

Antifungal effect of polymeric films containing the essential oil of *Schinus terebinthifolius* on *Candida albicans* biofilms

Efecto antifúngico de películas poliméricas que contienen aceite esencial de *Schinus terebinthifolius* en las biopelículas de *Candida albicans*

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Abstract

Introduction: *Candida albicans* is a commensal microorganism in humans, that may cause oral candidiasis in people with immune deficiencies or ill-fitting dentures. *C. albicans* is becoming more resistant to drugs used in its treatment. This makes it necessary to develop new medicines that do not damage the host cells. There are many studies on the antimicrobial activity of leaves, stem and roots of *Schinus terebinthifolius*, but few studies attend its fruits. The aim of this study is to investigate the antifungal effect of polymeric films of hydroxypropyl methylcellulose (HPMC) containing the essential oil of the fruits of *S. terebinthifolius* against biofilms of *C. albicans*.

Materials and methods: The oil was prepared from the fruits of *S. terebinthifolius* and incorporated into polymeric films of HPMC using concentrations of 0.146, 0.073 e 0.037 g.mL⁻¹. The minimum inhibitory concentration (MIC) was determined by micro dilution, wherein the minimum inhibitory concentration was 0.78%. The antifungal effect of the films was tested using agar in petri dishes and acrylic resin blocks, both contaminated with *C. albicans*.

Results: The polymeric films containing essential oil had average inhibition zones of 24 mm, 21 mm and 20 mm for the concentrations 0.037, 0.073 and 0.146 g.mL⁻¹, respectively.

Conclusion: The treated resin blocks showed no fungal growth afterwards, with the exception of the control blocks. The use of antimicrobial films containing natural products like *S. terebinthifolius* in the treatment of diseases such as oral candidiasis associated to prosthetic seems promising and with a decreased possibility of side effects.

Keywords: candidiasis, biological products, antifungal agents, oils, *anacardiaceae*, microbial sensitivity tests, dentures.



Efecto antifúngico de películas poliméricas que contienen aceite esencial de *Schinus terebinthifolius* en las biopelículas de *Candida albicans*

Resumen

Introducción: *Candida albicans* es un microorganismo comensal en humanos que puede causar candidiasis oral en personas con deficiencias inmunitarias o dentaduras mal ajustadas. *C. albicans* se está volviendo más resistente a los medicamentos utilizados para su tratamiento. Esto hace que sea necesario desarrollar nuevos medicamentos que no dañen las células huésped. Existen muchos estudios sobre la actividad antimicrobiana de hojas, tallo y raíces de *Schinus terebinthifolius*, pero pocos estudios se centran en sus frutos. El objetivo de este estudio es investigar el efecto antifúngico de las películas poliméricas de hidroxipropilmetilcelulosa (HPMC) que contiene el aceite esencial de los frutos de *S. terebinthifolius* en biopelículas de *C. albicans*.

Materiales y métodos: el aceite se preparó a partir de los frutos de *S. terebinthifolius* y se incorporó en películas poliméricas de HPMC con concentraciones de 0,146, 0,073 y 0,037 g.mL⁻¹. La concentración mínima inhibitoria (MIC) se determinó mediante microdilución, cuya concentración inhibitoria mínima fue de 0,78%. El efecto antifúngico de las películas se probó con agar en placas de Petri y bloques de resina acrílica, ambos de ellos contaminados con *C. albicans*.

Resultados: las películas poliméricas que contenían aceite esencial presentaron zonas de inhibición promedio de 24 mm, 21 mm y 20 mm para las concentraciones 0,037, 0,073 y 0,146 g.mL⁻¹, respectivamente.

Conclusión: los bloques de resina tratados no mostraron crecimiento fúngico posterior, con la excepción de los bloques de control. El uso de películas antimicrobianas que contienen productos naturales como *S. terebinthifolius* en el tratamiento de enfermedades como la candidiasis oral asociada a la prótesis parece prometedor y con menor posibilidad de que se desarrollen efectos secundarios.

Palabras clave: candidiasis, productos biológicos, agentes antifúngicos, aceites, *anacardiaceae*, pruebas de sensibilidad microbiana, dentaduras.

Efeito antifúngico de filmes poliméricos que contém óleo essencial de *Schinus terebinthifolius* nos biofilmes de *Candida albicans*

Resumo

Introdução: *Candida albicans* é um microorganismo comensal em humanos que pode causar candidíase oral em pessoas com deficiências imunológicas ou próteses mal ajustadas. *C. albicans* está se tornando mais resistente aos medicamentos usados, pelo qual é necessário desenvolver uns novos que não prejudiquem as células hospedeiras. Existem muitos estudos sobre a atividade antimicrobiana de folhas, caules e raízes de *Schinus terebinthifolius*, mas poucos estudos consideram seus frutos.

Objetivo: investigar o efeito antifúngico dos filmes poliméricos de hidroxipropilmetilcelulose (HPMC) contendo o óleo essencial dos frutos de *S. terebinthifolius* em biofilmes de *C. albicans*.

Materiais e métodos: preparou-se o óleo a partir dos frutos de *S. terebinthifolius* e incorporou-se em filmes poliméricos de HPMC com concentrações de 0,146, 0,073 e 0,037 g.mL⁻¹. A concentração inibitória mínima (CIM) foi determinada através de microdiluição, cuja concentração inibitória mínima foi de 0,78%. O efeito antifúngico dos filmes foi testado com ágar em placas de Petri e blocos de resina acrílica, ambos contaminados com *C. albicans*.

Resultados: os filmes poliméricos contendo óleo essencial apresentaram zonas de inibição médias de 24 mm, 21 mm e 20 mm para as concentrações 0,037, 0,073 e 0,146 g.mL⁻¹, respectivamente.

Conclusão: os blocos de resina não apresentaram crescimento fúngico posterior, com exceção dos blocos de controle. O uso de filmes antimicrobianos contendo produtos naturais, tais como *S. terebinthifolius*, parece promissor para o tratamento de doenças como a candidíase oral associada à prótese. Estes filmes reduzem a possibilidade de desenvolvimento de efeitos colaterais.

Palavras-chave: candidíase, produtos biológicos, antifúngicos, óleos, *anacardiaceae*, testes de sensibilidade aos antimicrobianos, próteses.



Introduction

Candidiasis is the most common fungal disease in humans. Amongst the *Candida* species, *Candida albicans* is the most frequent in the oral cavity. In the past, candidiasis was considered only an opportunistic infection, which affected patients debilitated by other diseases. However, today it is known that oral candidiasis can occur in relatively healthy people as a result of a complex interaction between host and microorganism [1]. Oral candidiasis is becoming prevalent in immunocompromised patients, [2] as well as in patients with ill-fitting dentures, and this occurrence has been strongly associated with denture-related stomatitis. Denture stomatitis is known to have a multifactorial etiology, but the presence of the fungi *C. albicans* is always strongly associated with it [3,4].

Dentures have been used by some populations in a time range of 25 to 48 months and present some difficulties to maintain: level of education and motivation are correlated with older adult population [5]. Even so, these are important predisposing factors of *candida* contamination because the acrylic has microporosities, most of which usually could cause candidiasis when associated with sub-optimal hygiene, acting as reservoirs of infections due to their porous nature [6]. It is believed that the ability of *Candida* spp., especially *C. albicans*, to adhere to acrylic surfaces may be important in the pathogenesis of the disease as adherence is apparently the initial step in microbial colonization and subsequent invasion of host surfaces.

Although diverse antifungal agents for the treatment of candidiasis have been used, failure of therapy is commonly found. The efficacy of treatment is dependent upon many factors such as the saliva and the cleansing action of the oral muscles. Thus, the therapeutic dose of antimicrobial agents does not reach the effective therapeutic concentration. In this manner, patients experience only a subtherapeutic antifungal concentration during treatment and the concentration of the drug may vary in different niches of the mouth. [7]

Due to the limited range of synthetic antifungal drugs available, coupled with *C. albicans* increase in drug resistance, as well as collateral effects experienced by some patients, some Brazilian plants claimed to have medicinal properties

and over the past few years, they have been and are still being studied to determine their possible antimicrobial properties. The essential oil extracted from some of these medicinal plants as well as the extract derived from *Schinus terebinthifolius* have been tested against some clinically relevant fungi, including *Candida* species, and antifungal activity has been reported against the microorganisms tested [8,9].

The Anacardiaceae family comprises more than 76 genera and 600 species with the genera *Schinus*, [10,11] being the most studied. It is native to South America and found mainly on the coast of Brazil. [12] *Schinus* genus includes about 29 species, [13] and the main species are *S. terebinthifolius* and *S. molle*. The essential oil of this plant is concentrated mostly in the fruits [14] and its properties are attributed to the presence of high levels of monoterpenes [15]. *S. terebinthifolius* is used in folk medicine (alternative), with experimentally proven effects such as healing, anti-inflammatory, antipyretic, antiseptic (bactericidal and bacteriostatic), anti-allergic, anti-fungal, analgesic effects, purifying agent and hemostatic as well. It has also been used to treat sexually transmitted diseases, uterine inflammation, urinary and respiratory tract infections, skin ulcers, rheumatism and gastrointestinal disorders [16-22]. Many parts of this plant such as the bark, leaves, roots and fruits have been studied, as well as various antimicrobial properties, which have been reported for many microorganisms such as *Candida* spp., *Botrytis* spp. and *Staphylococcus* spp. [23-26].

The aim of this study was to evaluate the antifungal properties of polymeric films of hydroxypropyl methylcellulose containing the essential oil of *Schinus terebinthifolius* against biofilms of *Candida albicans*.

Materials and methods

S. terebinthifolius essential oil extraction

Because *S. terebinthifolius* is a native plant and due to the difficulty in finding producers, we chose to always buy the pepper samples from the same distributor. The extraction of the essential oil was done by the hydrodistillation method. The fruits were grinded and placed in dichloromethane

(CH₂Cl₂) for five days to extract the oil. After this period, the mixture was brought to a rotary evaporator at a temperature of 40 °C to evaporate the dichloromethane, thereby yielding the pure essential oil which prevents the loss of compounds sensitive to high temperatures [27].

Determination of the Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined following guidelines from the Clinical and Laboratory Standards Institute. [28] *C. albicans* strains (ATCC 18804) were incubated in Sabouraud Dextrose broth at 34 °C in aerobic conditions for 24 h. After that, the incubated strains were diluted with 0.9% sterile saline solution to obtain an inoculum of 10⁸ CFU/mL and dispersed into a 96 well plate containing Sabouraud Dextrose broth. With an initial concentration of 0.072 g·mL⁻¹ of the *S. terebinthifolius* essential oil, a serial dilution was made in triplicate. A negative control was made using only Sabouraud broth; while a positive control was made using Sabouraud broth and the inoculum of *C. albicans* strains, to ascertain the viability of the fungi strains as well as to use it as a standard for comparison. The plates were incubated for 24 h in aerobic conditions at 34 °C; this was defined as the minimum inhibitory concentration in absence of fungal growth.

Polymeric films of HPMC containing the essential oil of *S. terebinthifolius* formation

Three concentrations of the *S. terebinthifolius* essential oil were used to make the HPMC films: 0.146, 0.073 and 0.037 g·mL⁻¹. For example, to make an HPMC film containing a concentration of 0.146 g·mL⁻¹ *S. terebinthifolius* essential oil, 0.3 g of the powder of HPMC were dissolved in a total volume of 5 mL of distilled water (75%) and propylene glycol (25%). 5 mL of the pure essential oil of *S. terebinthifolius* and 20 µL of citric acid, a conservative, were added to the solution and mixed thoroughly while being heated at a temperature of 60 °C for 30 minutes. A control film was made by dissolving 0.3 g of HPMC powder into 7.5 mL of distilled water, 2.5 mL of propylene glycol and 20 µL of citric acid. All concentrations were mixed for about 30 minutes at a temperature of 60 °C after

which the mixtures were poured into petri dishes and allowed to dry at a temperature of 37 °C for 24 h. The dried films were then cut into square pieces of dimensions 4 x 4 mm each.

Antifungal test using polymeric films of HPMC containing *S. terebinthifolius* essential oil

Strains of *C. albicans* were previously cultivated a day before and adjusted to an inoculum of 10⁸ CFU/mL. 100 µL of this inoculum was spread on different petri dishes containing Sabouraud Dextrose Agar each, in sixplicate. The HPMC films with the different concentrations of the essential oil of *S. terebinthifolius* previously prepared, and the control films were placed on the three contaminated agar surfaces, each petri dish containing the four different films. The dishes were incubated for 24 h in aerobic conditions at 34 °C. The inhibition zone was defined as the largest diameter, the mean of the data was determined and the results were evaluated applying Kruskal-Wallis test.

Antifungal test using polymeric films of HPMC containing essential oil of *S. terebinthifolius* in resin blocks

The antifungal activity of the films was also tested using acrylic resin blocks previously contaminated with strains of *C. albicans*. Strains of *C. albicans* were initially incubated in Sabouraud Dextrose broth in aerobic conditions at 34 °C for 24 h. After 24 h, sterilized square acrylic resin blocks of size 6 x 6 x 4 mm were placed into the test tube containing the already cultivated strains of *C. albicans*. Enough Sabouraud Dextrose broth was added to the test tube to cover all the blocks. The content of the test tube was homogenized and the test tube was incubated for another 24 h. Following those 24 h, each contaminated acrylic resin block was washed gently with sterile saline solution to remove unattached strains while not disturbing the biofilm formed around the blocks.

These blocks were covered on every side with the polymeric films containing the different concentrations of *S. terebinthifolius*, stored in Sabouraud Dextrose broth and incubated for 24 h in aerobic conditions at 34 °C. Each concentration was coated around three different contaminated acrylic

resin blocks. The control films were coated around three contaminated blocks and three sterilized blocks each. After 24 h, the blocks were removed from the films and placed in a different sterile test tube containing Sabouraud Dextrose broth and incubated for another 24 h in aerobic conditions at 34 °C.

After the 24 h, the blocks were each placed gently in sterile test tubes containing sterile saline solution. The blocks were placed in an ultrasonic machine for 10 minutes to shake off the unattached fungal strains. Afterwards, blocks were placed on petri dishes containing sterile Sabouraud Dextrose Agar and incubated for 24 h at 34 °C in aerobic conditions to check for fungal growth.

Citotoxicity study

3T3-L1 cells (murine fibroblast) were cultured in DMEM (Dulbecco's Modified Eagle Medium) high glucose supplemented with 10% FBS (fetal bovine serum) and 1 % antibiotic / antimycotic solution. These cells were maintained in an incubator at 37 °C with 5% CO₂.

Polymeric HPMC films containing the essential oil in three concentrations were used to obtain the eluates. To achieve this, the HPMC films with dimensions of 4 x 4 mm were put into culture medium each (the same used for the cultivation of the cells to be tested). The supernatant was collected after 24 h of its release in an incubator at 37 °C with 5% CO₂. This material was kept frozen in a -20 °C freezer until the completion of the cell cytotoxicity assays.

The treatments were distributed in triplicate in serial dilutions starting from a 1:1 dilution totaling 18 points, only dilutions with significant results are represented graphically. The treatments lasted for 24 h and the assessment of cellular cytotoxicity was performed using the MTT colorimetric assay according to the manufacturer's recommendations. The readings of the plates were made by a reader at a wavelength of 570 nm. The construction of graphs and statistical analysis were performed with the aid of GraphPad Prism 6. The results were expressed as mean ± standard error of the mean and compared with the control groups. Analysis of variance were performed, one-way ANOVA followed by Tukey's as a post-test, the differences were considered significant at $p < 0.05$.

Results

Essential oil extraction of *S. terebinthifolius* and Minimum Inhibitory Concentration

In this study, the antifungal activity of the essential oil obtained from the fruits against *C. albicans*, using dichloromethane as a solvent, was observed. The minimum inhibitory concentration found in this study was 0.0078 g/mL.

Antifungal test using polymeric films of HPMC containing the essential oil of *S. terebinthifolius*

All the polymeric films of HPMC containing the different concentrations of the essential oil of *S. terebinthifolius* had an inhibitory effect on *C. albicans* strains. However, the 0.146 g.mL⁻¹ concentration was the most significantly active (Table 1).

Table 1. Inhibition zones after placing the polymeric films of HPMC containing the different concentrations of essential oil of *Schinus terebinthifolius* against *C. albicans*.

Polymeric films with different concentrations of essential oil	Inhibition zones
0.146 g.mL ⁻¹	25 ± 1.2 mm*
0.073 g.mL ⁻¹	21 ± 1.7 mm
0.037 g.mL ⁻¹	20 ± 0.7 mm

* Statistical difference ($p < 0.05$)

Source: own work

Antifungal films inhibition of HPMC containing the essential oil of *S. terebinthifolius* in association with contaminated acrylic resin blocks

None of the acrylic resin blocks contaminated with the strains of *C. albicans* had any fungal growth after they were treated with the polymeric films of *S. terebinthifolius*, washed with saline solution and incubated on Sabouraud Dextrose Agar for 24 h. Only the control blocks, which have not been treated with the polymeric films containing the essential oil, showed fungal growth.

Cytotoxicity assay

The cytotoxicity of polymeric films of HPMC containing the different concentrations of the essential oil of *S. terebinthifolius* was evaluated by cell viability assay. The fibroblasts maintained cell viability above 50% in dilution 1:64 in the concentration of 0.146 g.mL⁻¹, in dilution 1:32 in the concentration of 0.073 g.mL⁻¹ and in dilution 1:16 in the concentration of 0.037 g.mL⁻¹ (Fig. 1). The 0.037 g.mL⁻¹ in dilution 1:16 was not cytotoxic and the difference was significant in relation to the other two groups analyzed (*p<0.05).

Discussion

There are many studies about the antifungal effect of the essential oils from the leaves, bark [29] and roots of *S. terebinthifolius*. Johann et al. [30] showed that the leaves of *S. terebinthifolius* were able to inhibit the growth of three different strains of *C. albicans* isolated from the mouth of patients with denture related stomatitis, with the MIC showing to be 7.81 g/mL. Johann et al. [31] then noted that the MIC of the essential oil of the leaves and bark of *S. terebinthifolius* against three strains of *P. brasiliensis* was 30 µg/mL for all the strains tested. Alves et al. [29] had a MIC of 0.3125 g/mL against *C. albicans* using the tincture from the bark of *S. terebinthifolius*. However, there are very few studies about the antifungal effect of the essential oil from its fruits.

In this study, an antifungal activity of the essential oil obtained from the fruits was found against *C. albicans* using dichloromethane as a solvent. This result contrasts the results of a study by Degáspari et al. [32] where the alcoholic and aqueous extract of the fruits of *S. terebinthifolius* were tested against some microorganisms including *C. albicans* and inhibition zones were only observed with the alcoholic extract against two bacteria only. Neither the alcoholic nor the aqueous extract in that study exhibited any antifungal activity against *C. albicans*. The antifungal activity encountered in our study could be due to differences in both the strains of fungi and fruits used, since composition of the fruits especially vary in accordance with many factors, such as the place and season of harvest. Our positive results may have also been due to the polymeric films of HPMC used in our study,

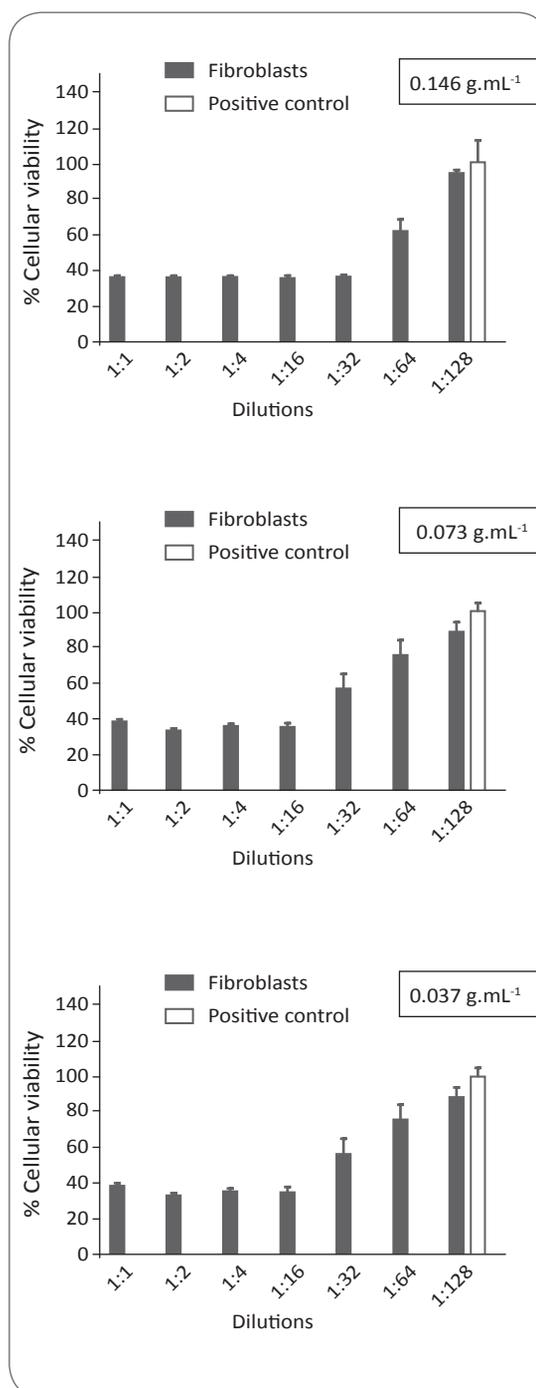


Figure 1. Cytotoxicity effect of eluate of HPMC films containing essential oil from mature fruits of *S. terebinthifolius* in concentrations of 0.146 g.mL⁻¹, 0.073 g.mL⁻¹, 0.037 g.mL⁻¹ in 3T3-L1 fibroblasts (*p<0.05).

Source: own work

which ensured a slow, prolonged and steady liberation of the active compounds of the essential oil, exposing the fungi to it for a long period.

Oliveira et al. [23] tested the antifungal activity of the essential oil of the fruits of *S. terebinthifolius* against *Colletotrichum gloeosporioides* and an inhibition of 79.07% of the fungal growth was noted using a concentration of 0.5%. In addition, it was also noted that the higher the concentration, the higher the inhibition of fungal growth. Our study proved the opposite with the greatest inhibition of fungal growth occurring with the lowest concentration used which was 12.5%. These results further demonstrate that the essential oil of *S. terebinthifolius* in conjunction with the polymeric films of HPMC does have an antifungal effect against biofilms of *C. albicans*.

Some reasons for the popularity of HPMC for extended release coatings, are that the water-soluble HPC may act as a pore former, [33] soluble in water and able to accommodate high levels of drug loading. Moreover, the variations in processing show little effect on component composition [34]. Due to the oil's viscosity, getting a homogenous solution most of the time proved difficult. All the same, the results from this study proved promising since the use of the polymeric films helped maintaining a steady supply of the active compounds from the essential oil, ensuring that the oil was in contact with the fungi for a prolonged period.

Conclusion

This study proved that the polymeric films containing the essential oil were able to inhibit the growth of the strains of *C. albicans*, as well as the biofilms attached to the contaminated acrylic resin blocks, showing to be a promising method for treating denture-related Candidiasis, considering the antifungal activity of this plant as well as its vast availability in nature.

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