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POTENCIAL ANTIMICROBIANO E INFLUÊNCIA DE SOLUÇÕES DESINFETANTES NA RESINA ACRÍLICA UTILIZADA EM PRÓTESES OCULARES

ANTIMICROBIAL POTENTIAL AND EFFECT OF DISINFECTANT SOLUTIONS ON ACRYLIC RESIN FOR OCULAR PROSTHESIS

POTENCIAL ANTIMICROBIANO E INFLUENCIA DE LAS SOLUCIONES DESINFECTANTES EN LA RESINA ACRÍLICA UTILIZADA PARA PRÓTESIS OCULARES

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Resumo

Introdução: A desinfecção de próteses oculares é essencial; entretanto, os agentes disponíveis podem comprometer as propriedades dos materiais acrílicos ou apresentar atividade antimicrobiana limitada. **Objetivo:** Avaliar in vitro a atividade antimicrobiana do óleo essencial de alecrim (OEA), óleo essencial de melaleuca (OEM), peróxido de hidrogênio (H_2O_2) e polihexametileno biguanida (PHMB), bem como seu impacto nas propriedades físicas da resina acrílica termopolimerizável (RA) utilizada em próteses oculares e a ação sobre biofilmes. **Métodos:** Sessenta corpos de prova de RA incolor foram confeccionados. Determinaram-se as concentrações inibitórias mínimas (CIM) frente a *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli* e *Enterococcus faecalis*. Com base na CIM, foram realizados ensaios de biofilme de *S. aureus* em discos de RA com concentrações de 10× CIM. Avaliaram-se rugosidade superficial (Ra), microdureza Knoop (MH) e alteração de cor (ΔE) após 0, 16 e 48 ciclos de imersão. Os dados foram analisados por ANOVA e teste de Tukey (α = 0,05). **Resultados:** Todos os agentes apresentaram atividade antimicrobiana. O OEA não apresentou CIM contra *P. aeruginosa*, enquanto PHMB, OEM e H_2O_2 inibiram todas as espécies testadas. No biofilme, PHMB, OEA e OEM reduziram aproximadamente 2 log na formação de *S. aureus*, enquanto H_2O_2 eliminou o crescimento detectável. Após 48 ciclos, OEA e OEM reduziram MH e aumentaram Ra, enquanto o OEA e H_2O_2 causaram alterações de cor clinicamente aceitáveis. PHMB não alterou significativamente nenhuma propriedade. **Conclusão:** Todas as substâncias reduziram o biofilme de *S. aureus*, sendo H_2O_2 o único agente com inibição completa. O PHMB preservou as propriedades da resina acrílica, sendo promissor para desinfecção de próteses oculares.
Palavras-chave: prótese ocular; biofilme; polihexametileno biguanida; óleos essenciais; resina acrílica.

Abstract

Introduction: The disinfection of ocular prostheses is essential; however, available agents may compromise the properties of acrylic materials or exhibit limited antimicrobial activity. **Objective:** To evaluate in vitro the antimicrobial activity of rosemary essential oil (REO), tea tree essential oil (TTO), hydrogen peroxide (H_2O_2), and polyhexamethylene biguanide (PHMB), as well as their impact on the physical properties of heat-polymerized acrylic resin (AR) used in ocular prostheses and their effect on biofilms. **Methods:** Sixty colorless AR specimens were fabricated. Minimum inhibitory concentrations (MICs) were determined against *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterococcus faecalis*. Based on MICs, biofilm assays of *S. aureus* were performed on AR discs using 10× MIC concentrations. Surface roughness (Ra), Knoop microhardness (KHN), and color change (ΔE) were evaluated after 0, 16, and 48 immersion cycles. Data were analyzed using ANOVA and Tukey's test (α = 0.05). **Results:** All agents exhibited antimicrobial activity. REO showed no MIC against *P. aeruginosa*, whereas PHMB, TTO, and H_2O_2 inhibited all tested species. In biofilm assays, PHMB, REO, and TTO reduced *S. aureus* biofilm formation by approximately 2 log units, while H_2O_2 eliminated detectable growth. After 48 cycles, REO and TTO reduced KHN and increased Ra, whereas REO and H_2O_2 induced clinically acceptable color changes. PHMB did not significantly alter any evaluated property.
Conclusion: All substances reduced *S. aureus* biofilm, with H_2O_2 being the only agent achieving complete inhibition. PHMB preserved acrylic resin properties and appears promising for ocular

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prosthesis disinfection.
Keywords: ocular prosthesis; biofilm; polyhexamethylene biguanide; essential oils; acrylic resin.

Resumen

Introducción: La desinfección de las prótesis oculares es esencial; sin embargo, los agentes disponibles pueden comprometer las propiedades de los materiales acrílicos o presentar una actividad antimicrobiana limitada. **Objetivo:** Evaluar in vitro la actividad antimicrobiana del aceite esencial de romero (AER), aceite esencial de árbol de té (AAT), peróxido de hidrógeno (H_2O_2) y polihexametileno biguanida (PHMB), así como su impacto en las propiedades físicas de la resina acrílica termopolimerizable (RA) utilizada en prótesis oculares y su acción sobre biopelículas. **Métodos:** Se fabricaron sesenta especímenes incoloros de RA. Se determinaron las concentraciones inhibitorias mínimas (CIM) frente a *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli* y *Enterococcus faecalis*. Con base en las CIM, se realizaron ensayos de biopelícula de *S. aureus* en discos de RA utilizando concentraciones de 10× CIM. Se evaluaron la rugosidad superficial (Ra), la microdureza Knoop (MH) y el cambio de color (ΔE) después de 0, 16 y 48 ciclos de inmersión. Los datos se analizaron mediante ANOVA y la prueba de Tukey (α = 0,05). **Resultados:** Todos los agentes presentaron actividad antimicrobiana. El AER no presentó CIM contra *P. aeruginosa*, mientras que PHMB, AAT y H_2O_2 inhibieron todas las especies evaluadas. En la formación de biopelícula, PHMB, AER y AAT redujeron 2 log la biopelícula de *S. aureus*, mientras que H_2O_2 eliminó el crecimiento detectable. Después de 48 ciclos, el AER y el AAT redujeron la MH y aumentaron la Ra, mientras que el AER y el H_2O_2 causaron cambios de color aceptables. PHMB no alteró significativamente ninguna propiedad evaluada. **Conclusión:** Todas las sustancias redujeron la biopelícula de *S. aureus*, siendo H_2O_2 el único agente con inhibición completa. PHMB preservó las propiedades de la resina acrílica y se muestra prometedor para la desinfección de prótesis oculares.
Palabras clave: prótesis ocular; biopelícula; polihexametileno biguanida; aceites esenciales; resina acrílica.

1. Introduction

The human eye is the organ responsible for vision and contributes to harmonious facial appearance. It is formed by a complex physiology and is located within a bony cavity, protected by adjacent structures such as membranes, eyelids, and eyelashes (Swindell et al., 2008; Nalawade et al., 2013).

Malformations or interruptions in ocular globe development may result from genetic or environmental alterations, or from a combination of both. More than 90 associated genes have already been identified, and several gestational conditions, such as vitamin A deficiency, alcohol consumption, and viral infections including measles, have been linked to congenital ocular anomalies such as cataracts, microphthalmia, and anophthalmia (Mattos et al., 2006; Matías-Pérez et al., 2017; Harding et al., 2019).

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In addition, structural alterations to the eyeball may be acquired through trauma involving sharp or penetrating instruments such as pencils, needles, knives, glass fragments, or occupational accidents, among other causes (Amornvit et al., 2014).

In these scenarios, the ocular prosthesis is often the only rehabilitative option for patients. First developed by French physician Ambroise Paré in the sixteenth century, ocular prostheses evolved from spherical shapes to shell-like designs and from metals such as gold and silver to glass and porcelain. Currently, most ocular prostheses are fabricated from acrylic resin, either custom-made after impression of the anophthalmic cavity or as prefabricated stock eyes (Osborn et al., 2010; Modugno et al., 2013; Shrivastava et al., 2020).

The widespread use of acrylic resin prostheses underscores the need to establish effective disinfection protocols, because bacteria are capable of proliferating on these surfaces and mechanical cleaning alone cannot adequately remove biofilm. Therefore, new disinfectant solutions have been evaluated with the aim of achieving effective antimicrobial activity without altering acrylic properties, while remaining cost-effective and biocompatible (Heidrich et al., 2018).

Disinfection is particularly important due to the space between the posterior surface of the ocular prosthesis and the anatomical base of the cavity, which favors the accumulation of fluids and creates an environment conducive to the proliferation of bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, which commonly colonize the ocular region. (Nagay et al., 2018; Andreotti et al., 2018; Rezeck et al., 2020).

Essential oils (EOs) have emerged as potential disinfectants against bacteria and fungi. They are volatile liquids extracted from plant materials that can disrupt bacterial cell membrane integrity, leading to cell death (Oliva et al., 2018; Kozics et al., 2019).

Among the essential oils described in the literature, Rosemary essential oil (REO) and Tea Tree essential oil (TTEO) stand out. TTEO is derived from the

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leaves of Melaleuca alternifolia, native to Australia, whereas rosemary (Rosmarinus officinalis) is easily recognized by its characteristic aroma. Both exhibit broad-spectrum antimicrobial action, largely attributed to components such as 1,8-cineole and eugenol (Oliva et al., 2018; Oliveira et al., 2019).

Another disinfectant substance available on the market is Polyhexamethylene Biguanide (PHMB), an alkaline agent widely used in products for disinfection of equipment, floors, and walls. It is also found in topical wound treatment solutions and gels due to its antimicrobial properties (Santos et al., 2011; Kramer et al., 2019).

Other chemical agents such as sodium hypochlorite, chlorhexidine, and hydrogen peroxide have also been reported in the literature for prosthesis disinfection, with variation in product concentration and immersion times to achieve effective bactericidal action (Osborn et al., 2010; Goiato et al., 2010; Hashizume et al., 2015).

Given the need for ocular prosthesis disinfectants that are active against local microorganisms and do not adversely affect acrylic resin properties, the aim of this study was to evaluate in vitro the antimicrobial activity of REO, TTEO, H_2O_2, and PHMB by MIC and *S. aureus* monobiofilm assays, as well as their influence on microhardness, surface roughness, and color of heat-polymerized acrylic resin after different immersion intervals (16 and 48 cycles of immersion followed by washing).The first null hypothesis was that the tested substances would not exhibit antibacterial potential against the evaluated strains. The second null hypothesis was that immersion and rinsing cycles would not affect acrylic resin properties.

2. Materials and methods

Sample Size Calculation

To determine the number of samples to be included in the present study, a sample size calculation was performed. For this purpose, studies from the literature (Heidrich et al., 2018; Nagay et al., 2018; Andreotti et al., 2018; Kambala et al.,

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2020) presenting outcome characteristics similar to those proposed in this study (microhardness, roughness, color, and bacterial frequency) were selected.

The following statistical formula was applied:

$$MSD = t5\% \sqrt{\frac{2*MSE}{n}}$$

Where MSD represents the minimum significant difference intended to be observed (a 10% difference was selected), t(5%) corresponds to a tabulated value of 2, and MSE represents the observed dispersion measure, with the standard deviation being selected.

After performing the statistical test and adopting a 10% safety margin, the inclusion of 5 specimens per group was determined to be necessary for the present study (n), totaling 60 specimens for the complete study, with 25 allocated for the acrylic resin property tests and 35 for the *S. aureus* biofilm test. The confidence interval used was 95%, and the significance level was 5%.

The primary outcome variables included MIC values against the tested microorganisms, reduction of Staphylococcus aureus biofilm viability, and changes in the physical properties of the acrylic resin (microhardness, roughness, and color alteration).

Disinfectant substances and experimental groups

The evaluated substances were: Rosemary essential oil 90% (REO, Rosmarinus officinalis, WNF, Sao Paulo, Brazil); Tea Tree essential oil 90% (TTEO, Melaleuca alternifolia, WNF, Brazil); Hydrogen Peroxide 10% (H_2O_2, Sao Paulo, Brazil); and Polyhexamethylene Biguanide 2% (PHMB, LONZA, Salto, SP, Brazil).

Essential oils were diluted in polysorbate 80 (Tween 80; Dinâmica Química Contemporânea Ltda®, Campinas, SP, Brazil) at a 1:9 ratio (polysorbate:oil) to allow solubilization before testing. Polysorbate 80 was also evaluated separately at

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the same concentrations used in the essential oil dilutions and was included as a solvent control to assess possible interference of the surfactant on microbial growth.

The experimental groups consisted of the tested substances at concentrations corresponding to the MIC values described in Table 1. In addition, the following control groups were included: (i) growth control, containing bacterial inoculum and culture medium without test substances; (ii) sterility control, containing only culture medium; and (iii) sterility control of the substances, containing culture medium supplemented with each test substance, without bacterial inoculum.

Table 1: Distribution of groups according to MIC.

Substance	MIC
REO 90%	11.25% a 0.005%
TTEO 90%	11.25% a 0.005%
H_2O_2 10%	0.5% a 0.0001%
PHMB 2%	0.05% a 0.00001%
Polysorbate 80	1.25% a 0.0002%

Bacterial strains and culture conditions

The following bacterial strains were used: *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* INCQS 00197, *Pseudomonas aeruginosa* ATCC 25619, *Escherichia coli* ATCC 10536, and *Enterococcus faecalis* ATCC 29212. Bacteria were cultivated on Tryptone Soy Agar (TSA; Difco Co., Detroit, MI, USA) and incubated aerobically.

Minimum inhibitory concentration (MIC) assay

MIC determination followed the Clinical and Laboratory Standards Institute (CLSI) guideline for dilution methods for antimicrobial susceptibility testing of bacteria that grow aerobically. Bacterial cultures of each strain were grown on TSA

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for 24 h. A suspension was prepared by transferring 3–5 colonies into 9 mL of Mueller–Hinton broth (MHB; Difco) or Brain Heart Infusion broth (BHI; Difco) for *S. epidermidis*.

The inoculum turbidity was adjusted to the 0.5 McFarland standard (absorbance 0.135 at 660 nm using a Multiskan GO spectrophotometer, Thermo Scientific, USA), corresponding to approximately 1–2 × 10^8 CFU/mL.

Serial two-fold dilutions of each test substance were prepared in triplicate in 96-well polystyrene microplates, using 100 µL of MHB per well. From the standardized bacterial suspension, 50 µL were diluted in 10 mL of the respective broth, and 100 µL of this adjusted suspension were added to each well, resulting in a final inoculum of 5 × 10^4 CFU/mL.

Plates were incubated aerobically for 24 h and then analyzed by spectrophotometry to determine microbial growth. To aid visual confirmation, wells were stained with 0.01% resazurin solution (INLAB, São Paulo, Brazil). The dye acts as an indicator of metabolic activity since bacteria are able to convert resazurin into a fluorescent product, resorufin, this is an easy-to-apply test measured by colorimetry (Chen et al., 2017). The MIC was defined as the lowest concentration in which no visible bacterial growth was observed either by visual inspection or by resazurin color change.

Acrylic resin specimen preparation

Sixty heat-polymerized colorless acrylic resin specimens (VIPI - Produtos Odontológicos, Pirassununga, SP, Brazil) were fabricated. Discs were produced from a condensation silicone mold (Zetaplus – Zhermack; Badia Polesine, Italy) and invested in type III dental stone within monomaxillary flasks.

After stone crystallization, excess material was removed and acrylic resin was proportioned and manipulated according to the manufacturer's instructions. Flasks were closed and pressed in a bench hydraulic press at 0.5 tons for 1 h. Polymerization was carried out in a conventional water bath at 73°C for 9 h, as recommended by the manufacturer.

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After polymerization, specimens were deflasked and finished sequentially under water cooling with 400-, 600-, and 1200- wet sandpaper on an automatic polishing unit (Aropol, Arotec Ind e Com, São Paulo, Brazil). Dimensions were adjusted to approximately 6 mm in diameter × 2 mm thickness (25 specimens) and 10 mm × 3 mm (35 specimens), measured with a digital caliper.

Between each abrasive step, specimens were ultrasonically cleaned in water for 2 min and, after polishing, were immersed in distilled water for 3 min in an ultrasonic bath.

Specimens intended for roughness, microhardness, and colorimetry tests were stored individually in labeled Eppendorf tubes filled with distilled water, according to experimental group.

Immersion protocol and evaluation times

The experiment comprised 25 acrylic specimens (6 mm × 2 mm) for physical property assessment, with five specimens assigned to each group: Control (distilled water), REO, TTEO, H_2O_2, and PHMB. Properties were evaluated at three time points: Time 1 (immediately after fabrication); Time 2 (after 16 immersion and washing cycles, simulating approximately 4 months of weekly use); and Time 3 (after 48 cycles, equivalent to 1 year of weekly use) (Heidrich et al., 2018, with modifications).

Each cycle consisted of immersion in the assigned solution for 10 min, followed by individual washing with 2.5 mL neutral detergent and 7.5 mL distilled water for 180 s in an ultrasonic tank to remove residual product.

The remaining 35 specimens (10 mm × 3 mm) were used for *S. aureus* inhibition of biofilm formation assay.

Surface roughness evaluation

Mean surface roughness (Ra) was measured with a contact profilometer (Surftest SJ301 Mitutoyo, Suzano, SP, Brazil) with specimens positioned horizontally. Three parallel readings were obtained per specimen under the

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following parameters: sampling length 0.25 mm, evaluation length 2.5 mm, pre- and post-measurement 0.5 mm, traverse speed 0.5 mm/s, single measurement mode, Ra parameter. A maximum surface roughness value of 0.2 µm has been considered ideal for acrylic resin (Heidrich et al., 2018).

Knoop microhardness evaluation

Knoop microhardness (MH) was measured using a microhardness tester (HMV 2T Shimadzu, Tokyo, Japan). Three indentations were made per specimen, spaced 1.0 mm apart, using a 25 g load applied for 10 s. Indentation lengths were automatically converted to Knoop hardness numbers (kg/mm^2) by the device software, and mean values were calculated for each specimen.

Colorimetric evaluation

Color measurements were obtained using a reflectance spectrophotometer (CM-2600d, Konica Minolta, Osaka, Japan). Measurements were taken under standardized conditions using the CIE L*a*b* color system and D65 illumination with 100% ultraviolet radiation and specular component included.

L* represents lightness (0 = black, 100 = white), a* positive values indicate red and negative values indicate green, while b* positive values indicate yellow and negative values indicate blue. Color differences (ΔE) between time points were calculated and interpreted using the National Bureau of Standards (NBS) system:

$$NBS = \Delta E \times 0.92$$

According to NBS, values < 3 correspond to perceptible but clinically acceptable changes (Kambala et al., 2020).

***S. aureus* biofilm formation inhibition assay on acrylic resin discs**

Monospecies biofilms of *S. aureus* were grown on acrylic resin discs to evaluate biofilm inhibition at 10× MIC concentrations of each substance. The assay was adapted from (Graziano et al. 2015).

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S. aureus was reactivated on TSA and incubated for 24 h at 37°C. Colonies were suspended in BHI broth, vortexed, and adjusted spectrophotometrically at 660 nm to an absorbance of 0.1, corresponding to approximately 1.0–2.0 × 10^8 CFU/mL.

Resin discs were disinfected externally with 70% alcohol and sterile gauze, mounted on sterile metallic devices, and placed into 24-well plates. For each well, 300 µL of bacterial inoculum were added, along with the test substance at 10× MIC and BHI broth containing 1% glucose, to a final volume of 3 mL per well.

The experimental groups consisted of the tested substances at 10× MIC concentrations. Polysorbate 80 (Tween 80), used for essential oil solubilization, was evaluated separately at the same concentrations used in the experimental groups and included as a solvent control. Positive control wells contained only 300 µL of inoculum and 2.7 mL of BHI broth supplemented with 1% glucose, without antimicrobial substances. Sterility controls consisted of wells containing only culture medium and wells containing culture medium plus test substances, without bacterial inoculum.

Plates were incubated at 37°C for 24 h. After incubation, discs were transferred to 24-well plates containing 3 mL of 0.9% NaCl solution to remove non-adherent bacteria cells by immersion. Then, each disc was placed in a sterile polystyrene tube with 5 mL of sterile saline and vortexed at maximum speed for 2 min to detach biofilm cells. These washing and dilution steps were performed to minimize possible residual antimicrobial effects during microbiological quantification.

From the resulting suspension, 100 µL aliquots were serially diluted in sterile saline (1:10, 1:100, 1:1.000, and 1:10.000). Ten microliters of each dilution were plated on TSA and incubated at 37°C for 24 h. After incubation, colonized plates were selected, and colony-forming units (CFU/mL) were calculated.

Statistical analysis

The data were tested for normality and homogeneity of variance. For independent analytical situations, one-way ANOVA followed by Tukey's post-hoc test was applied. Dependent analytical comparisons were analyzed using two-way

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ANOVA followed by Tukey's post-hoc test. The level of significance was set at α = 0.05.

3. Results and Discussion

Overall, microhardness values decreased with increasing numbers of immersion cycles in REO, TTEO, and H_2O_2 groups. Two-way ANOVA revealed statistically significant differences among substances (p = 0.000175), among times (p < 0.0001), and in the interaction between substance and time (p < 0.0001).

PHMB did not significantly alter microhardness over time and at Time 3 remained similar to the control and H_2O_2 groups. Specimens immersed in H_2O_2 showed significant changes only at Time 3, although MH values remained above 15 kg/mm². REO and TTEO caused the greatest reductions in MH at Time 3, with mean values below 15 kg/mm², suggesting a higher risk of material weakening. REO presented the greatest overall variation (Table 2).

Table 2. Knoop microhardness values (kg/mm^2) according to substances and analysis time after immersion and rinsings.

Substances	Time 1	Time 2	Time 3	Substance factor
Control group	17.58 ± 0.70 Aa	17.51 ± 0.60 Aa	18.01 ± 0.64 Aa	A
PHMB	17.65 ± 0.51 Aa	17.47 ± 0.42 Aa	17.33 ± 1.06 Aab	A
TTEO	17.57 ±0.61 Aa	15.95 ± 0.83 Bb	14.78 ± 0.28 Cc	Bc
H_2O_2	17.76 ± 0.97 Aa	17.57 ± 0.99 Aa	16.31 ± 0.82 Bb	Ab
REO	17.55 ± 0.74 Aa	15.74 ± 0.88 Bb	14.36 ± 0.32 Cc	C
Time factor	A	B	C	

* Different uppercase letters (in rows) indicate statistically significant differences among groups for the time factor. Different lowercase letters (in columns) indicate statistically significant differences among groups for the substance factor, p < 0.05.

Surface roughness (Ra) tended to increase after 16 immersion cycles (Time 2) for all experimental groups. ANOVA showed no significant differences between substances (p = 0.079), only between times (p < 0.0001) and the interaction

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between substance and time (p = 0.0083). No group showed significant variation at Time 2 compared to Time 1. At Time 3, REO showed the highest Ra values, exceeding the 0.2 µm limit considered ideal.

PHMB did not differ statistically from the control, while TTEO and H_2O_2 were similar to one another. Under the substance factor, the control group was comparable to TTEO and H_2O_2, differing from REO, which produced the highest roughness values (Table 3).

ANOVA revealed that two test substances significantly affected color stability at the longest immersion period. There were significant differences among substances (p = 0.037), among times (p = 0.022), and in substance-time interaction (p = 0.033).

Table 3. Surface roughness values (µm) according to substances and analysis time after immersions and rinsings.

Substances	Time 1	Time 2	Time 3	Substance factor
Control group	0.23 ± 0.01 Aa	0.24 ± 0.01 Aa	0.26 ± 0.02 Bab	Ab
PHMB	0.23 ± 0.01 Aa	0.22 ± 0.03 Aa	0.23 ± 0.04 Aa	A
TTEO	0.22 ± 0.01 Aa	0.24 ± 0.01 Aa	0.28 ± 0.01 Bbc	Ab
H_2O_2	0.23 ± 0.01 Aa	0.24 ± 0.01 Aa	0.27 ± 0.04 Bbc	Ab
REO	0.22 ± 0.01 Aa	0.24 ± 0.01 Aa	0.30 ± 0.01 Bc	B
Time factor	A	A	B	

* Different uppercase letters (in rows) indicate statistically significant differences among groups for the time factor. Different lowercase letters (in columns) indicate statistically significant differences among groups for the substance factor, p < 0.05.

Spectrophotometric data showed that REO and H_2O_2 were the only substances that produced significant ΔE values at Time 3 when compared to earlier times and to other substances. Although they did not differ from each other at Time 3, REO produced higher ΔE values overall. The control, PHMB, and TTEO did not

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cause clinically perceptible color changes at any time and remained statistically similar for the substance factor (Table 4).

Table 4. Colorimetry values according to substances and analysis time after immersions and rinsing.

Substance	Time 2	Time 3	Substance factor
Control group	0.97 ± 0.69 Aa	1.16 ± 0.66 Aa	A
PHMB	1.06 ± 0.59 Aa	0.93 ± 0.32 Aa	A
TTEO	1.21 ± 0.69 Aa	0.88 ± 0.56 Aa	A
H_2O_2	0.76 ± 0.59 Aa	2.20 ± 1.5 Bb	Ab
REO	0.80 ± 0.38 Aa	3.07 ± 1.60 Bb	B
Time factor	A	A	

* Different uppercase letters (in rows) indicate statistically significant differences among groups for the time factor. Different lowercase letters (in columns) indicate statistically significant differences among groups for the substance factor, $p < 0.05$.

According to the NBS scale, the control group, PHMB, and TTEO showed slight color changes (0.5–1.5), whereas H_2O_2 and REO produced perceptible but clinically acceptable changes (1.5–3). Therefore, all substances were considered clinically acceptable in terms of color stability (Table 5).

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Table 5: Colorimetry values according to the NBS formula.

Substance	Time 3 (mean)	NBS
Control group	1.16	1.06
PHMB	0.93	0.85
TTEO	0.88	0.80
H_2O_2	2.20	2.02
REO	3.07	2.82

*Time 3 – equivalent to 48 immersions; NBS – National Bureau of Standards

Regarding the disinfection test, firstly, the minimum inhibitory concentrations of each substance were identified, with the 5 bacteria tested (Table 6).

Table 6: Minimum inhibitory concentrations (%) according to bacteria and substances evaluated.

Bacteria	PHMB (%)	TTEO (%)	H_2O_2 (%)	REO (%)
S aureus	0.0001	0.17 oil 0.01 polysorbate	0.003	1.4 oil 0.15 polysorbate
S epidermidis	0.0007	2.81 oil 0.31 polysorbate	0.003	5.62 oil 0.62 polysorbate
P aeruginosa	0.0007	0.35 oil 0.03 polysorbate	0.007	X
E coli	0.0001	0.35 oil 0.03 polysorbate	0.007	1.4 oil 0.15 polysorbate
E faecalis	0.0003	0.35 oil 0.03 polysorbate	0.015	2.81 oil 0.31 polysorbate

*Polysorbate 80; *X – did not present MIC.

Based on the results obtained, the concentrations of each substance for the formation of *S. aureus* biofilm were calculated at a concentration of 10 x MIC (Table 7).

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Table 7. Concentration of each substance required for effectiveness against *S. aureus* biofilm.

Substances (solution)	Quantity used
S. aureus control	300 µl
Polysorbate 80	45 µl
PHMB 2%	1,5 µl
H_2O_2 10%	9 µl
TTEO 90%	57 µl
REO 90 %	467 µl

*The amount of polysorbate used was 0.15%, corresponding to the higher value when comparing the MICs of rosemary and tea tree oils.

All substances tested were effective against *S. aureus* biofilm formation (Table 8), with polysorbate 80 acting statistically similarly to the positive control ($p = 0.99$).

Table 8: Results obtained from the disinfection test (Mean and standard deviation - log CFU/sample).

	S aureus control	Polysorbate	PHMB	H_2O_2	TTEO	REO
Mean	6.83 ± 0.11 a	6.66 ± 0.24 a	4.36 ± 0.89 b	0 c	4.30 ± 0.69 b	4.48± 0.30 b

*Different lowercase letters indicate statistically significant differences among groups, $p < 0.05$

PHMB, TTEO, and REO did not vary statistically from each other ($p > 0.99$). H_2O_2 was the substance that showed the greatest effectiveness against bacterial biofilm, being statistically different from the other liquids tested ($p < 0.0001$).

However, when evaluating only the concentrations of the antibacterial substances tested, it can be concluded that PHMB and H_2O_2 were the most effective, showing effectiveness at concentrations approximately 1000 times lower than essential oils.

In the absence of fully biocompatible, broad-spectrum, and material-safe disinfectants for ocular prostheses, this study evaluated the antimicrobial efficacy and effects on acrylic resin of four different agents: REO, TTEO, H_2O_2, and PHMB.

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Both null hypotheses were rejected: all substances showed antibacterial activity against *S. aureus* biofilm, and the essential oils and H_2O_2 significantly altered resin properties under the present conditions.

The test agents acted differently on the acrylic specimens over time. Ten-minute immersion cycles may have favored absorption of disinfectant molecules by the resin due to sorption phenomena common to polymeric materials. Combined with intrinsic characteristics of each solution, this may explain the observed physical changes in microhardness, roughness, and color (Goiato et al., 2009; Hashizume et al., 2015; Heidrich et al., 2018).

Although there is no universally accepted minimum value for ocular acrylic microhardness, values below 15 kg/mm² have been used as a reference threshold in artificial tooth studies and indicate a higher likelihood of fracture and surface degradation (Goiato et al., 2009). In the present work, REO and TTEO led to MH values below 15 kg/mm² at Time 3, suggesting that long-term exposure to high concentrations of these oils may compromise material durability. H_2O_2 also reduced MH over time but maintained values above 15 kg/mm², indicating less severe weakening.

The simultaneous increase in roughness and reduction in microhardness in the H_2O_2 group may be associated with its acidic pH and oxidative mechanism, which can promote surface erosion and microstructural changes such as microporosity (Hashizume et al., 2015). Interestingly, although H_2O_2 is known as a bleaching agent, the current findings indicated a reduction in whiteness and increased color change, possibly due to differences in response between dental polymers and industrial substrates where H_2O_2 whitening is typically evaluated (Trautmann et al., 2020).

Essential oils produced reduction in microhardness values at Times 2 and 3, and increase in roughness at Time 3, which may have been exacerbated by the relatively high concentration of polysorbate 80 used as an emulsifier. Polysorbate 80 is an amphiphilic surfactant whose hydrophobic tail interacts with oils, while the hydrophilic head interacts with water (Nielsen et al., 2016). As the sample was

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immersed in an oil solution with polysorbate 80, it is plausible that part of this solution was incorporated into the resin, which resulted in changes in the resin's properties. These observations suggest that future studies should test lower concentrations of both essential oils and surfactant, aiming to maintain antimicrobial efficacy while reducing the impact on material properties. In addition, clinical studies should be conducted to prevent potential irritation in the ocular region. Due to the sensitivity of ocular tissues and adjacent regions, a rigorous assessment of biocompatibility is an essential prerequisite before translating any experimental protocol into clinical use, ensuring the preservation of tissue integrity and patient safety in both the short and long term.

In addition, it is recommended that ocular prostheses be thoroughly rinsed with sterile saline solution prior to insertion into the ocular cavity, in order to prevent the presence of chemical agent residues, thereby reducing the risk of ocular and periocular irritation. (Rezeck et al., 2020).

Finally, regarding color, the REO caused statistically significant ΔE at the longest immersion time, when compared to the substance factor. This may be related to its intrinsic color, described as colorless to slightly yellow in supplier reports, as well as its higher density compared with TTEO. Tea tree oil, in contrast, did not significantly affect color under the same conditions. Heidrich et al. (2018) also reported increases in roughness after long-term immersion in rosemary oil solutions but did not observe color changes when compared to the control group, likely due to differences in concentrations (8% vs. 90% in the present study) and immersion protocols (30 min vs. 10 min).

Among all tested agents, PHMB was the most stable in terms of its effects on acrylic resin. Although a small statistical difference in MH was observed at Time 3, PHMB remained similar to the control group when the substance factor was evaluated, indicating minimal impact on material properties. Its high solubility in water and non-corrosive behavior may have enabled disinfection without significant penetration or alteration of the acrylic structure. Similar stability has been reported for chlorhexidine sprays, which did not affect microhardness of acrylic materials

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(Goiato et al., 2009), although these studies used shorter contact times (1 minute) than those simulated here.

MIC results confirmed the high potency of PHMB, with effective inhibition at very low concentrations for all tested species, followed by H_2O_2 and TTEO. REO exhibited the highest MIC values and failed to inhibit *P. aeruginosa*, a known resistant organism. Even so, all agents achieved meaningful reductions in *S. aureus* biofilm formation. A reduction of approximately 2 log units, as observed for REO, TTEO, and PHMB, represents a 100-fold reduction, while complete elimination by H_2O_2 highlights its strong antibiofilm potential.

The relationship between surface roughness and microbial adhesion is complex. Although high roughness may favor biofilm retention, it should not be used as the sole factor determining bacterial adherence (Goiato et al., 2010; Andreotti et al., 2018). This statement corroborates with the study carried out by Nagay et al. (2018) found *S. aureus* adhesion on acrylic surfaces with Ra of 0.038 μm, emphasizing that minimal roughness does not guarantee absence of biofilm. In the present study, even though REO increased roughness beyond the ideal threshold, it still reduced *S. aureus* biofilm formation through its antimicrobial effect, indicating that chemical action can counteract predisposition to colonization.

In this sense, the essential oil findings are consistent with previous reports showing that tea tree oil at 2% exerts bactericidal activity against methicillin-resistant *S. aureus*, with bacteriostatic effects at concentrations of 0,5 and 1% (Oliva et al., 2018), and that rosemary oil exhibits bacteriostatic and bactericidal properties at lower minimum inhibitory concentrations (MICs), of 0.03% and 0.06% respectively, results lower than those of this work whose MIC was 1.4% (Wang et al., 2012). The differences in MICs compared to previous studies may be related to variations in the chemotype of the oil, the choice of emulsifier, and the assay design.

Finally, the PHMB widely used in wound care, has been described as an effective bactericidal agent with a favorable safety profile when used at solutions up to 2% and being in drug form at concentrations of 0.01 to 0.5%, and not being

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absorbed systemically when applied topically (Kramer et al., 2019). Its negligible systemic absorption when applied topically supports its potential use in ocular prosthesis hygiene, provided formulations are designed for periocular safety and patient tolerance.

Despite the relevant findings, this study has some limitations. First, this was an in vitro study using flat acrylic resin discs, which do not fully reproduce the clinical conditions of ocular prostheses. In addition, the disinfectant substances were evaluated against a monospecies *S. aureus* biofilm. Although *S. aureus* is an important pathogen associated with ocular prosthesis contamination, multispecies biofilms may better represent the clinical microbiota found on these devices. Furthermore, cytotoxicity and clinical evaluations were not performed. Therefore, future studies involving multispecies biofilms, cytotoxicity assays, and clinical investigations are necessary to better establish the safety and effectiveness of the tested substances.

Taken together, the present results indicate that PHMB and H_2O_2 are promising agents for disinfection of ocular prostheses, with PHMB offering the important advantage of causing minimal changes to acrylic resin properties. Essential oils also show potential but require optimization of concentration and surfactant content to minimize detrimental effects on the prosthetic material. Future research should evaluate these agents in clinical protocols, including their effect on patient comfort, mucosal compatibility, and long-term prosthesis esthetics, as well as exploring formulations applied to iris-painted acrylic specimens to better simulate clinical reality.

4. Conclusion

Based on the results obtained in this study and the methodology used, the following conclusions can be drawn:

1- PHMB was the only disinfectant that did not significantly affect the microhardness, roughness, and color of the acrylic resin-based samples during the evaluated periods. Furthermore, it exhibited antimicrobial activity against *S. aureus*,

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S. epidermidis, P. aeruginosa, E. coli, and *E. faecalis*, representing a promising antimicrobial agent for the disinfection of ocular prostheses;

2- REO was the only substance that did not show antimicrobial activity against all the bacteria tested, as it was not effective against *P. aeruginosa*;

3- All tested substances reduced the formation of *S. aureus* biofilm on acrylic resin discs, with H_2O_2 being the only agent capable of completely preventing detectable biofilm growth, although it caused changes in the properties of the tested samples at time point 3;

4-The immersion cycles negatively influenced the surface properties of the acrylic resin. REO and TTEO produced the greatest reductions in microhardness and increases in surface roughness, suggesting limited applicability and emphasizing the need for further investigations involving different formulations, including the solvent.

In this context, it is important to emphasize that the present manuscript describes an *in vitro* study. Therefore, further investigations are required to evaluate other biofilm species commonly found in the ocular region, as well as to perform cytotoxicity assays and clinical studies involving the tested substances, in order to ensure the safety of patients using ocular prostheses.

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