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ANTIMICROBIAL ACTIVITY OF BIOPOLYMER ASSOCIATED WITH ESSENTIAL OILS

ATIVIDADE ANTIMICROBIANA DE BIOPOLÍMEROS ASSOCIADOS A ÓLEOS ESSENCIAIS

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ABSTRACT: The objective of this study was to develop chitosan-based biopolymers associated with essential oils with antimicrobial properties. Chitosan was prepared from a solution, followed by the addition of essential oils at specific concentrations, and the materials were subjected to organoleptic analysis, gas chromatography, Fourier-transform infrared spectroscopy (FTIR), and swelling degree evaluation. Antimicrobial activity was assessed using the agar disk diffusion method with microbial suspensions. The major compounds identified by chromatography in oregano and tea tree essential oils were carvacrol and 3-carene, respectively. The chitosan membranes associated with the tested oils showed hydrophilicity in the polymeric matrix according to the swelling degree analysis, while FTIR results indicated interactions between the chitosan matrix and the incorporated oils. Organoleptic analysis demonstrated high homogeneity of the films, and in the evaluation of antimicrobial activity, the film-forming emulsions exhibited inhibition zones against the tested microorganisms. These analyses demonstrate the feasibility of developing a biopolymer with promising potential for biomedical applications.
KEYWORDS: Chitosan. Essential Oils. Melaleuca. *Origanum vulgare*. Products with Antimicrobial Action.

RESUMO: O objetivo foi desenvolver biopolímeros à base de quitosana associado aos óleos essenciais com propriedades antimicrobianas. A quitosana foi preparada a partir de uma solução, após foi adicionado óleos essenciais em determinadas concentrações. Seguidos de análises organolépticas, cromatografia gasosa, FTIR e grau de inchamento. A atividade antimicrobiana foi realizada através do método disco-difusão em ágar, utilizando suspensões microbianas. Os compostos majoritários referentes à cromatografia frente aos óleos de orégano e melaleuca foram, respectivamente, o carvacrol e o 3-Carene. A membrana de quitosana associada aos óleos analisados no grau de inchamento apresentaram hidrofilicidade na matriz polimérica. Foi observado no FTIR interação na matriz de quitosana com os óleos testados. Na análise organoléptica os filmes apresentaram alta homogeneidade. As emulsões filmogênicas, referentes à atividade antimicrobiana os filmes apresentaram zona de inibição frente aos microrganismos testados. As análises demonstraram a possibilidade em desenvolver um biopolímero tornando promissor para a aplicabilidade biomédica.
PALAVRAS-CHAVE: Melaleuca. Óleos Essenciais. *Origanum vulgare*. Produtos com Ação Antimicrobiana. Quitosana.

INTRODUCTION

Serious complications are evidenced by healthcare-associated infections (HAIs), compromising patients' health and safety risks, as well as increasing the chances of morbidity and death[1]. Strategies that can be used as preventable harm to minimize infections in hospital settings have become a hot topic in contemporary society[2,3].

Patient safety must be determined by the quality of subsidies for safe health care that aim to reduce the risks of non-essential harm associated with health care[4]. Due to their clinically serious condition and invasive medical interventions, patients in intensive care units (ICUs) are more susceptible to infectious processes[5].

In view of the above, initiatives are being created to reduce the incidence of HAIs in healthcare institutions by implementing a *structured bundle.* This approach aims to reduce and prevent health-related infections, and the correct implementation of the *structured bundle* must be precise, ensuring that it maintains effective protection for the patient[6].

Although commercially available dressings on the market today are efficient, there are difficulties in disposing of these contaminated materials, in addition to the exacerbated production that this hospital waste generates for the environment. In view of this, new technologies are being implemented in order to reduce the waste that is generated in the hospital environment with the use of biopolymers[7].

A promising natural polymer is chitosan, derived from the basic deacetylation of chitin found in the exoskeleton of arthropod crustaceans and mollusks. It has applications in the biomedical field such as antibacterial action - against both

Gram-positive and Gram-negative bacteria - and antifungal action and can be used in polymeric films, It can be used in polymeric films, gels and products such as nanoparticles, due to its hydrophilic character and its characteristic biological features, as well as being biodegradable and can be used in the manufacture of catheter covers and wound dressings8. Allied to natural polymers are essential oils, volatile substances in complex, lipophilic, odoriferous and liquid mixtures that are extracted from aromatic plants. Furthermore, the composition of essential oils will depend on their specificity, depending on their species and the part of the plant from which they were extracted[9].

Extracted from the small leaves of the *Origanum vulgare* herb*,* oregano essential oil has an antimicrobial action against microorganisms. Thymol and Carvacrol are the main effective compounds for antimicrobial action[10].

Although the mechanism of oregano essential oil has not been fully elucidated, it is able to penetrate the peptidoglycan layer and act on the cytoplasmic membrane of Gram-positive bacteria. Another promising essential oil is *Melaleuca alternifolia, which* is extracted by steam distillation or hydrodistillation[11].

The main product extracted from melaleuca essential oil is TTO - *tea tree oil*, recognized for being an antimicrobial and healing agent[12]. The mechanism of bactericidal action of *Melaleuca alternifolia* oil is due to the impairment of the cell membrane, reducing intracellular material and causing the inability to maintain homeostasis and cellular respiration[13].

In view of the above problem, the aim of this study was to develop chitosan- based biopolymers associated with essential oils with antimicrobial properties.

METHODOLOGY

This is an *in vitro* experimental study, the aim of which was to evaluate the efficacy of chitosan-based biopolymers associated with essential oils against microorganisms frequently related to HAIs. A biodegradable matrix was used in order to investigate the potential of these compounds as a therapeutic alternative for biomedical applications. It should be noted that, as this was an in vitro study, no analysis was made of the large-scale applicability or economic impact of the use of the material developed.

The sample was made up of high viscosity, medium molecular weight chitosan polymers, the solvent 1% acetic acid and TWEEN® 80 (polysorbate surfactant), which were purchased from Sigma Aldrich®. The essential oils of *Melaleuca alternifolia* and *Origanum vulgare* were purchased commercially from dōTERRA. For the antimicrobial activity tests, ATCC (American Type Culture Collection) strains of *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 14990), *Escherichia coli* (ATCC 25922) *Candida albicans* ATCC (750) were used.

The essential oils were added according to the model proposed by Wang and collaborators (2011), with adaptations. To the chitosan solution prepared, following the previous description, 0.5% of the emollient glycerol (Sigma Aldrich®) and 0.5% of the surfactant Tween 80 (Sigma Aldrich®) were added, since the hydrophobic nature of the oils does not allow homogenization, followed by 30 minutes of stirring. To this solution, add 1% (v/v) melaleuca (*Melaleuca alternifolia) and* 1% (v/v) oregano *(Origanum vulgare*) in combination. The chitosan solution associated with the essential oil(s) was kept stirring for 90 minutes. The emulsions were then placed in an ultrasonic cleaner (Unique®, model Ultrasonic Cleaner) for 10 minutes to remove bubbles. The emulsions were then weighed into Petri dishes (15g) and dried in an oven with forced air circulation (Tecnal TE-394/3) at 35 °C for 72 hours. At the end, the plates containing the chitosan films incorporated with the essential oils were covered with aluminum foil and kept in a desiccator until they were used for analysis[14].

CHEMICAL COMPOSITION OF THE OILS

The chemical composition of the essential oils *of Origanum vulgare* and *Melaleuca alternifolia* purchased commercially was analyzed using gas chromatography coupled with mass spectrometry (GC-MS) on a QP2010 Plus (Shimadzu®) apparatus. A fused silica capillary column (30 m x 0.25 mm x 0.25 µm) was used and the temperature was set at 60-250°C, increasing by 4°C/ minute. A Split injector mass detector (SPL) with an injection volume of 1 µL was used. Helium gas was used as the carrier gas. The constituents were identified using the library stored in the equipment's software database.

INTERACTION OF CHITOSAN WITH ESSENTIAL OILS

The chemical composition of chitosan, as well as the associations with essential oils and isolates, were characterized by analysis using infrared spectroscopy on a Perkin Elmer® Spectrum 400 infrared spectrometer. The samples were measured right at the center of the biopolymers, which were positioned directly in the ATR (Attenuated Total Reflectance) accessory in the region between 4,000 and 650 cm-1.

DEGREE OF SWELLING

The gravimetric and dimensional methods were used to analyze the degree of swelling. The gravimetric method considers the initial dry mass of a film sample (2 x 2 cm) and the mass after 24 hours immersion in distilled water. First, the dry film was weighed (mi;g) and then immersed in a beaker with approximately 50 mL of distilled water. After 24 hours of immersion, the film was removed and the excess water removed with absorbent paper; the film was weighed again (mf;g). The swelling percentage was calculated using the following equation:

Degree of swelling %= $(mf - mi).\ mi.\ 100$

In the dimensional method, the initial and final dimensions of the film samples were compared after immersion in distilled water for 24 hours. The initial dimension of the samples analyzed was 2 x 2 cm. This analysis was carried out in duplicate and the mean and standard deviation were calculated.

ORGANOLEPTIC ANALYSIS

The chitosan polymers containing melaleuca oil and oregano oil were evaluated qualitatively in terms of their color, appearance and homogeneity through visual and tactile analysis.

As for appearance, the following characteristics were analyzed: transparent (when you can clearly see what's behind); non-transparent (when you can't distinguish what's behind); plastic texture (similar to cellophane); silky texture (oily, glides more easily), rough texture (not smooth, not pleasant to the touch); malleable (elastic, flexible, bendable); partially malleable (reduced elasticity and flexibility). Regarding homogeneity, they were classified as: homogeneous (uniform composition whose components are not easily distinguished) and partially homogeneous (composition with less uniformity, being able to distinguish more concentrated components in some places).

ANTIMICROBIAL ACTIVITY

To assess the antimicrobial activity of the coatings developed, standard strains of the bacteria *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) and the yeast *Candida albicans* (ATCC 750) were used. The antimicrobial activity test was carried out by the agar disc diffusion method, using the solutions/emulsions, which were added to sterile filter paper discs (5mm in diameter); and the antimicrobial coatings, in which the films were cut into discs (6 mm in diameter) with the aid of a perforator previously sanitized with 70% alcohol. Antimicrobial activity was assessed according to the method proposed by Archana, Dutta and Dutta, with adaptations. The tests were carried out in duplicate and the average of the halos was calculated on Mueller Hinton agar for bacteria and *Sabouraud* dextrose agar for yeast[15].

Initially, microbial suspensions of the standard strains were prepared using sterile saline at 0.5 on the *Mc Farland* scale. For antibacterial activity, *Mueller Hinton* agar was used, where bacterial suspensions of the standard strains of *S. aureus and E. coli* were sown on the agar using a sterile *swab*. After sowing, sterile filter paper discs were added to test the pure oils, the solutions of pure chitosan and chitosan associated with the surfactant, and the emulsions containing chitosan associated with the surfactant and the individual essential oil, as well as the combination of two oils, in search of synergism.

The samples were distributed as follows: (1) 3 µL of the pure essential oil; (2) 10 µL of the solution and the pure chitosan film discs; (3) 10 µL of the solution and the chitosan film discs containing glycerol and Tween; (4) 10 µL of the emulsion and the chitosan film discs containing the isolated antimicrobial agent and (5) 10 µL of the emulsion and the chitosan film discs containing the associated antimicrobial agents. For antifungal activity, *Sabouraud* dextrose agar and the standard strain of *C. albicans* were used, and the solutions and films were tested according to the technique described for antibacterial activity. To check the antimicrobial activity, the plates were incubated in a bacteriological oven for 18 hours at

37°C for the bacteria and 48 hours at 28°C for the yeast. The antimicrobial activity of the solutions and films will then be measured by measuring the inhibition halo using a caliper.

The quality control of the standard strains studied was carried out using the agar disc diffusion technique, using Cefoxitin (*S. aureus*) and (*S. epidermidis*), Ampicillin (*E. coli*) discs, according to BrCAST (2021)[16]. All will be incubated in an oven, according to the specifications for each microorganism, and the inhibition halos will then be read for each antimicrobial drug tested. For pure essential oils, inhibition zones greater than 15.0 mm in diameter characterize the essential oils as very active, between 10.0-15.0 mm as active compounds and less than 10.0 mm as inactive compounds[17].

The project is part of a larger project entitled "Synthesis and characterization of biopolymers with antimicrobial activity for the development of catheter covers" registered with the National Genetic Heritage Management System (SISGEN) under the number A98EBF9.

No comparative statistical analysis was carried out between the essential oils, since each one has different physicochemical characteristics, which would make a direct and reliable comparison between them impossible. Therefore, the data will be presented with the respective variation between the triplicates, expressed as the standard deviation

RESULTS

GAS CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRY

Percentage identification of the substances obtained from the Melaleuca alternifolia and Origanum vulgare essential oil samples are shown in Figures 1 and 2, respectively.

FOURIER TRANSFORM INFRARED SPECTROSCOPY

The FTIR of the chitosan films and those associated with the essential oils of Melaleuca alternifolia and Origanum vulgare are shown in figure 3.

DEGREE OF SWELLING

The degree of swelling of the films is shown in Table 1.

ORGANOLEPTIC ANALYSIS

Organoleptic and qualitative analysis of the characteristics of the chitosan films are shown in Box 1.

MICROBIOLOGICAL ANALYSIS

The results of the antimicrobial activity of the essential oils isolated from *Melaleuca alternifolia* and *Origanum vulgare*, as well as the solutions and films, against the microorganisms tested, are shown in Table 2.

DISCUSSION

The presence of Aromadendrene, α-Guaiene, α-Terpineol and 2- Methylisoborneol as the majority compounds of Melaleuca essential oil in the analysis of gas chromatography coupled with mass spectrometry[18]. The high percentages of carvacrol (69.1%) and p-cymene (18.8%) compared to oregano essential oil. Based on the results of the research into the incorporation of essential oil oregano essential oil showed Carvacrol as the main component, while melaleuca essential oil showed 3-Carene as the main compound[11] (Figures 1 and 2).

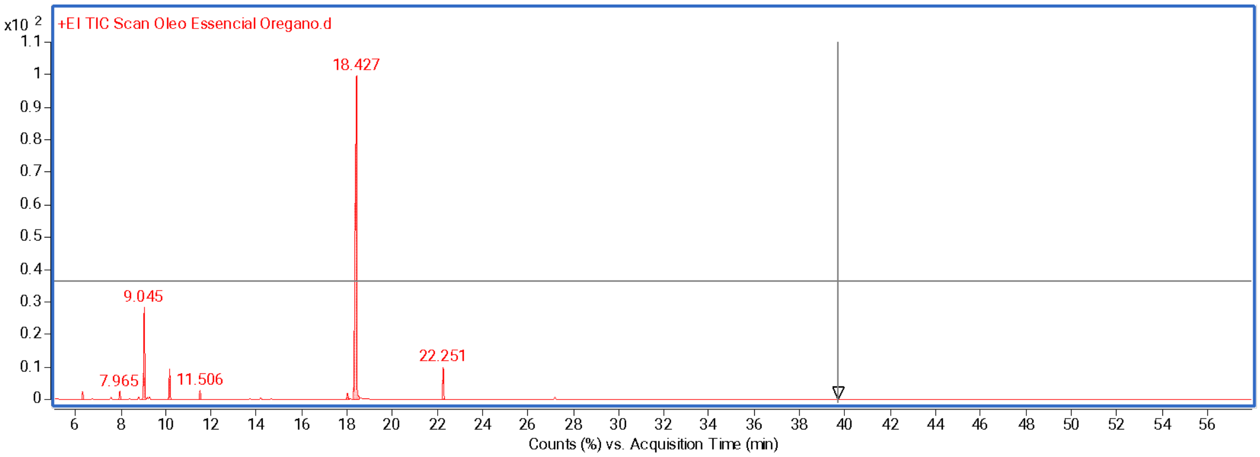

Figure 1. Chromatogram obtained for the oregano essential oil sample. Caption: 1- Carvacrol; 2- O- ymene; 3- gamma- Terpinene; 4- Caryophyllene; 5- Linalool; 6-beta- Myrcene.

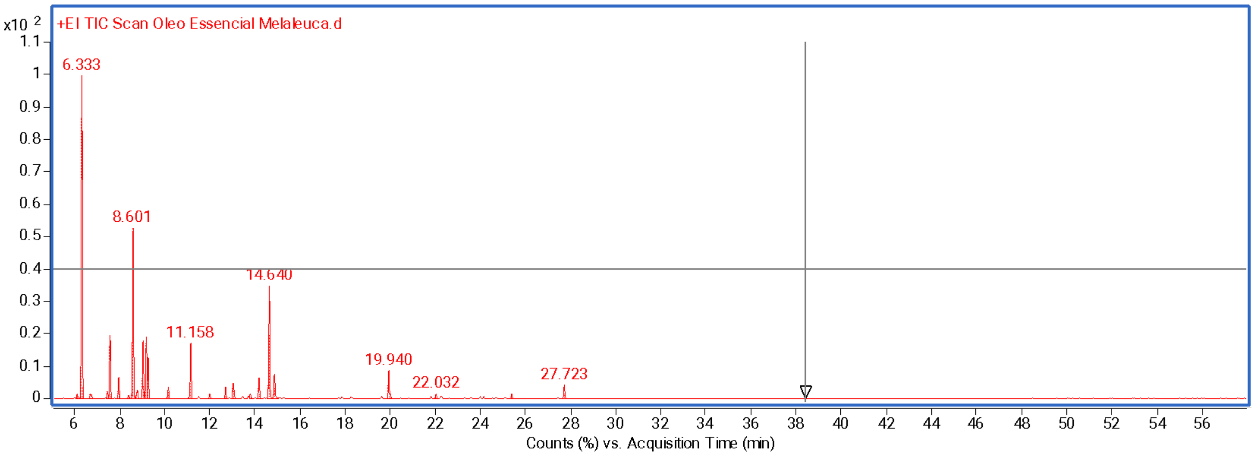

Figure 2. Chromatogram obtained for the melaleuca essential oil sample. Caption: 1- 3- Carenene ; 2- L- alpha- Terpineol; 3- D-Limonene; 4- o- Cymene; 5- I- methylethylidene; 6- Eucalyptol; 7- beta- Myrcene; 8- L- alpha- Terpineol- 4-ol; 9- Alpha terpinyl acetate; 10- gamma- Terpinene.

In relation to Fourier transform infrared spectroscopy, the 3652 cm-1band is characteristic of chitosan (Nh2 OH), and the 2928 cm-1band identifies the superposition of chitosan tween+glycerol (CH2). At 1175 cm-1and 1168 cm-1the vibration of Carvacrol, characteristic of oregano essential oil, was identified. At a frequency of 1150 cm-1, the highest peak was observed in the association of *Melaleuca alternifolia* and *Origanum vulgare* essential oils. The 1660 cm-1frequency refers to the -C=C- stretching found in the oregano molecule[19]. For Mendes (2022), the frequencies 1446 and 1357 cm-1indicate a bending vibration of the -CH2 bond, while other bands are clear around 1250 cm-1and 1020 cm-1which denote C-O stretching in the aromatic groups. In addition, the presence of the major component of *Melaleuca alternifolia* essential oil, terpinene, pinem and sesquiterpene alcohols[12] (Figure 3).

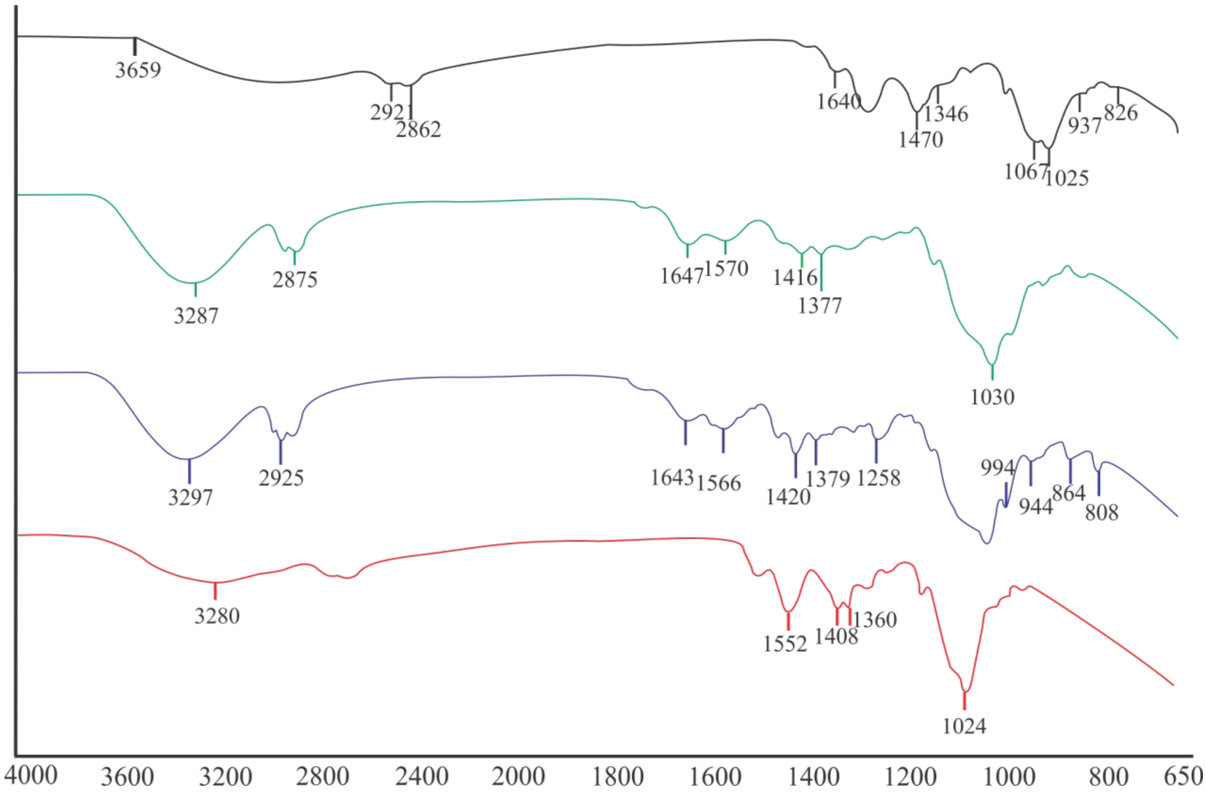

Figure 3. Fourier transform infrared spectroscopy (FTIR). Caption**: Black color** FQ/TG (Chitosan film 1.75% + Tween 80 + Glycerol); **Green color** (Chitosan film 1.75% + Tween 80 + Glycerol + melaleuca essential oil 2% (v/v); **Blue color** (Chitosan film 1.75% + Tween 80 + Glycerol + oregano essential oil 2% (v/v); **Red color** FQ/TG-OEO+ OEM (Chitosan film 1.75% + Tween 80 + Glycerol + oregano essential oil 1% + melaleuca essential oil 1%).

Origanum vulgare essential oil combined with *Melaleuca alternifolia* essential oil showed changes in the polymer matrix of the chitosan film in both films tested. The results presented for the polymeric matrix of the films tested with oregano essential oil, referring to the analysis of the swelling samples, showed C and absorption of the polymeric matrix[20], as shown in Table 1.

Table 1. Degree of swelling of chitosan films associated with isolated oregano, tea tree and associated oils.

Film	Degree of swelling (%)
FQ/TG/OEO	106,8
FQ/TG/OEM	122,2
FQ/TG/OEO+OEM	105,53

Caption: FQ/TG/OEO: Chitosan film 1.75% + oregano essential oil 2% (v/v); FQ/TG/OEM: Chitosan film 1.75%+ melaleuca essential oil 2% (v/v); FQ/TG/OEO + OEM (Chitosan film 1.75% + oregano essential oil 1% (v/v) + melaleuca essential oil 1% (v/v)).

The pure chitosan film and the films containing the essential oil of *Melaleuca alternifolia* and *Origanum vulgare* showed similar results, being flexible, malleable and transparent to visible light. Qualitative and organoleptic analysis[21,22] showed that the films had a characteristic and predominant odor of the oils tested, as well as changes according to the concentrations they were incorporated into, as shown in Box 1.

According to the results presented, the inhibition halos against the microorganisms were 20.11 mm for *S. aureus* and 16.33 mm for *E. coli*[23]*.* Oregano essential oil showed antimicrobial action against Gram-positive and Gram-negative bacteria, such as *Escherichia coli* and *Staphylococcus aureus.* In this sense, the same occurred with the results of the research, with halos of inhibition against the three bacteria tested[24]. Inhibition halos were only found at 25% of the concentration of *Melaleuca alternifolia* essential oil, with inhibition halos ranging from 5 to 20 mm for the standard strain of *C. albicans*[25], as shown in Table 2.

Box 1. Organoleptic and qualitative analysis of the characteristics of chitosan biopolymers.

Sample	Color	Appearance	Odor	Homogeneity
Pure chitosan	Colorless	Plastic texture, partially malleable	Smooth	Homogeneous
FQ/TG	Slightly yellowish	Silky texture, malleable	Smooth	Partially homogeneous
FQ/TG /OEO	Yellowish	Silky, malleable texture	Predominantly oregano oil	Partially homogeneous
FT/TG/OEM	Dark yellow	Silky texture, pliable	Predominantly melaleuca oil	Partially homogeneous
FT/TG/OEO+OEM	Dark yellow	Silky texture, malleable	Predominantly oregano oil, mild melaleuca	Homogeneous

Caption: FQ (pure chitosan film 1.75%); FQ-TG (Chitosan film 1.75%+ Tween 80 + Glycerol); FQ/TG /OEO Chitosan film 1.75%+ Tween 80 + Glycerol + oregano essential oil 2% (v/v); FQ/TG /OEM Chitosan film 1.75%+ Tween 80 + Glycerol + melaleuca essential oil 2% (v/v); FQ/TG- OO+ OM (Chitosan film 1.75% + Tween 80 + Glycerol + oregano essential oil 1% (v/v) + melaleuca essential oil 1% (v/v).

Table 2. Antimicrobial activity of oregano essential oil antibiotics, solutions and films produced against the microorganisms tested.

	INHIBITION HALL (mm)*			
	S. aureus	*S. epidermidis*	*E. coli*	*C. albicans*
CONTROL				
OEO	IC	IC	31,77 ± 2,76	47,86 ± 1,52
OEM	7,25 ± 0,24	8,6 ± 0,36	7,09 ± 0,26	8,09 ± 0,28
OEO + OEM	7,70 ± 0,27	NA	8,69 ± 0,29	12,28 ± 0,35
Oxacilin	35,0 ± 0,32	32,0 ± 0,16	NA	NA
Cefoxitin	NA	NA	20,0 ± 0,11	NA
Itraconazole	NA	NA	NA	21,5 ± 0,21
SOLUTIONS/EMULSIONS				
QT Pure	6,0 ± 0,13	5,9 ± 0,08	5,9 ± 0,13	5,8 ± 0,07
QT/TG	6,0 ± 0,11	6,1 ± 0,07	5,8 ± 0,16	5,9 ± 0,12
QT/TG/OEO	9,9 ± 0,86	9,1 ± 0,68	8,5 ± 0,81	10,2 ± 0,37
QT/TG/OEM	IC	8,6 ±0,36	11,0 ±0,5	13,6 ±2,46
QT/TG/OEO+OEM	12,7 ± 0,53	8,1 ± 0,56	10,1 ± 0,46	9,0 ± 0,83
FILME				
QT Pura	ACMAF	ACMAF	ACMAF	ACMAF
QT/TG	ACMAF	ACMAF	ACMAF	ACMAF
QT/TG/OEO	ACMAF	ACMAF	ACMAF	ACMAF
QT/TG/OEM	ACMAF	ACMAF	ACMAF	ACMAF
QT/TG/OEB	ACMAF	ACMAF	ACMAF	ACMAF

* Mean and standard deviation. **Caption:** ACMAF (Absence of microbial growth below the film); CI (Complete inhibition); NA (Not applicable); OEO (Oregano essential oil 2% (v/v); OEM (Melaleuca essential oil 2% (v/v); OEO+OEM (Oregano essential oil 1%(v/v) + Melaleuca 1%(v/v); QT (Chitosan); QT/TG (Chitosan 1.75% + Tween 80 + Glycerol); QT/TG/OEO (Chitosan 1.75% + Tween 80 + Glycerol + Oregano essential oil 2% (v/v); QT/TG/OEM (Chitosan 1.75% + Tween 80 + Glycerol + Melaleuca essential oil 2% (v/v); QT/TG/OEO + OEM (Chitosan 1.75% + Tween 80 + Glycerol + Oregano essential oil 1% (v/v) + Melaleuca essential oil 1% (v/v).

CONCLUSION

Given the significant impact of HAIs, the search for new biodegradable materials that can act as effective protective barriers is becoming urgent, especially for wounds and the insertion of medical devices such as catheters. In this context, this study investigated the application of chitosan biopolymers associated with the essential oils of *Melaleuca alternifolia* and *Origanum vulgare*, focusing on their physicochemical properties and antimicrobial activity. The results showed that carvacrol, the main compound in oregano essential oil, and 3-carene, present in melaleuca oil, were effective in inhibiting the microorganisms tested, reinforcing the potential of these compounds as natural therapeutic agents.

FTIR analysis showed the presence of bands characteristic of chitosan and its additives (glycerol and Tween), as well as functional groups corresponding to the essential oils used, confirming their incorporation into the polymer matrix. Evaluation of the degree of swelling showed good hydrophilicity of the films, a desirable characteristic for dressings aimed at maintaining the moist environment necessary for healing. From an organoleptic point of view, oregano oil had a predominant odor, which may be relevant to the product's acceptance in its clinical application.

This study contributes directly to health promotion by offering a therapeutic alternative that combines safety, efficacy and sustainability, while minimizing the unnecessary use of antibiotics and, consequently, the risk of microbial resistance - one of the greatest challenges facing contemporary public health. The development of dressings with antimicrobial action incorporated from natural compounds represents an innovative strategy in line with global infection prevention guidelines.

From a practical point of view, the biomaterials developed have shown promise for various clinical applications, such as dressings for acute and chronic wounds, catheter coatings and post-operative protective barriers. The association of chitosan with natural essential oils results in a product with stable physicochemical properties, effective antimicrobial activity and lower environmental impact, strengthening the role of science in the development of technologies aimed at health, preventing infections and improving the population's quality of life. Therefore, the analyses demonstrated the possibility of developing a biomaterial based on chitosan associated with oregano and melaleuca essential oils that has physiochemical properties and a potential inhibitory effect against the bacteria and fungi that cause HAIs that were tested, making them promising for biomedical applicability.

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