

IN VITRO ACTIVITY OF *SCHINUS TEREBINTHIFOLIUS* (BRAZILIAN PEPPER TREE) ON *CANDIDA TROPICALIS* GROWTH AND CELL WALL FORMATION

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ABSTRACT

The aim of this study was to evaluate the *in vitro* antifungal activity of *Schinus terebinthifolius* (Brazilian pepper tree) tincture on planktonic *Candida tropicalis* (ATCC 40042), which is a microorganism associated to oral cavity infections. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) were determined through the microdilution technique. Possible action of the tincture on fungal cell wall formation was also studied by adding an osmotic protector (0.8M sorbitol) to the microplates. Nystatin was used as standard con-

trol and tests were performed in triplicate. *S. terebinthifolius* was found to have MIC and MFC values of 625 µg/mL on the strain assayed, whereas nystatin showed MIC and MFC of 6.25 µg/mL. Results suggest that *S. terebinthifolius* tincture acts on fungal cell walls, since the sorbitol test indicated a MIC of 1.250 µg/mL. It may be concluded that *S. terebinthifolius* has potential *in vitro* antifungal activity against *C. tropicalis* strains, and probably acts by inhibiting fungal cell wall formation.

Key-words: *Candida tropicalis* - Anacardiaceae - natural products.

ATIVIDADE IN VITRO DE *SCHINUS TEREBINTHIFOLIUS* (AROEIRA) SOBRE O CRESCIMENTO E FORMAÇÃO DA PAREDE CELULAR DE *CANDIDA TROPICALIS*

RESUMO

O objetivo deste estudo foi avaliar *in vitro* a atividade antifúngica da tintura de *Schinus terebinthifolius* (aroeira) sobre *Candida tropicalis* (ATCC 40042) planctônica, que é um microrganismo associado a infecções na cavidade oral. A Concentração Inibitória Mínima (MIC) e a Concentração Fungicida Mínima (CFM) foram determinadas por meio da técnica de microdiluição. Também foi investigada uma possível ação da tintura na formação da parede celular de fungos, adicionando-se um protetor osmótico (sorbitol 0,8M) nas microplacas. Nistatina foi utilizada como controle e os testes foram realizados em triplicata. A tintura de *S. terebinthi-*

folius apresentou MIC e MFC de 625 µg/mL sobre a cepa ensaiada, enquanto que a nistatina mostrou MIC e MFC de 6,25 µg/mL. Os resultados sugerem que *S. terebinthifolius* atua sobre a parede celular fúngica, uma vez que o teste com sorbitol indicou uma MIC de 1.250 µg/mL. Pode-se concluir que *S. terebinthifolius* apresentou, *in vitro*, atividade antifúngica potencial contra a cepa de *C. tropicalis*, e provavelmente atua inibindo a formação da parede celular fúngica.

Palavras-chave: *Candida tropicalis* - Anacardiaceae - produtos biológicos.

INTRODUCTION

Candida species are commonly found in the oral cavity of healthy individuals. These yeasts usually inhabit the host as saprophytic microorganisms, establishing an ecological balance in the oral microbiota. Changes in such balance and the presence of predisposing factors result in the disruption of organic integrity and the modification of the yeast-like conformation into a fusiform shape, which is pathogenic and causes candidiasis¹⁻³.

Oral candidiasis may be caused by different species of the genus *Candida*, including *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. guilliermondii*⁴.

Mycotic studies have drawn attention to the increasing importance of non-*albicans* species, especially *C. tropicalis*, as a pathogenic microorganism that is responsible for several diseases in human beings. *C. tropicalis* has been described as the major cause of candidiasis induced by non-*albicans* species, and

has frequently been found in samples from the oral cavity of HIV⁺ patients, showing oropharyngeal candidiasis in 18% of patients wearing prosthesis, but present in healthy individuals as well⁵.

Considering the group of non-*albicans* species, *C. tropicalis* has become an emerging worldwide pathogen. The major factors responsible for such emergence are: (1) increasing use of antifungal agents; (2) increasing number of immunocompromised individuals; (3) prolonged use of catheters; (4) use of broad-spectrum antibiotics; and (5) complications in the treatment of subclinical underlying conditions along with intolerance to antifungal drugs. In addition, drug resistance in immunocompromised patients who survive longer has been increasing together with mortality rate⁶.

Taking into account the resistance of *Candida* genus yeasts to current antifungal agents, it may be inferred that studies searching for new plant antifungal compounds are increasingly relevant. It is possible to observe the antibiotic potential of plant species, and consequently, the feasibility of applying them to the prevention and treatment of fungal diseases⁷.

Schinus terebinthifolius is a small to medium-sized tree widely present in many continents, including America, Africa and Oceania⁸. It is native to South America, especially Brazil, Paraguay and Argentina, and commonly known as beach-, white- or red-pepper tree, among other designations. It belongs to Anacardiaceae family and is well known for its astringent, antiseptic and anti-inflammatory activities according to folk medicine and use in cosmetic formulations⁹.

The Brazilian pepper tree has also shown an anti-ulcer effect, and is used as an antiseptic and in the treatment of stomatitis. Those properties are owed

to its stabilizing effect on the membrane and anticholinergic and anti-histaminic action¹⁰.

The aim of this study is to evaluate the antifungal activity of *S. terebinthifolius* tincture (Brazilian pepper tree) on *Candida tropicalis*, establishing a possible mechanism of antimicrobial action.

MATERIALS AND METHODS

Research Center

Microbiological assays were performed at the Oral Microbiology Laboratory of the Nucleus of Tropical Medicine, Center for Health Sciences, Federal University of Paraiba, Joao Pessoa, Brazil.

Fungal strain and preparation of inoculum

The *Candida tropicalis* strain (ATCC 40042) was provided by the National Institute of Quality Control in Health at Oswaldo Cruz Foundation (NIQCH).

The yeast was routinely grown in Sabouraud dextrose agar plates incubated at 35°C for 24h from stock cultures in SDB-glycerol and stored at 4°C during the procedures. In the antimicrobial trial, fungal inoculum was standardized to approximately 5.0 x 10⁶ CFU/ml according to the 0.5 McFarland turbidity range (CLSI Clinical and Laboratory Standards Institute - NCCLS).

Study product

The study product was a tincture from the stem bark of *Schinus terebinthifolius*, which was provided by a compounding pharmacy in Joao Pessoa, Paraiba, Brazil. The product, detailed in Table 1, met all required specifications concerning quality control according to the technical survey provided by the supplier.

Determination of the Minimum Inhibitory Concentration (MIC)

The MIC determination of the *S. terebinthifolius* tincture was performed by the microdilution technique proposed by Ellof (1998)¹¹, using 96-well U-bottom microtiter plates (ALAMAR[®], Diadema, Sao Paulo, Brazil). Initially, 100 µL of Sabouraud Dextrose Broth (SDB, HIMEDIA[®], Sao Paulo, Brazil) doubly concentrated were placed in each well on the plates. Then 100 µL of the *S. terebinthifolius* tincture were added at an initial concentration of 5,000 µg/mL. Serial dilutions were prepared from these concentrations by taking 100 µL from the most concentrated well and placing it in the following well. Concentrations then ranged from

Table 1: Characterization of the experimental product.

Scientific name	<i>Schinus terebinthifolius</i>
Common name	Brazilian pepper tree
Origin	Stem bark
Pharm. Form	Tincture
Initial Concentration	100 mg.mL ⁻¹
Physical-chemical characteristics	pH: 4.99, Soluble in water, Density: 0.910 g/mL Extractor liquid: hydroalcoholic solution Alcohol strength: 60° GL, Dry residue: 2.0%
Phytochemical profile	Triterpenes; flavonoids; steroids; tannins and saponins.



5,000 µg/mL to 39 µg/mL. Finally, 10 µL aliquots of inoculum were placed into the wells of each column. At the same time, yeast viability was checked and yeast susceptibility verified by using powdered nystatin at an initial concentration of 100 µg/mL, ranging up to 0.78 µL/mL.

Tests were performed in triplicate, and the plates were incubated at 35°C for 48 hours. The MIC of the tincture on the yeast strain was determined visually. The formation or non-formation of cell clusters (“buttons”) at the bottoms of the wells was considered. MIC was taken as the lowest concentration of the study product capable of producing visible inhibition of the growth of the yeast strain used in the microbiological assay¹¹.

Determining Minimum Fungicidal Concentration (MFC)

After the MIC determination, the inhibitory and two following higher concentrations as well as the positive controls were sub-cultured on plates containing Sabouraud Dextrose Agar, in triplicate. The MFC readings were based on the growth of the controls after 24 hours incubation at 35°C. MFC was taken as the lowest drug concentration that hindered visible growth of the sub-culture.

Effect of *S. terebinthifolius* on fungal cell wall

MIC of *S. terebinthifolius* tincture in presence of sorbitol (0.8M) was determined using the microdilution technique¹¹, in triplicate. To each well, we added 100 µL SDB previously supplemented with sorbitol, with molecular weight 132.17 g (Compounding pharmacy, Joao Pessoa, Paraiba, Brazil), both doubly concentrated. Subsequently, 100 µL of the tincture were also placed to the wells in the first row of the plate. Through a serial dilution at a ratio of two, concentrations were obtained ranging from 5,000 µL/mL to 39 µL/mL of the *S. terebinthifolius* tincture and, in relation to sorbitol, a final concentration of 0.8 M was obtained in each well. Finally, 10 µL of inoculum were added to each well.

Microorganisms were controlled by placing 100 µL of doubly concentrated DSB plus sorbitol (0.8M) and 10 µL of inoculum in the wells. Sterility was controlled by using the same culture medium and sorbitol without fungal suspension. Plates were incubated at 35° C for 48h, after which they were read¹².

Data were tabulated and analyzed using descriptive statistics.

RESULTS

Table 2 shows the MIC and MFC of *S. terebinthifolius* tincture on *Candida tropicalis*. MIC was considered as the lowest product concentration capable of inhibiting fungal growth, whereas MFC was the lowest concentration capable of eliminating the fungus.

Table 3 shows the MIC values of the *S. terebinthifolius* tincture in presence or absence of sorbitol (osmotic protector/O.8M) on *C. tropicalis*. This study shows *S. terebinthifolius* might act upon fungal cell wall synthesis.

DISCUSSION

Plants represent a substantial source of biologically active natural products, many of which have been used as models for the synthesis of a number of drugs. In Brazil, there are about 100,000 plant species cataloged, but only 8% have been studied chemically, and it is estimated that only 1,100 species have been assessed as regards therapeutic properties¹³.

Traditional, folk or popular usages are not enough for ethical validation of medicinal plants as safe and effective drugs, i.e., medicinal plants do not differ from any other synthetic xenobiotic. Their recommendation or the official authorization for use must be based on experimental evidence, which attests that the risks to which users are exposed are outweighed by the expected benefits. Their use must be previously validated, i.e., their expected action must be proven and their potential toxicity to humans must be evaluated, as required for any drug¹⁴.

Brazil has been involved in establishing guidelines for using medicinal plants or drugs, meeting safety

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) in µg/mL of the *Schinus terebinthifolius* (Brazilian pepper tree) tincture on *Candida tropicalis*. Values are expressed in µg/mL.

Strain	<i>S. terebinthifolius</i>		Nystatin	
	MIC	MFC	MIC	MFC
<i>C. tropicalis</i> (ATCC 40042)	625	625	6.25	6.25

Table 3: Action of *Schinus terebinthifolius* tincture on *C. tropicalis* cell wall. Values are expressed in µg/mL.

Strain	<i>Schinus terebinthifolius</i>	
	MIC – without sorbitol	MIC – with sorbitol
<i>C. tropicalis</i> (ATCC 40042)	625	1250





requirements and considering the policy established for the rational use of drugs¹⁵.

The findings of our research are of great importance because the *Candida* species evaluated has been indicated as pathogenic in cases of oral candidiasis, especially in immunocompromised patients^{16,17}.

The susceptibility of strains of *C. albicans*, *C. tropicalis* and *C. krusei* to nystatin was evaluated in the study by Alves and Cury (1992)¹⁸. They found MIC and MFC values ranging from 0.5 to 8.0 µg/mL and 8 to 64.0 µg/mL, respectively. These data were similar to what was found in this study, wherein nystatin had MIC and MFC values of 6.25 µg/mL against *C. tropicalis*.

Freires et al. (2011)¹⁹ studied the action of 10% *S. terebinthifolius* tincture on *C. tropicalis* by means of the disc diffusion test. They found mean zones of inhibition measuring 25.32 mm promoted by the tincture, compared to 33.32 mm corresponding to the control (nystatin).

A study by Alves et al. (2009)²⁰ using the agar diffusion method found *in vitro* antifungal activity of *S. terebinthifolius* hydroalcoholic extract against *C. albicans*, with MIC of 1:8. On *C. tropicalis* and *C. krusei*, MIC was 1:16.

Nevertheless, in the abovementioned studies^{19,20} antifungal activity was assayed by solid medium diffusion method, which differs from this study, in which microdilution was used, hampering comparisons between studies.

The hydroalcoholic extract of Brazilian pepper was found to have a healing effect on cystotomies in rats²¹. Soares et al. (2007)²² demonstrated effective *in vitro* action of the tincture at 20% in the decontamination of toothbrushes contaminated with *Streptococcus mutans*. These findings highlight the anti-inflammatory and antimicrobial properties of *S. terebinthifolius* established in the literature.

A recent study²³ evaluated the anti-inflammatory and healing effects of the hydroalcoholic extract of *S. terebinthifolius* at 30% orabase. To do so, 60 Wistar rats were submitted to electrically produced sores on the skin and back. By means of macroscopic and microscopic examination, the authors showed that if the product was used daily, it had both anti-inflammatory and healing activity.

MIC values of the *S. terebinthifolius* tincture in presence or absence of sorbitol against *C. tropicalis* were also recorded, suggesting that sorbitol protects cells from the tincture's inhibitory effects, once changes

occurred in the MIC of the product under evaluation. The control using sorbitol guaranteed the reliability of the results and methods employed since the strain was capable of growing in presence of sorbitol and absence of the experimental product. These outcomes suggest that the antifungal activity of *S. terebinthifolius* tincture somehow involves its direct interaction with the fungal cell wall.

The test with sorbitol performed in this study, is based on the extent and capacity of the damage that substances with antifungal activity have on the components of the fungal cell wall. If the product acts somehow on fungal cell wall, it will cause cellular lysis in absence of an osmotic stabilizer. This assay therefore compares the MIC of antifungal agents in absence and presence of 0.8 M sorbitol, which is, as mentioned above, an osmotic protector used to stabilize fungal protoplasts²⁴.

According to findings of this research, *S. terebinthifolius* may have a mechanism of action on fungal cell wall, since tincture MIC was 625 µg/mL in absence of sorbitol, and 1,250 µg/mL in its presence, proving that *S. terebinthifolius* activity was reduced in presence of sorbitol, which hampered the effect of the test product against fungal cell wall. Protection with sorbitol is a test that has a wide range of possibilities, detecting not only agents which interfere with the synthesis of cell wall polymers and surrounding areas, but also regulatory mechanisms involved in these processes, which are complementary to microscopic observation of malformations detected in fungal strains analyzed previously²⁵.

This study used stem bark because it is the part used in most studies testing antimicrobial and anti-inflammatory activities^{19,22,26}. *S. terebinthifolius* bark contains numerous chemical compounds, such as flavonoids, tannins, and triterpenes. These constituents are considered to be responsible for the antibacterial, antifungal and anti-inflammatory action, making the plant widely used in the treatment of infectious and inflammatory diseases²⁷. Tannins have the ability to precipitate proteins from surface cells of tissues and mucous membranes, creating a protective layer (tannin complex / protein) on the damaged skin or mucosa, in addition to having high antimicrobial activity²⁸. Flavonoids have proven anti-inflammatory and antioxidant activity²⁹, and triterpenes have an anti-inflammatory property as they act as phospholipase A2 competitive inhibitors³⁰.





The clinical efficacy of *S. terebinthifolius* tincture was tested to treat denture stomatitis. Authors selected 18 users of removable prostheses diagnosed with denture stomatitis type II and presence of candidiasis associated with prosthesis, confirmed by clinical and mycological examinations. All patients were instructed to sanitize the prosthesis using a brush and dentifrice, and then apply the product on the palatal mucosa and prosthesis surface 3 times daily for 15 consecutive days. They concluded that the tincture was effective in the treatment of denture stomatitis, providing remission of the inflammatory process and infection by *Candida* spp. Their results were similar to the traditional therapy for this disease. Nevertheless, this clinical trial refers to the need for further studies researching the long-term success of the therapy with this plant²⁶.

It is worth noting that this study is an initial assessment of the determination of the antifungal activity

of *S. terebinthifolius* tincture, which points to the need for other pre-clinical trials, including toxicological evaluation.

The findings of this study allow the continuity of research on *S. terebinthifolius*, in view of its potential antifungal activity against *C. tropicalis* and thus applicability in the medical/dental fields. Accordingly, it is shown to be an alternative for the treatment of oral candidiasis that may overcome the shortcomings of the synthetic agent (i.e. Nystatin) regarding cost, substantivity, treatment extension, adverse effects and above all, microbial resistance. Clinical studies are suggested to further research the advantageous effects of *S. terebinthifolius* on the treatment of superficial candidiasis.

Given the above, *Schinus terebinthifolius* (Brazilian pepper tree) has *in vitro* antifungal activity (fungistatic and fungicidal) against *Candida tropicalis*, and probably acts by inhibiting fungal cell wall formation.

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