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Denture disinfection using *Salvia officinalis L.*: microbial load and selected properties of PMMA

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Denture use may aggravate the occurrence of oral infections, considering it enhances microbial adherence. **Aim:** This study assessed the reduction of microbial loads of *Candida albicans*, *Staphylococcus aureus*, and *Klebsiella oxytoca* by disinfecting the polymethylmethacrylate (PMMA) of complete dentures with hydroalcoholic extract of *Salvia officinalis*. Additionally, the effect of such extract on the properties of PMMA was examined. **Methods:** Microorganisms were isolated from saliva samples collected from complete denture wearers. The hydroalcoholic extract of *S. officinalis* was produced according to the Brazilian Pharmacopoeia 5. The PMMA specimens (n=188) were immersed in microbial inoculum and incubated at 37°C for 16 hours per day. Then, they were subjected to a disinfection protocol for 30 days. The specimens were divided into five treatment groups: sterile saline solution (0.85%; control), 0.2% chlorhexidine digluconate, and hydroalcoholic extract of *S. officinalis* (0.2%, 0.8%, and 1.16%). Microorganism adherence to the PMMA surface was also assessed, as well as surface roughness (Ra in μm) and color stability of the PMMA (mean ΔE). Changes in microbial load and surface roughness after the disinfection protocol were verified with paired t-test. Substances at day 10, adherence, and color stability were compared by the Kruskall-Wallis and Mann-Whitney tests, and one-way ANOVA was used to compare substances at the beginning and end of the experiment (α=0.05). **Results:** The 1.16% *S. officinalis* extract significantly reduced the microbial load of all the microorganisms after 30 days of disinfection ($p<0.05$). The microbial load of *K. oxytoca* was also reduced at lower concentrations of the *S. officinalis* extract (0.2% and 0.8%) ($p<0.02$). Antimicrobial and anti-adherent effects against microorganisms isolated from the oral cavity were observed. There was no significant change in surface roughness ($p>0.05$) and color stability was significantly higher in the control group ($p<0.0001$). **Conclusions:** The hydroalcoholic extract of *S. officinalis* may be used as a disinfectant solution for dentures.

Keywords: Colony count, microbial. Denture cleansers. Plant extracts. Polymethyl Methacrylate.

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Introduction

Denture use may aggravate the occurrence of oral infections, considering it enhances microbial adherence, particularly in poorly sanitized conditions[1]. Among the pathogens particularly implicated in the etiology of oral infections, gram-positive cocci, enterobacteria, and *Candida albicans* have been frequently associated with several types of infections in denture wearers[2-4]. Disinfecting dentures is essential to reduce the number of microorganisms present on their surface and consequently to reduce the incidence of oral infections[5].

Microorganism adherence occurs through biofilm formation, especially on surfaces kept in direct contact with the oral mucosa[6]. Different methods have been proposed to disinfect the polymethylmethacrylate (PMMA) of dentures. Professional cleaning alternatives involve ultrasonic bath, immersion in glutaraldehyde, sodium hypochlorite, or alcohol-based solutions, and microwaving[7,8]. Some of these disinfection methods, although effective, may have a negative effect on the properties of PMMA[8].

Overnight denture immersion in cleaning solutions has also been indicated for disinfecting purposes. Immersion in chlorhexidine digluconate, alkaline peroxide, and sodium hypochlorite has been shown to produce a significant disinfecting effect[9,10]. However, the long-term contact of PMMA with these solutions has shown significant denture discoloration[10,11].

Therefore, it may be important to acknowledge natural substances with antimicrobial potential to work as denture disinfectants. These substances should be easily obtained and widely used by the population.[12] Plant extracts have shown significant results regarding antimicrobial potential in different conditions[3,12,13].

In Brazil, the use of medicinal plants is common. Among the various species within the rich Brazilian biodiversity, *Salvia officinalis* L. (Lamiaceae) - popularly known as 'sage' - is native of the Mediterranean region[14] and it has been widely used in dentistry. Sage oil presents antibacterial, antifungal, and antiviral activities[15]. Its leaves contain an essential oil (0.5-2.5%) composed of cineol (up to 15%), pinene, thujone, salvia, borneol, camphor, and other terpenoids, as well as flavonoids, tannins, acid triterpenes (ursolic and oleanolic acids), diterpenes, phenolic acids, amines, and vegetable acids[16]. In the modern European herbal medicine, a gargle of sage tea is commonly recommended to treat sore throat, oral inflammations, and gingivitis[15]. It presents wound-healing, antimicrobial, regenerative, protective, anti-inflammatory, and deodorant effects. Essential oils, decoctions, and infusions with sage heal the oral cavity and accelerate recovery.

The antimicrobial effects of several plant species have been tested against a wide variety of oral cavity microorganisms[13], but evaluating the amplitude of microbial reduction, especially on the denture surface, is still required[12]. Furthermore, it is worth noting the physical changes in dentures caused by the use of such extracts[17].

Therefore, this study aimed to assess the antimicrobial effect of the hydroalcoholic extract of *Salvia officinalis* and to analyze its influence on surface roughness and color stability of the PMMA. The hypothesis to be tested was that sage extracts would sig-

nificantly reduce the microbial load without significantly affecting the physical properties of the PMMA of dentures.

Materials and methods

This *in vitro* study was approved by the Institutional Research Ethics Committee, under protocol n. 1.752.675. Microbial samples were collected from 31 denture wearers selected by convenience at a private dental office located in the west region of the state of Santa Catarina, Brazil, in November/December 2016. Patients who wore complete dentures for at least one year, of any age and sex, and without oral lesion at the time of collection were included in the study. Those who underwent antimicrobial treatment were excluded.

Samples of saliva were seeded on plates of Sabouraud dextrose agar with chloramphenicol (Oxoid, Basingstoke, Hampshire, England), blood agar (Merck, Darmastadt, Germany), and MacConkey agar (Merck, Darmastadt, Germany), by the depletion technique. The plates were incubated in a bacteriological oven at temperature of 37°C for 24 hours. After this period, the plates were read. The identification of species considered the color aspects, morphology, and texture of the crop in the medium.

Microbial inoculums of *C. albicans*, *S. aureus*, and *K. oxytoca* were prepared from the cultures on the specific medium. Cell density was adjusted to a turbidity value equivalent to that of the McFarland standard solution (scale 1; approximately 3×10^8 cells/ml).

The plant materials of *S. officinalis* L. were collected in the city of Pinhalzinho, Santa Catarina, Brazil (26°49'19,16"S and 53°00'59,52"). The exsiccate was identified by a curator at the Municipal Botanical Museum Herbarium of Curitiba, Paraná, Brazil and deposited under registration number MBM 388402.

The chemical characterization of *S. officinalis* was previously performed by Roman Junior et al.[18] (2015) using high-performance liquid chromatography (HPLC). The tests were performed as described by Oliveira e Oliveira[19] (2013) with modifications. The analysis and quantification of rosmarinic acid in the aqueous extract of *S. officinalis* by HPLC was possible by replacing the elution gradient, which was proposed by Oliveira e Oliveira[19] (2013). Using the analytical curve ($y = 0.462x + 1.623$; $r = 0.999$), a concentration of 3.53% was obtained for the plant material (Figure 1). The hydroalcoholic extract was produced according to the Brazilian Pharmacopoeia 5[20]. Briefly, the leaves were reduced to small fragments and subjected to drying (25°C), protected from direct light and humidity. The dehydrated plant species was ground in a knife mill (Ciemlab®, CE430) and sieved to select particles of 425 μm (35 Tyler/Mesh). Aliquots of dry milled *S. officinalis* leaves (10 g) were extracted successively with ethanol (70%, 200 ml) by maceration during 5 days. After filtration, the solvents were eliminated under reduced pressure using a rotary evaporator and the resulting hydroalcoholic extracts from *S. officinalis* were lyophilized, weighed, and stored in a freezer at -20°C.

A sample size of four test specimens per group was calculated for the color stability test using G* Power 3.1.9.2 (Universität Düsseldorf, Germany), considering a Type I error of 0.05, 95% test power, one-tail, and effect size of 3.3, based on a ΔE^* value that was considered clinically visible[21].

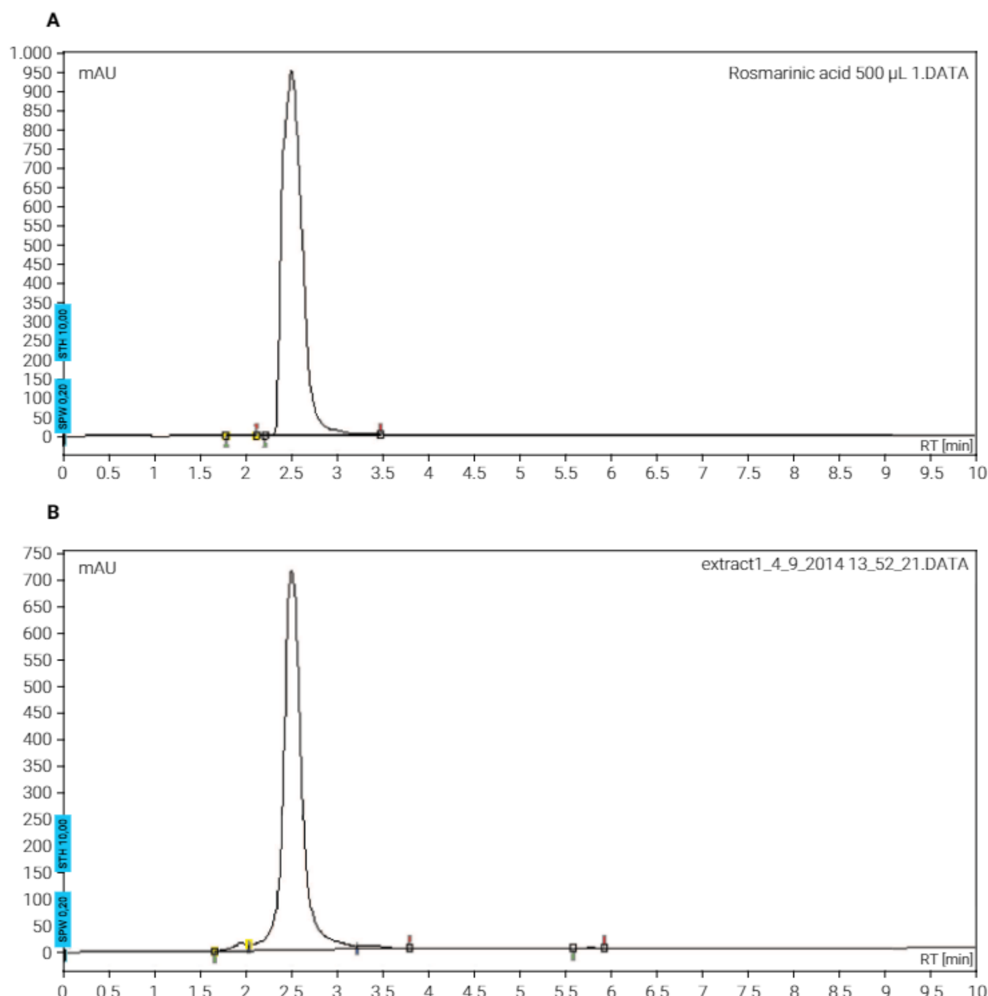

Figure 1. High-performance liquid chromatography of rosmarinic acid and aqueous extract of *Salvia officinalis* L. (Lamiaceae) leaves: A. rosmarinic acid (500 µg/ mL in MeOH) (Rf: 2.5 min); B. Aqueous extract of *S. officinalis* (10 mg/ ml). Varian® Chromatograph, Kromasil® ODS column (5 µm) C-18 reverse phase (25 × 4.5 mm) at a temperature of 24°C ± 2°C. Solvents used: MeOH: H_3PO_4 (0.1% v / v) isocratic mode for 10 min with flow rate of 1 mL/ min and detection at 300 nm (n = 3). Source: Roman Júnior et al.[18] (2015).

One hundred and eighty-eight disc-shaped specimens (10-mm diameter; 2-mm thick) were first prepared in wax and molded in gypsum plaster. Then, using the plaster molds, the discs were remade with thermopolymerizable polymethylmethacrylate (PMMA) resin (medium pink with veins; Clássico, Campo Limpo Paulista, São Paulo, Brazil). The discs were manually finished and consecutively followed by horsehair discs soaked in pumice stone (Asfer, São Caetano do Sul, São Paulo, Brazil). The specimens were polished with felt (Vipibril, Pirassununga, São Paulo, Brazil). Next, all specimens were exposed to ultraviolet light for 30 min on each side of the surface for disinfection.

The test specimens were immersed in liquid culture medium containing the microbial inoculum and incubated at 37°C for 16 hours/day[12]. Subsequently, they were sepa-

rated into groups and disinfected in sterile saline solution (0.85%; control), in a 0.2% chlorhexidine gluconate dental disinfectant (Riohex - Rioquímica, São José do Rio Preto, São Paulo, Brazil), or in hydroalcoholic extracts of *S. officinalis* at concentrations of 0.2%, 0.8%, and 1.16%. The specimens were immersed for 8 hours/day, simulating the nighttime, for a total of 30 days.

Surface roughness and color of the PMMA specimens were analyzed before and after the disinfection protocol (at 1 and 30 days). The color was analyzed with an Easyshade spectrophotometer (Vita Zahnfabrik, Bad Säckingen, Germany). Color change was calculated according to the CIE L*a*b* system[22] and the resulting ΔE^* was classified in three clinically significant intervals: $\Delta E^* < 1$ (undetectable color change); $1 < \Delta E^* < 3.3$ (clinically acceptable color change); and $\Delta E^* > 3.3$ (clinically unacceptable color change)[17].

Surface roughness was analyzed with an RP-200 roughness meter (Instrutherm, São Paulo, SP, Brazil) with three parallel readings along the length of 4 mm and cut-off value of 0.8 mm to determine the mean surface roughness (Ra - µm).

To analyze the adhesion capacity of microorganisms to the PMMA surface, one test specimen from each sample was washed in sterile distilled water at day 10 to remove the poorly adhered cells. The specimen was sonicated in an ultrasonic cell disruptor (Unique USC 800A) at a frequency of 40 kHz, for 15 min at room temperature. Next, the samples were seeded in Plate Count Agar (PCA; Merck, Darmastadt, Germany) or Sabouraud dextrose agar plate with chloramphenicol (Oxoid, Basingstoke, Hampshire, England) and incubated for 24 h at 37°C to count the colony forming units (CFU)[23].

The reduction of microbial load was evaluated by seeding and counting the CFUs on Sabouraud dextrose agar plate and PCA at 1 and 30 days of the protocol with the substances tested, so to observe whether there was a reduction in the number of CFUs over time. The culture medium was renewed during the experiment to prevent microbial reduction because of the lack of nutrients.

Normal data distribution was verified with the Anderson-Darling test. The before-after changes in microbial loads and surface roughness were compared by the paired t-test and Wilcoxon test. Substances at day 10 and color stability (ΔE) were compared by the Kruskal-Wallis and Mann-Whitney tests. The substances were compared at the beginning and end of the experiment using one-way ANOVA. The tests were performed with the Minitab 17.0 statistical package at 5% significance level.

Results

The microbial load of all microorganisms decreased at day 30. *S. aureus* significantly reduced in contact with both 1.16% *S. officinalis* solution and 0.2% chlorhexidine solution. Similar results were found for *C. albicans*. *K. oxytoca* was more sensitive to the disinfectant solutions, significantly decreasing CFU counts when in contact with all the concentrations of *S. officinalis* extract and 0.2% chlorhexidine solution (Table 1). The ANOVA revealed no statistically significant difference between concentrations of *S. officinalis* extract for the three microorganisms.

Table 1. Reduction in the microbial load of *Staphylococcus aureus*, *Candida albicans* and *Klebsiella oxytoca* (×10^7CFU/mL) in the experimental groups at the end of the disinfection protocol (N = 9)

Microorganism	Disinfectant	Day 1 Mean ± SD	Day 30 Mean ± SD	Δ	p
S. aureus	Control	37.00±2.65	36.33±4.16	0.67±1.53	0.529
	S. officinalis 0.2%	37.00±2.00	30.67±4.73	6.33±3.51	0.089
	S. officinalis 0.8%	35.33±2.08	31.00±3.61	4.33±3.06	0.133
	S. officinalis 1.16%	34.33±4.51	28.00±4.36	6.33±1.53	0.019*
	Chlorexidine 0.2%	36.33±1.53	6.63±0.42	29.70±1.11	0.0001*
C. albicans	Control	38.67±4.04	39.00±3.00	-0.33±1.16	0.667
	S. officinalis 0.2%	38.67±1.53	37.00±2.65	1.67±1.53	0.199
	S. officinalis 0.8%	40.00±2.00	35.00±4.36	5.00±2.65	0.082
	S. officinalis 1.16%	38.33±2.52	33.33±3.21	5.00±1.73	0.038*
	Chlorexidine 0.2%	40.67±2.08	7.90±0.30	32.77±2.31	0.002*
K. oxytoca	Control	34.33±3.06	34.33±4.04	0.00±1.00	1.000
	S. officinalis 0.2%	35.00±3.00	29.33±2.08	5.67±1.15	0.014*
	S. officinalis 0.8%	36.00±3.00	28.00±4.36	8.00±2.00	0.020*
	S. officinalis 1.16%	33.00±2.00	26.33±2.08	6.67±1.53	0.017*
	Chlorexidine 0.2%	36.00±3.61	7.43±0.40	28.57±3.30	0.004*

MO = microorganism. SD = Standard Deviation. Δ = Difference between means. * Statistically significant difference. Paired t-test ($p<0.05$).

Table 2. Adherence of microorganisms (x10^6 CFU/mL) on the PMMA surface among the experimental groups (N = 9)

Disinfectant	S. aureus	C. albicans	K. oxytoca
Control	47.330^c	36.667^b	43.000^b
S. officinalis 0.2%	3.800^b	3.167^a	2.933^a
S. officinalis 0.8%	3.767^b	2.967^a	3.900^a
S. officinalis 1.16%	4.133^b	2.800^a	3.800^a
Chlorexidine 0.2%	1.767^a	4.267^a	3.067^a

Statistically significant differences among groups are indicated by different overlapping letters (within columns), Kruskal Wallis and Mann Whitney tests ($p<0.05$).

In addition, significant reductions in microbial adherence to the PMMA surface were noted with all the disinfectant solutions when compared to the control group ($p<0.02$). *S. aureus* showed a considerably lower adherence under the action of 0.2% chlorhexidine. A greater reduction in *C. albicans* and *K. oxytoca* adherence was observed in all groups (Table 2).

Color stability by means of ΔE was significantly higher in the control group than in the other groups ($p<0.0001$), with no significant differences among the experimental groups. The ΔE values obtained for all experimental groups indicate a clinically unacceptable color change (Table 3).

A significant increase in PMMA roughness over 30 days was observed for 0.2% *S. officinalis* and no other group produced significant roughness changes at the end

Table 3. Mean color change (ΔE) in dentures caused by the disinfectant substances at the end of the disinfection protocol.

Disinfectant	Mean ± SD	Median	Minimum	Maximum
Control	2.78 ± 2.29[a]	1.74	0.29	9.03
S. officinalis 0.2%	6.94 ± 3.40[b]	6.29	1.32	15.01
S. officinalis 0.8%	7.54 ± 6.09[b]	6.15	0.01	22.98
S. officinalis 1.16%	7.26 ± 4.55[b]	6.19	1.02	17.35
Chlorexidine 0.2%	7.92 ± 5.48[b]	7.35	0.69	19.84

Different letters indicate statistically significant differences between groups (Kruskal Wallis and Mann Whitney, $p<0.05$).

Table 4. Mean roughness (Ra - µm) before and after the disinfection protocol.

Disinfectant	Day 1 Mean ± SD	Day 30 Mean ± SD	Δ	p
Control	0.1976 ± 0.0412[a]	0.2047 ± 0.0463[a]	-0.0071	0.432
S. officinalis 0.2%	0.1778 ± 0.0528[a]	0.2052 ± 0.0440[a]	-0.0274	0.031*
S. officinalis 0.8%	0.2065 ± 0.0379[a]	0.2073 ± 0.0507[a]	-0.0008	0.945
S. officinalis 1.16%	0.1865 ± 0.0442[a]	0.2056 ± 0.0556[a]	-0.0190	0.088
Chlorexidine 0.2%	0.1954 ± 0.0407[a]	0.1953 ± 0.0453[a]	0.0001	0.988

* Indicates a statistically significant difference before (Day 1) and after (Day 30) the disinfection protocol (verified by paired t-test). The letters in the columns indicate statistically similar values between groups (One-way ANOVA, $p>0.05$).

of the disinfection protocol (Table 4). There was no statistically significant difference in roughness at the end of the disinfection protocol among the experimental groups (Table 4).

Discussion

This study tested the potential use of a natural substance that is easily available for simulated nighttime denture disinfection. In this sense, using strains isolated from the oral cavity of individuals provides a reliable representation of the clinical scenario and the virulence potential of these microorganisms. Our results revealed the potential use of the S. officinalis extract as disinfecting solution for dentures, proving true an antimicrobial and non-adherent effect against oral microorganisms, and not affecting substantially the surface roughness of the denture PMMA. Additionally, it should be noted that the results of this study are unique, considering most *in vitro* studies have used agar diffusion or broth dilution[24-27].

An antimicrobial potential of the hydroalcoholic extract of *S. officinalis* against *C. albicans*, *S. aureus*, and *K. oxytoca* was revealed. This was expressed by reduction of microbial load. The antimicrobial effect is attributed to the oleanolic and ursolic acids, which are the two triterpenes present in *S. officinalis*[25]. Although comparisons between the various concentrations of the extract did not present any statistically significant difference, superior antimicrobial effects were observed at the concentration

of 1.16%, suggesting that its effect is concentration-dependent. However, *K. oxytoca* was more sensitive to the effect of the extract, showing considerable reduction in CFU counts at all concentrations (Table 1). Our results, therefore, confirm the antimicrobial effect of *S. officinalis* against gram-positive and gram-negative bacteria and fungi, assigning this effect to terpenes and terpenoids[13,24,27]. This is promising, considering that the action of *S. officinalis* in soft tissue lesions against *S. aureus*, *K. oxytoca*, and *C. albicans* has been previously shown[23-25].

Microbial adherence is responsible for biofilm formation in poorly sanitized dentures. Therefore, disinfecting substances that present a non-adherent effect are expected. Microbial adhesion tests on the PMMA surface showed a 2-log CFU/mL reduction in all experimental groups. the experimental solutions presented a significant non-adherent effect on the microoganisms tested in comparison with the saline solution, regardless of the concentration. For *C. Albicans* and *K. oxytoca* this effect was even similar to chlorhexidine digluconate, meaning that both substances present mechanisms to interfere on microbial adherence.

The fact that *K. oxytoca* was more sensitive to the extract contradicts a previous study[26] in which gram-positive bacteria are generally more sensitive than gram-negative bacteria. Another study showed no antimicrobial effect of *S. officinalis* against *K. oxytoca* when the authors tested the essential oil[25]. However, the present study tested the hydroalcoholic extract, which reinforces the idea that the antimicrobial action of *S. officinalis* against gram-negative bacteria depends on the type of extract used[26]. The antimicrobial effect of the various concentrations of *S. officinalis* extract was similar to that of the 0.2% chlorhexidine solution, although, when compared individually, chlorhexidine presented superior results.

Treatment with 0.2% chlorhexidine solution for 5 min has been shown to produce death of >4 log10 of all tested microorganisms, including *C. albicans*[28]. Also, 0.2% chlorhexidine gluconate solution is effective in reducing microbial biofilm, suggesting that denture immersion in the solution at nighttime is sufficient to avoid the recurrence of the infection[29]. However, this product has been shown to cause discoloration of the PMMA of dentures, making it inappropriate for daily use.

The treatment with 0.2% chlorhexidine solution was the most effective in reducing *S. aureus* adherence, whereas in the case of *K. oxytoca*, the anti-adherent effect of all concentrations of *S. officinalis* extract was similar to that of chlorhexidine solution, which is considered a positive result. The adherence of *C. albicans* was considerably lower when exposed to the extract at concentration of 1.16%, indicating the anti-adherent effect of this substance (Table 2).

Data on the anti-adherent activities of the hydroalcoholic extract of *S. officinalis* are scarce, particularly in regards to clinical isolates such as those tested in the present study. It is known that *S. aureus* presents several virulence determinants, which facilitate its adhesion to biotic and abiotic surfaces[30]. This may explain the higher adherence of this microorganism when compared to the others in the groups treated with *S. officinalis*.

Non-adherent effect of *S. officinalis* has been identified against three strains of *C. albicans* on the PMMA surface, even though the essential oil of the plant was used[24].

It also has been shown that the anti-adherent activity of *S. officinalis* is dose-dependent, and the action may be attributed to the monoterpenes present in the plant, which interact with the lipid components of the cell membrane[24]. This would increase its permeability and affect the electrolyte balance, consequently interfering with biofilm formation[30]. It was also observed that *S. officinalis* at 2.78 g/L caused reductions between 89% and 96% in *C. albicans* adhesion to the PMMA surface when compared to the 96% to 98% reduction observed with 0.2% chlorhexidine[24].

Color changes in PMMA specimens were not significantly different between the experimental groups at the end of the disinfection protocol, with all concentrations of *S. officinalis* producing a color change similar to that of the chlorhexidine solution (Table 3). The staining of PMMA resin by the disinfectant solutions is due to the accumulation of extrinsic pigments, but there is still controversy in the literature regarding color changes caused by chlorhexidine solution. Moffa et al.[31] (2011) claimed to have stained this material with 2% chlorhexidine over a period of 6 months of exposure. Further studies should be dedicated to understanding the effects of the contact with *S. officinalis* extract, with particular emphasis on examining color change and antimicrobial effects of the extract on PMMA at shorter exposure times.

No significant increase in surface roughness was observed over the 30-day disinfection period (except for 0.2% *S. officinalis*). Therefore, the effect of *S. officinalis* was comparable to that of 0.2% chlorhexidine. The roughness findings of our study are similar to those reported by previous ones[32]. However, the lack of studies on *S. officinalis* extract and its potential implications on PMMA materials prevents deeper comparisons with other studies. Considering that roughness acts as a facilitator for microbial adhesion and biofilm formation, the absence of any significant change in surface roughness in this study may be perceived as favorable.

There is a clear demand for the efficient disinfection of dentures, which helps preventing lesions caused by pathogenic microorganisms in the oral cavity. In this sense, *S. officinalis* extract has shown potential to be used as a disinfectant solution for dentures by reducing the microbial load on the surface without significantly affecting its physical properties when compared to the standard disinfectant.

It may be concluded that the hydroalcoholic extract of *S. officinalis* is a potential disinfectant solution for dentures. The study hypothesis was partially confirmed, since color change resulting from the disinfection procedure, although, not different from the standard disinfectant, was clinically detectable. Further studies focusing on the action mechanism and potential toxicity effects on humans are still required. Clinical trials are essential to confirm the effectiveness results of the extract found in this *in vitro* study.

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