus*, *Candida albican*s and *Candida glabrata*. The null hypothesis was that the experimental dentifrices would have properties similar to the negative control.

Methodology

The dentifrices were obtained (Laboratory of Pharmacognosy, Department of Pharmaceutical Sciences - School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo) and evaluated (Laboratory of Oral Rehabilitation, Department of Dental Materials and Prosthodontics, School of Dentistry of Ribeirão Preto, University of São Paulo) in accordance with previous methodology.[10] Five denture-specific dentifrice formulations were obtained with 1% natural extracts: *Bowdichia virgilioides Kunth* (BvK), *Copaifera officinalis* (Co), *Eucalyptus citriodora* (Ec), *Melaleuca alternifolia* (Ma) and *Pinus strobus* (Ps). All oils were produced by steam distillation or cold pressing (Figure 1). The dentifrices were prepared according to the previously mentioned methodology. Hydroxyethyl cellulose, glycerin, EDTA, sodium saccharin, essential oils and water were briefly homogenized and left to stand until gel formation. The other components were then added and mixed with the gel. After obtaining a homogeneous dentifrice, it was dispensed and stored in appropriate tubes. The dentifrices were stored in white aluminum tubes identified by numbers (GP Pharma, São José do Rio Preto, SP, Brazil) by a researcher who was not involved in the following steps of the evaluation.

Organoleptic and physicochemical properties

After obtaining the dentifrices, the organoleptic properties were evaluated at time intervals of 15, 30, 60 and 90 days after the initial assessment.[17,25]

Four blinded examiners evaluated the organoleptic properties (color, odor, flavor and appearance) and one examiner evaluated the physicochemical properties (density, consistency, pH, rheological features and abrasiveness), in accordance with Santos, et al.[10] (2021). For abrasiveness, heat-polymerized acrylic resin specimens (90 mm × 30 mm × 4 mm; n=96) (Clássico, Artigos Odontológicos Ltda., São Paulo, SP, Brazil) were obtained by the conventional method of inclusion, packaged, water polymerized and polished.[4] They were then divided into eight groups (n=12): 1. Distilled water (Negative Control - NC); 2. Placebo dentifrice (without essential oil - Pl); 3. Trihydral dentifrice (Perland Pharmacos Ltd., Brazil; Positive Control - PC); 4. BvK dentifrice; 5. Co dentifrice; 6. Ec dentifrice; 7. Ma dentifrice; and 8. Ps dentifrice. Brushing was performed with a mechanical machine (Mavtec, Ribeirão Preto, SP, Brazil) and soft brushes (Tek; Johnson & Johnson, Brazil) for 125 minutes (44,500 cycles), simulating 2.5 years of brushing. The samples were weighed before and after brushing, and the change in mass (mg) was classified as low (up to 20 mg), medium (from 21 to 40 mg) or high (over 41 mg).[6,10,12,26]

Evaluation of the Antimicrobial Activity of Specific Dentifrices

A multispecies biofilm model consisting of *Streptococcus mutans* (Sm - ATCC 25175), *Staphylococcus aureus* (Sa - ATCC 6538), *Candida albicans* (Ca - ATCC 90028) and *Candida glabrata* (Cg - ATCC 2001) was used. Antimicrobial activity was evaluated by reduction of microbial load in terms of colony-forming units per mL (CFU/mL), biofilm metabolic activity (XTT assays) and cell viability (epifluorescence microscopy). The strains were obtained from the collection of the Oral Rehabilitation Laboratory of the School of Dentistry of Ribeirão Preto, University of São Paulo.

A total of 160 circular samples (13 mm x 5 mm) of heat-polymerized acrylic resin (Clássico, Artigos Odontológicos Ltda., São Paulo, SP, Brazil) (n=72 for microbial load count, n=72 for biofilm metabolic activity, n=16 for cell viability and total biofilm quantification) were obtained by conventional method[10] and immersed in distilled water at 50°C for 24 hours to eliminate residual monomer. The samples were sterilized in a microwave oven (650W; power 8; 6 min; Consul Facilite, Manaus, AM, Brazil) and divided into eight brushing groups (n=9), as previously described. To compare the groups and demonstrate the sterility conditions of the samples, two additional groups (contaminated and uncontaminated - 18 specimens) were obtained.

Preparation and standardization of the inoculum, biofilm formation and contamination of the samples followed previous methodology.[10] To ensure reproducibility, the following steps were conducted at three different times, in triplicate. for each assay.

Following previous methodology, the samples were subjected to manual brushing[27] with experimental dentifrices on soft bristle brushes by a calibrated researcher. The test was performed in a laminar flow chamber (Pachane, Pa 400-ECO, Piracicaba, São

Components	Manufacturer	Function
Hydroxyethylcellulose	Fagron Rubber Industry Products Ltd.a, Guarulhos, SP, Brazil	Thickener
Glycerin	Ely Martins, Ribeirão Preto, SP, Brazil	Humectant
EDTA	Fagron P Rubber Industry Products Ltd.a, Guarulhos, SP, Brazil	Chelating Agent
Sodium benzoate	Labsynth Ltd.a, Diadema, São Paulo, SP, Brazil	Preservative
Cocamidopropyl betaine	Fagron Rubber Industry Products Ltd.a, Guarulhos, SP, Brazil	Surfactant
Oils*	Laszo Group	Antimicrobial active
	Oshadhi Brazil	
	Sítio das Melaleucas	
Silica (Tisoxil 73)	Rhodia Solvay Group, São Paulo, SP, Brazil	Abrasive
Silica (Tisoxil 43 B)	Rhodia Solvay Group, São Paulo, SP, Brazil	Thickener
Titanium dioxide	Fagron Rubber Industry Products Ltd.a, Guarulhos, SP, Brazil	Pigment (white)
Menthol aroma	Givaudan of Brazil Ltd.a, São Paulo, SP, Brazil	Flavoring
Distilled water	-	Vehicle

**Bowdichia virgilioides Kunth*, *Copaifera officinalis*, *Eucalyptus citriodora*, *Melaleuca alternifólia* or *Pinus strobus*.

Figure 1- Basic composition of experimental dentifrice

Paulo, Brazil).[28] Briefly, acrylic resin plates (Clássico, Artigos Odontológicos Ltda. São Paulo, SP, Brazil) (90 x 20 mm) with three cavities (14 mm x 3 mm) were placed on top of each other in a Petri dish. Two plates were sterilized in a microwave oven (650W; power 8; 6 min; Consul Facilite, Manaus, AM, Brazil). The contaminated specimens (3) were placed in the cavities and manually brushed with soft, sterilized (ultraviolet radiation, laminar flow chamber; 1 hour) toothbrushes (Tek, Johnson & Johnson Ind. Com. Ltda., S. J. dos Campos - SP, Brazil). The brush bristles were moistened in PBS for 10 seconds and 3 mm of dentifrice were applied. Brushing was performed over the entire surface of the specimens with unidirectional and horizontal movements for 20 seconds. After brushing the first surface, the sample was rotated, and the second surface was brushed according to the established parameters. One brush was used for each set of three samples.

Microbial load count

The assay was performed according to previous methods.[9,10] After brushing and rinsing, each specimen was aseptically removed, washed three times with PBS and transferred to tubes containing 10 mL of Letheen medium (Himedia) and sonicated (200W; 40KHz) (Altsonic, Ribeirão Preto, São Paulo, Brazil) for 20 minutes. Then, serial dilutions (10^{-1} to 10^{-4}) of the resulting suspension were seeded on Petri dishes containing specific medium. After incubation in a microbiological oven (De Leo Equipamentos Laboratoriais, Porto Alegre, RS, Brazil) at 37°C for 48 hours and *S. mutans* in a microaerophilic environment in an anaerobic jar (Permution, Curitiba, PR, Brazil), the number of colonies was counted and the CFU/mL value was calculated based on the dilution that gave 1-300 colonies, according to the following equation: CFU/mL = (number of colonies) × 10 n/q, in which: n is the absolute value of the dilution (0, 1, 2 or 3) and q is the volume of the plated suspension (0.025 mL). The CFU/mL values were converted to $\log^{10}$. Several readings resulted in zero CFU counts; therefore, the microbial count data obtained were expressed as $\log^{10}$ (CFU + 1).

Biofilm metabolic activity

The metabolic activity of the microorganisms was assessed using the XTT (2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (XTT) tetrazolium salt reduction assay (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

After brushing, the specimens were washed four times with 10 mL of PBS and individually transferred to 12-well sterile plates containing XTT solutions, and the plates were incubated (dark/37°C/2 hours) in a microbiological oven. Colorimetric reading was then performed in a spectrophotometer at 492 nm, normalized to 690 nm. Quantification was performed in triplicate by spectrophotometry using a Multiskan GO microplate reader (Thermo Scientific, Waltham, MA, USA), and the mean colorimetric alterations were calculated in absorbance units. The microplate had 03 "blank" wells (PBS with glucose + XTT + Menadione), which served as a "standard" for the quantifications. The alterations were calculated by subtracting the mean of the readings of these standards from the mean of the readings of the wells of the experimental groups.

Cell viability and total biofilm quantification

Quantification of live cell area and total biofilm area was performed in duplicate using the Live/Dead® BacLight™ kit (Invitrogen, Eugene, OR, USA), which contains two fluorophores, SYTO9 (green) and propidium iodide (PI, red). After brushing and rinsing, the specimens were transferred to a new 12-well culture plate (Techno Plastic Products, Trasadingen, Switzerland). In a laminar flow chamber, in the dark, 1.5 mL of the dye mixture, 2.5 µL of component A solution (Sturayto 9 - green) and 2.5 µL of component B solution (propidium iodide - red) were pipetted into 15 mL of PBS. They were incubated for 15 minutes at room temperature in a light-protected environment, washed with PBS and again protected from light.

For each specimen, two images (excitation wavelength [nm]: 490/536, emission wavelength [nm]: 525/617) were captured in 20 fields, totaling 40 images for each group. Nikon NIS-Elements software (Nikon Microscope Solutions, Nikon Instruments Inc, Tokyo, Japan) was used for area quantification. The area marked in green corresponds to the total biofilm area. The live cell area was calculated as the difference between the green and red (dead cells) pigmented areas. The percentages (%) were calculated using the following equations:

Live cell % = (Live cell area/Total image area) × 100;

Total biofilm % = (Biofilm area/Total image area) × 100.

Statistical analysis

The organoleptic characteristics are presented by descriptive analysis. The values of density, pH, consistency and viscosity are presented in a table. The data were analyzed using the Wald test in a generalized linear model, followed by the Kruskal-Wallis test, Dunn's test (Mass Change) and the Bonferroni test (UFC and XTT). The Wald test in Generalized Estimating Equations and the Bonferroni test were used to analyze cell viability. All analyses were performed at a 5% significance level using IBM SPSS Statistics for Windows 21.0 software (IBM Corp.).

Results

Organoleptic and physicochemical properties

The experimental dentifrices showed no changes in organoleptic properties and were classified as "normal" at baseline (day 0) and after 15, 30, 60 and 90 days, demonstrating stability. Table 1 shows the physicochemical properties (density, pH, consistency and rheological properties). The results showed that for density and consistency, the highest values were those of the Pl dentifrice and the lowest values were those of the Ps dentifrice. All dentifrices showed pH (>7), but the highest pH values were those of the Ma and Co dentifrices and the lowest values were those of the Pl dentifrice. The data on rheological properties were obtained by viscosity values (ascending and descending curves) that allowed the hysteresis area to be formed. The values obtained for the hysteresis area were higher for Ma and lower for BvK. There were significant differences in abrasiveness between the groups ($p<0.001$). The NC group showed the lowest mass loss, while Ma and Co showed the highest abrasiveness. NC, Eu, Ps, Pl and PC showed low abrasiveness (Table 2).

Microbial load

PC was the most effective against *S. mutans* and *S. aureus*. Compared to the contaminated group, the microbial load of the three microorganisms was reduced in all groups. Compared to the NC group, there was microbial load of *S. mutans* when using the

Table 1- Physical-chemical properties of dentifrices

Groups	Density (g/mL)	pH	Consistency (mm)	Viscosity		Hysteresis Area
				Curve Ascending	Curve Downward	
Pl	1.25	7.04	57.33	387.654.321	323.045.267	1.70
BvK	1.11	7.37	43.00	484.567.901	516.872.428	0.60
Co	1.16	7.16	44.33	581.481.481	678.395.062	3.40
Ec	1.14	7.24	42.33	516.872.428	549.176.955	1.02
Ma	1.15	7.38	43.67	387.654.321	323.045.267	4.33
Ps	1.05	7.19	40.00	1.938.271.605	2.067.489.712	0.84

Table 2- Descriptive statistics of mass loss (mg) and statistical comparisons

Groups	Mean	SD	Median	95% CI (Range)	p Value *	Abrasiveness classification
NC	-0,08[a]	0.25	-0.10	-24.55 / -0.07	<0.001	Low
Eu	-10,84[ab]	5.79	-11.70	-14.52 / -7.16		Low
Ps	-10,90[ab]	7.04	-9.55	-15.37 / -6.42		Low
Pl	-12,31[ab]	3.33	-13.00	-14.43 / -10.19		Low
PC	-19,70[bc]	17,33	-16.35	-30.71 / -8.8		Low
BvK	-25,77[bc]	11.99	-21.90	-33.39 / -18.15		Medium
Ma	-26,46[c]	8.10	-28.00	-31.61 / -21.31		Medium
Co	-28,47[c]	5.86	-29.35	-32.20 / -24.74		Medium

Kruskal-Wallis test followed by the Dunn test*; SD: Standard deviation; CI–Confidence Interval for Mean; Range (minimum; maximum); Negative Control: brushing without dentifrice (water); Positive Control: Trihydral Commercial toothpaste; equal letters indicate statistical similarity ($p > 0.05$).

Table 3- Comparison of the microbial load [log10 (CFU+1)] for *S. mutans*, *S. aureus*, *C. albicans*, and *C. glabrata*

S. mutans			***S. aureus***			***C. albicans***			***C. glabrata***		
Groups	**Mean ± SD (Median)**	**p***	**Groups**	**Mean ± SD (Median)**	**p***	**Groups**	**Mean ± SD (Median)**	**p***	**Groups**	**Mean ± SD (Median)**	**p***
	95% CI (Range)			**95% CI (Range)**			**95% CI (Range)**			**95% CI (Range)**	
PC	0.66 ± 1.02 (0.00) c -0.12;1.45 (0.00; 2.45)	<0.001	PC	3.66 ± 0.62 (3.59) c 3.18; 4.13 (2.75; 4.55)	<0.001	PC	0.84 ± 1.26 (0.00) b -0.13; 1.81 (0.00; 2.64)	<0.001	PC	1.20 ± 1.21 (1.61) b 0.27; 2.13 (0.00; 2.90)	<0.001
NB	4.36 ± 0.36 (4.42) bc 4.08; 4.63 (3.68; 4.86)		NB	7.23 ± 0.50 (7.24) a 6.85; 7.62 (6.65; 7.99)		NB	4.51 ± 0.53 (4.63) a 4.10; 4.92 (3.83; 5.20)		NB	4.15 ± 0.74 (3.74)a 3.58; 4.72 (3.37; 5.26)	
PI	2.94 ± 0.39 (3.03) bc 2.64; 3.24 (2.38; 3.37)		PI	5.73 ± 0.33 (5.70) bc 5.48; 5.98 (5.41; 6.30)		PI	0.72 ± 1.08 (0.00) b -0.11;1.55 (0.00; 2.30)		PI	1.53 ± 1.21 (2.08) b 0.60; 2.45 (0.00; 3.08)	
NC	3.59 ± 0.40 (3.73) ab 3.28; 3.90 (2.94; 4.05)		NC	5.84 ± 0.60 (5.84) ab 5.38; 6.30 (4.70; 6.64)		NC	1.56 ± 1.25 (1.91) ab 0.60; 2.51 (0.00; 3.02)		NC	2.35 ± 0.27 (2.38) ab 2.14; 2.56 (1.91; 2.72)	
Ma	3.18 ± 1.31 (3.49) ab 2.18; 4.19 (0.00; 4.82)		Ma	6.04 ± 0.73 (6.08) ab 5.47; 6.60 (4.96; 7.08)		Ma	0.86 ± 1.09 (0.00) b 0.02; 1.70 (0.00; 2.90)		Ma	1.02 ± 1.25 (0.00) b 0.05; 1.98 (0.00; 2.81)	
BvK	3.25 ± 0.94 (3.44) ab 2.53; 3.97 (1.91; 4.70)		BvK	5.21 ± 1.39 (5.68) bc 4.14; 6.28 (1.61; 6.18)		BvK	0.89 ± 1.07 (0.00) b 0.06; 1.71 (0.00; 2.38)		BvK	1.19 ± 1.14 (1.91) b 0.94; 2.58 (0.00; 2.88)	
Co	3.30 ± 0.44 (3.30) ab 2.97; 3.64 (2.56; 4.09)		Co	5.69 ± 0.34 (5.72) bc 5.43; 5.95 (5.02; 6.11)		Co	1.45 ± 1.11 (2.08) ab 0.59; 2.30 (0.00; 2.45)		Co	1.76 ± 1.06 (2.08) ab 0.87; 2.61 (0.00; 3.60)	
Ps	2.79 ± 0.62 (2.51) bc 2.31; 3.26 (2.08; 3.69)		Ps	5.59 ± 0.63 (5.60) bc 5.11; 6.08 (4.81; 6.66)		Ps	0.68 ± 1.05 (0.00) b -0.13; 1.49 (0.00; 2.60)		Ps	0.78 ± 1.18 (0.00) b -0.13; 1.69 (0.00; 2.83)	
Ec	3.13 ± 0.65 (3.27) abc 2.63; 3.63 (1.61; 3.71)		Ec	5.49 ± 0.67 (5.71) bc 4.97; 6.00 (3.83; 6.20)		Ec	0.65 ± 1.01 (0.00) b -0.13; 1.42 (0.00; 2.60)		Ec	1.74 ± 1.13 (1.91) ab 0.87; 2.61 (0.00; 3.60)	

Wald test followed by the Bonferroni test*; SD: Standard deviation; CI–Confidence Interval for Mean; Range (minimum; maximum); Negative Control: brushing without dentifrice (water); Positive Control: Trihydral Commercial toothpaste; equal letters indicate statistical similarity ($p > 0.05$).

Table 4- Comparison of the metabolism activity of the biofilm

	Mean ±SD (Median)	**CI (Minimum; Maximum)**
CP	0.00±0.01 (0.00)^{c}	0.00; 0.01 (0.00; 0.01)
Eu	0.20±0.11 (0.17)^{bc}	0.11; 0.28 (0.08; 0.38)
PI	0.30±0.21 (0.40)^{bc}	0.14; 0.47 (0.04; 0.62)
BvK	0.30±0.14 (0.28)^{bc}	0.20; 0.40 (0.16; 0.51)
Co	0.48±0.23 (0.42)^{ab}	0.31; 0.65 (0.26; 0.93)
Ma	0.50±0.25 (0.49)^{ab}	0.31; 0.70 (0.21; 0.84)
CN	0.5 ±0.37 (0.37)^{ab}	0.24; 0.82 (0.20; 1.27)
Ps	0.54±0.20 (0.47)^{ab}	0.39; 0.69 (0.34; 1.00)
NB	2.61±0.40 (2.72)^{a}	2.31; 2.92 (1.74; 2.97)

Wald test followed by the Bonferroni test*; SD: Standard deviation; CI–Confidence Interval for Mean; Range (minimum; maximum); equal letters indicate statistical similarity ($p > 0.05$).

Table 5- Comparison of the cell viability

	Total Biofilm %					Live cell %				
	Mean	SD	Median	CI		Mean	SD	Median	CI	
NB	26.84	22.03	16.00^{Aa}	16.53	37.15	2.48	4.55	0.00^{Ba}	0.35	4.61
CN	2.95	2.93	1.80^{Ab}	1.57	4.32	2.00	2.46	1.00^{Aa}	0.85	3.15
Pl	1.38	1.69	1.00^{Abc}	0.59	2.17	0.32	0.69	0.05^{Aa}	-0.01	0.64
CP	0.56	0.45	0.40^{Ac}	0.34	0.77	0.38	0.45	0.10^{Aa}	0.16	0.59
BvK	0.58	0.48	0.35^{Ac}	0.36	0.80	0.30	0.29	0.20^{Ba}	0.16	0.44
Co	1.03	1.95	0.40^{Abc}	0.12	1.94	0.28	0.67	0.10^{Aa}	-0.03	0.59
Eu	0.99	1.80	0.25^{Abc}	0.14	1.83	0.02	0.04	0.00^{Ba}	0.00	0.03
Ma	0.81	0.80	0.45^{Abc}	0.43	1.18	0.32	0.63	0.05^{Aa}	0.03	0.61
Ps	0.58	1.23	0.10^{Ac}	0.00	1.15	0.28	1.01	0.00^{Ba}	-0.20	0.75

Wald test in Generalized Estimating Equations and Bonferroni test*; SD: Standard deviation; CI–Confidence Interval for Mean; Range (minimum; maximum); “A” compare columns and “a” compare lines; equal letters indicate statistical similarity (p > 0.05).

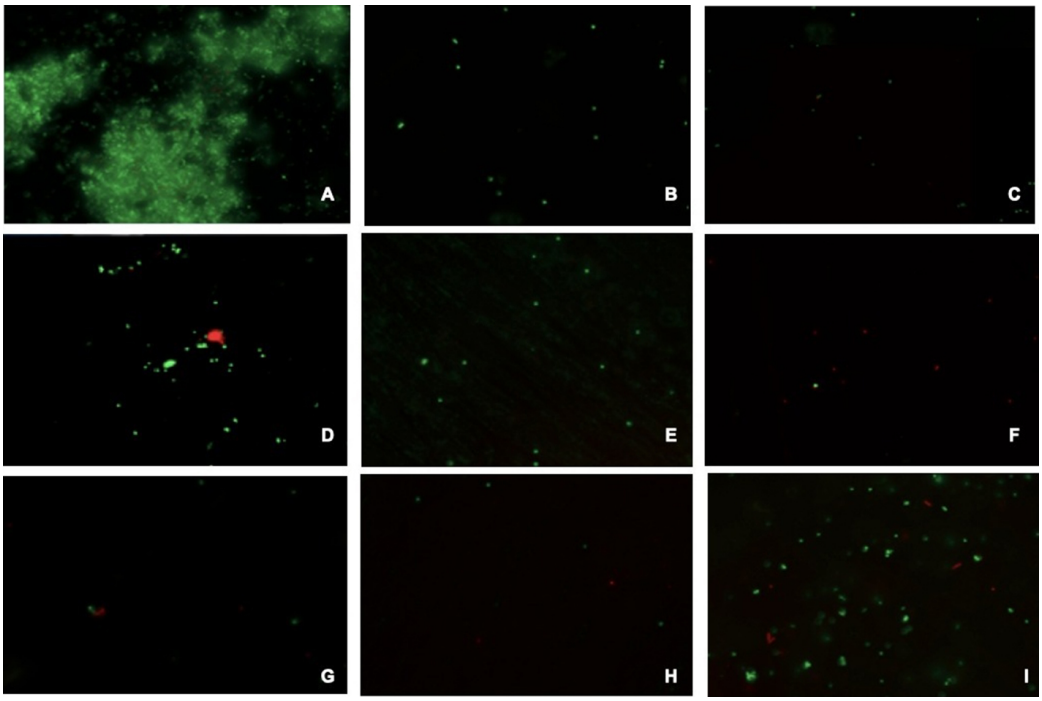

Figure 2- Illustrative sequence of images of the total biofilm (live cells in green and dead cells in red) on the surface of the heat-polymerized acrylic resin: NB (A), NC (B), PC (C), Placebo Dentifrice (D), *Bowdichia virgilioides* Kunth Dentifrice (E), *Copaifera officinalis* Dentifrice (F), *Eucalyptus citriodora* Dentifrice (G), *Melaleuca alternifolia* Dentifrice (H), *Pinus strobus* Dentifrice (I)

dentifrices Pl, Ec and Ps; of *S. aureus* when using Pl, Bvk, Ec and Ps; and of *C. albicans* and *C. glabrata* when using Pl, BvK, Ec, Ma and Ps (Table 3).

Biofilm metabolic activity (XTT assay)

The Trihydral (PC) dentifrice was the most efficient in reducing biofilm metabolic activity, followed by the Eu, Pl and BvK dentifrices. Co, Ma and Ps showed intermediate action between Eu, Pl and BvK and the no brushing group, which showed greater metabolic activity.

Cell viability by epifluorescence microscopy

The dentifrices with BvK and Ps were similar to PC in reducing the total biofilm (p= 1.000). There was no significant difference between the groups in terms of live cells. In the NB, BvK, Eu and Ps groups, there was a difference between the percentage of total biofilm and the percentage of live cells. There was no significant difference between the groups in terms of live cells (Table 5). Figure 2 shows an illustrative sequence of the images obtained by epifluorescence microscopy.

Discussion

The null hypothesis was partially accepted, as the experimental toothpastes were found to have adequate organoleptic and physicochemical properties, promoting low or moderate abrasiveness; however, not all the toothpastes were similar to the positive control in terms of reducing microbial load, metabolic activity and biofilm viability.

Over 90 days, the organoleptic properties indicated that the formulations were stable[10] and suitable for clinical use.[17] The feasibility of using the formulations depends on physicochemical properties. The evaluation of physicochemical properties indicated that the dentifrices were suitable for use in the cleaning of dentures.[10,17] The values obtained for density and consistency were acceptable for dentifrices.[7,13] The pH values (>7) obtained were considered safe for toothpastes, as the dentifrices had a natural pH characteristic of neutral products.[7,10,13] An acidic pH influences the viscosity and action of active ingredients. In terms of rheological properties, the dentifrices had low viscosity, considered suitable for denture toothpastes. The values obtained for the hysteresis area showed moderate degree of thixotropy and rate of active ingredient release.[19]

The hysteresis area indicates the thixotropy of a material. Specifically in relation to dentifrices, thixotropy with a moderate hysteresis area may be desirable to facilitate brushing, uniform distribution of the product and its active ingredients, and to allow the product to adhere to the surface after brushing.[19] To our knowledge, there are no standard values for the ideal amount of thixotropy, thus, the relationship between viscosity and hysteresis area depends on the objectives and indication for use of the product. The *Copaifera officinalis* and *Melaleuca alternifolia* dentifrices showed the largest areas of hysteresis, which may indicate greater difficulty in terms of spreading during use and uniform distribution of the active ingredient. Regarding abrasiveness, the *Eucalyptus citriodora* and *Pinus Strobus* dentifrices showed the lowest values of mass loss compared to the positive control toothpaste. However, the Eu, Ps, Pl and Trihydral dentrifices were classified as having low abrasiveness,[6,10,12,26] favoring less biofilm accumulation.

The multispecies biofilm was used to promote a complex configuration of the biofilm in terms of its formation. The results showed that there was a reduction in the microbial load of all microorganisms after the use of the dentifrices compared to the group that was contaminated but not exposed to brushing, which suggests that brushing, regardless of the auxiliary agent, promotes the reduction of biofilm.[13] The Trihydral dentifrice was the most promising, promoting a reduction in microbial load, in cellular metabolism and in the percentage of total biofilm and live cells. This dentifrice contained Chloramine T, which is capable of destroying cellular material or interrupting cellular processes important for the survival of microorganisms via oxidative reactions.[14,15] It also reduces the formation of pseudohyphae and inhibits the formation of chlamydospores,[29] promoting oxidative reactions that allow micromorphological changes in fungal strains and are fundamental for reducing the metabolic activity of cells.

The dentifrices containing *Eucalyptus citriodora*, *Bowdichia virgilioides Kunth* and *Pinus strobus* showed good antimicrobial results compared to Trihydral. The activity potential of oils may be related to the action of molecules on the cell membrane and the promotion of changes in permeability, as well as the presence of flavonoids that inhibit microbial growth.[30] *Eucalyptus* essential oil contains compounds such as aldehyde and citronellal, so the interaction between them and the nitrogen present in proteins and nucleic acids inhibits the growth of microorganisms.[20] The antibacterial activity of the tannins, flavonoids and alkaloids present in BvK essential oil are responsible for the antimicrobial effect on the biofilm. *Pinus strobus* (Ps) essential oil contains the bioflavonoid Pinostrobin, an agent with potential antimicrobial effect.[16,21,30] However, Ps showed regular results in terms of metabolic activity, with intermediate values between the no brushing group and Co and Ma.

The dentifrices containing *Eucalyptus citriodora, Bowdichia virgilioides Kunth (BvK)* and *Pinus strobus (Ps)* showed good antimicrobial results compared to Trihydral (PC). Although we did not find significant differences in the percentage of live cells among the groups, which can be considered a negative result, all groups showed a reduction in the total percentage of biofilm, which this can impact the microbial load and therefore the virulence of biofilm. Furthermore, BvK and PS dentifrices promoted a similar reduction in the total percentage of biofilm compared to the PC group, which this can be considered a good result.

The effects in terms of microbial load reduction were more significant for *C. albicans* and *C. glabrata*. This result is important because *C. albicans* is the most prevalent microorganism in the biofilm on complete dentures, followed by *C. tropicalis* and *C. glabrata*, species directly associated with the presence of denture stomatitis.[1] Components such as caryophyllene oxide, trans-caryophyllene, spathulenol, &-pinene and humulene, present in some essential oils, interact with the lipid bilayer of the fungal cell membrane, causing an increase in cell permeability and leakage of intracellular contents, resulting in the death of the microorganism.[22]

Regarding cell viability, it is important to highlight that the results indicate that there was a significant difference between the percentage of total biofilm and live cells only in NB, BvK, Eu and Ps. The result of the NB group was not expected, but a hypothesis can be raised due to the large cell aggregate that may have impacted cell viability. For the other groups, brushing with toothpastes or water may have caused a reduction in the percentage of biofilm but did not affect the viability of the remaining cells.

The brushing pattern proved to be a limitation of the study, as individual brushing patterns cannot be replicated in the laboratory. Future clinical studies capable of identifying the biofilm pattern collected from complete denture users, as well as antimicrobial evaluation of dentifrices, are needed to confirm these laboratory findings. It is important to highlight that the formulations analyzed in this study were evaluated for their organoleptic and physical-chemical properties, as well as their antimicrobial activity. The literature shows that essential oils have a non-cytotoxic effect, considering the wide use of oils in personal hygiene and medical products.[9,10,20,22,23,24,30] Therefore, in this study, no cytotoxicity test was performed for the oils evaluated. The presence of significant results for the placebo toothpaste also proved to be a limitation. However, in the literature, the shear stress resulting from the mechanical brushing process is considered to be the main factor responsible for reducing biofilm,[2,3,8] and this effect can also be enhanced by the presence of components such as abrasives or detergents in the toothpaste.[10] Future research should also evaluate the mechanism of action of essential oils, as existing commercial formulations have safe synthetic active ingredients with a well-studied mechanism of action.

Conclusion

Eucalyptus citriodora, Bowdichia virgilioides Kunth and *Pinus strobus* dentifrices showed adequate organoleptic and physicochemical properties, in addition to promising anti-biofilm activity. The *Bowdichia virgilioides Kunth* dentifrices showed medium abrasiveness and should be used with caution

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

All data generated and analyzed in this study are included in this published article.

Authors' contributions

Santos, Andrezza: Formal analysis (Equal); Investigation (Equal); Methodology (Equal); Writing – review & editing (Equal). **Pompeo, Fernanda Thaís:** Investigation (Equal); Methodology (Equal); Writing – review & editing (Equal). **Mendes, Filipe Ferreira:** Investigation (Equal); Methodology (Equal). **Watanabe, Evandro:** Investigation (Equal); Writing – review & editing (Equal). **Macedo, Ana Paula:** Data curation (Equal). **Pagnano, Valeria:** Validation (Equal); Writing – review & editing (Equal). **Paranhos, Helena de Freitas Oliveira:** Conceptualization (Equal); Formal analysis (Equal); Funding acquisition (Equal); Project administration (Equal); Writing – original draft (Equal); Writing – review & editing (Equal). **Silva-Lovato, Cláudia** Conceptualization (Equal); Formal analysis (Equal); Funding acquisition (Equal); Project administration (Equal); Writing – original draft (Equal); Writing – review & editing (Equal).

References

1- Budtz-joggensen, E. The significance of *Candida albicans* in denture stomatitis. Scand J Dent Res. 1974;82:151-90.

2- Paranhos HF, Silva-Lovato CH, Souza RF, Cruz PC, Freitas KM, Peracini A. Effects of mechanical and chemical methods on denture biofilm accumulation. J Oral Rehabil. 2007;34(8):606-12. doi: 10.1111/j.1365-2842.2007.01753.x

3- Salles MM, Oliveira VC, Macedo AP, Nascimento C, Silva-Lovato CH, Paranhos HF. Brushing associated with oral irrigation in maintaining implants and overdentures hygiene - a randomized clinical trial. Odontology. 2021;109(1):284-94. doi: 10.1007/s10266-020-00543-7

4- Panariello BH, Izumida FE, Moffa EB, Pavarina AC, Jorge JH, Giampaolo ET. Effect of mechanical toothbrushing combined with different denture cleansers in reducing the viability of a multispecies biofilm on acrylic resins. Am J Dent. 2016;29(3):154-60.

5- Nejatian T, Nathwani N, Taylor L, Sefat F. Denture base composites: effect of surface modified nano- and micro-particulates on mechanical properties of polymethyl methacrylate. Materials (Basel). 2020;13(2):307. doi: 10.3390/ma13020307

6- Sorgini DB, Silva-Lovato CH, Muglia VA, Souza RF, Arruda CN, Paranhos HF. Adverse effects on PMMA caused by mechanical and combined methods of denture cleansing. Braz Dent J. 2015;26(3):292-6. doi: 10.1590/0103-6440201300028

7- Panzeri H, Lara EH, Paranhos HF, Lovato da Silva CH, Souza RF, Souza Gugelmin MC, et al. *In vitro* and clinical evaluation of specific dentifrices for complete denture hygiene. Gerodontology. 2009;26(1):26-33. doi: 10.1111/j.1741-2358.2008.00235.x

8- Andrade IM, Silva-Lovato CH, Souza RF, Pisani MX, Andrade KM, Paranhos HF. Trial of experimental toothpastes regarding quality for cleaning dentures. Int J Prosthodont. 2012;25(2):157-9.

9- Leite VM, Pinheiro JB, Pisani MX, Watanabe E, Souza RF, Paranhos HF, et al. *In vitro* antimicrobial activity of an experimental dentifrice based on *ricinus communis*. Braz Dent J. 2014;25(3):191-6. doi: 10.1590/0103-6440201302382

10- Santos AC, Oliveira VC, Macedo AP, Bastos JK, Ogasawara MS, Watanabe E, et al. Effectiveness of oil-based denture dentifrices-organoleptic characteristics, physicochemical properties and antimicrobial action. Antibiotics (Basel). 2021;10(7):813. doi: 10.3390/antibiotics10070813

11- André RF, Andrade IM, Silva-Lovato CH, Paranhos HF, Pimenta FC, Ito IY. Prevalence of *mutans streptococci* isolated from complete dentures and their susceptibility to mouthrinses. Braz Dent J. 2011;22(1):62-7. doi: 10.1590/s0103-6440201100010001

12- Pisani MX, Bruhn JP, Paranhos HF, Silva-Lovato CH, Souza RF, Panzeri H. Evaluation of the abrasiveness of dentifrices for complete dentures. J Prosthodont. 2010;19(5):369-73. doi: 10.1111/j.1532-849X.2010.00592.x

13- Paranhos HF, Panzeri H, Lara EH, Candido RC, Ito IY. Capacity of denture plaque/biofilm removal and antimicrobial action of a new denture paste. Braz Dent J. 2000;11(2):97-104.

14- Masten S, Haneke KE. Toxicological summary for Chloramine-T [127-65-1] and p - Toluenesulfonamide [70-55-3] [internet]. 2002 [cited 2024 Feb 27]. Available from: https://ntp.niehs.nih.gov/ntp/htdocs/chem_background/exsumpdf/chloraminet_508.pdf

15- Badaró MM, Bueno FL, Makrakis LR, Araújo CB, Oliveira VC, Macedo AP, et al. Action of disinfectant solutions on adaptive capacity and virulence factors of the *Candida spp*. biofilms formed on acrylic resin. J Appl Oral Sci. 2021;29:e20210024. doi: 10.1590/1678-7757-2021-0024

16- Nedumgottil BM. Relative presence of *Streptococcus mutans*, *Veillonella atypica*, and *Granulicatella adiacens* in biofilm of complete dentures. J Indian Prosthodont Soc. 2018;18(1):24-8. doi: 10.4103/jips.jips_183_17

17- Brazil. National Health Surveillance Agency. Cosmetic products stability guide [Internet]. 1st ed. Brasília: ANVISA; 2004 [cited 2024 Feb 27]. v. 1. Theme Series. Available from: http://antigo.anvisa.gov.br/documents/106351/107910/Cosmetic+Products+Stability+Guide/5f90ee5b-c77b-4c1e-91f9-5fa680b05022

18- Maslii Y, Ruban O, Kasparaviciene G, Kalveniene Z, Materiienko A, Ivanauskas L, et al. The Influence of pH values on the rheological, textural and release properties of carbomer polacril® 40P-based dental gel formulation with plant-derived and synthetic active components. Molecules. 2020;25(21):5018. doi: 10.3390/molecules25215018

19- Meyer AC, Naressi SC, Conde IP. Manual manipulated formulations dentistry. São Paulo: Ed. Santos; 2007.

20- Polito F, Fratianni F, Nazzaro F, Amri I, Kouki H, Khammassi M, et al. Essential oil composition, antioxidant activity and leaf micromorphology of five tunisian *eucalyptus* species. Antioxidants. 2023;12(4):867. doi: 10.3390/antiox12040867

21- Patel NK, Jaiswal G, Bhutani KK. A review on biological sources, chemistry and pharmacological activities of pinostrobin. Nat Prod Res. 2016;30(18):2017-27. doi: 10.1080/14786419.2015.1107556

22- Degroot A, Schmidt E. Essential oils: contact allergy and chemical composition. 1st ed. Boca Raton, FL: CRC Press; 2021.

23- Simões CA, Conde NC, Venâncio GN, Milério PS, Bandeira MF, Veiga VF Jr. Antibacterial activity of copaiba oil gel on dental biofilm. Open Dent J. 2016;10:188-95. doi: 10.2174/1874210601610010188

24- Wiatrak K, Morawiec T, Rój R, Kownacki P, Nitecka-Buchta A, Niedzielski D, et al. Evaluation of effectiveness of a toothpaste containing tea tree oil and ethanolic extract of propolis on the improvement of oral health in patients using removable partial dentures. Molecules. 2021;26(13):4071. doi: 10.3390/molecules26134071

25- Brazil. National Health Surveillance Agency. Brazilian pharmacopeia National Form [Internet]. 2nd ed. Brasília: ANVISA; 2012 [cited 2024 Feb 24]. Available from: https://www.gov.br/anvisa/pt-br/assuntos/farmacopeia/formulario-nacional/arquivos/8071json-file-1

26- Sorgini DB, Silva-Lovato CH, Souza RF, Davi LR, Paranhos HF. Abrasiveness of conventional and specific denture-cleansing dentifrices. Braz Dent J. 2012;23(2):154-9. doi: 10.1590/s0103-64402012000200011

27- Badaró MM, Leite-Fernandes VM, Martin LT, Oliveira VC, Watanabe E, Paranhos HF, et al. Antibiofilm activity of an experimental *ricinus communis* dentifrice on soft denture liners. Braz Dent J. 2019;30(3):252-8. doi:10.1590/0103-6440201902326

28- Badaró MM, Salles MM, Arruda CN, Oliveira VC, Souza RF, Paranhos HF, et al. *In vitro* analysis of surface roughness of acrylic resin exposed to the combined hygiene method of brushing and immersion in *ricinus communis* and sodium hypochlorite. J Prosthodont. 2017;26(6):516-21. doi: 10.1111/jopr.12436

29- Gow NA, Brown AJ, Odds FC. Fungal morphogenesis and host invasion. Curr Opin Microbiol. 2002;5(4):366-71. doi: 10.1016/s1369-5274(02)00338-7

30- Cui T, Luo W, Xu L, Yang B, Zhao W, Cang H. Progress of antimicrobial discovery against the major cariogenic pathogen *streptococcus mutans*. Curr Issues Mol Biol. 2019;32:601-44. doi: 10.21775/cimb.032.601