Activity of the aqueous extract of *Schinus terebinthifolius* Raddi on strains of the *Candida* genus

Atividade do extrato aquoso de Schinus terebinthifolius Raddi sobre cepas do gênero Candida

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Abstract

Objectives To evaluate the antifungal susceptibility profile of the aqueous extract of the bark of *Schinus terebinthifolius* Raddi against the strains of the genus *Candida*.

Methods By using the disk diffusion method, 50 samples of the genus *Candida* (*Candida albicans*; *Candida krusei*; *Candida glabrata*; and *Candida tropicalis*), isolated from patients receiving treatment at Hospital Santa Casa de Misericórdia de São Paulo, and 1 American Type Culture Collection (ATCC) sample of each species were tested against: the isolated aqueous extract of the bark of *Schinus terebinthifolius* Raddi, isolated nystatin, and the association of nystatin and the aqueous extract of *Schinus terebinthifolius* Raddi.

Results There were no significant differences regarding the different strains of *Candida* tested. In the presence of the aqueous extract of *Schinus terebinthifolius* Raddi, no inhibition halo was visible. Isolated nystatin formed an inhibition halo measuring respectively 18.50 mm and 19.50 mm for the *Candida albicans* species and the others referred to as non-*Candida albicans* (*Candida krusei*; *Candida glabrata*; and *Candida tropicalis*). The association of nystatin and the aqueous extract of *Schinus terebinthifolius* Raddi resulted in inhibition halos measuring 14.25 mm and 16.50 mm respectively. The comparisons of these results are statistically significant ($p < 0{,}001$).

Conclusion The aqueous extract of *Schinus terebinthifolius* Raddi showed no antifungal activity in vitro against the strains tested, whereas the association of nystatin and the aqueous extract of *Schinus terebinthifolius* Raddi caused a decrease in the inhibition halo when compared with isolated nystatin.

Keywords

- anacardiaceae
- candida
- nystatin
- medicinal plants
- phytotherapeutic drugs

Resumo

Objetivos Avaliar o perfil de susceptibilidade antifúngica do extrato aquoso das cascas de *Schinus terebinthifolius* Raddi frente às cepas do gênero *Candida*.

Métodos Por meio do método de difusão em disco, 50 amostras do gênero *Candida* (*Candida albicans, Candida krusei, Candida glabrata e Candida tropicalis*) provenientes de pacientes do Hospital da Santa Casa de Misericórdia de São Paulo, e 1 amostra

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American Type Culture Collection (ATCC) de cada espécie foram testadas frente ao extrato aquoso das cascas de *Schinus terebinthifolius* Raddi isolado, nistatina isolada, e a associação da nistatina ao extrato aquoso de *Schinus terebinthifolius* Raddi.

Resultados Não houve diferenças significantes em relação às diferentes espécies de cepas de *Candida* testadas. O extrato aquoso de *Schinus terebinthifolius* Raddi não formou halo de inibição. A nistatina isolada formou halo de inibição de 18,50 mm e 19,50 mm respectivamente para as espécies *Candida albicans* e as demais nomeadas como não *Candida albicans* (*Candida krusei, Candida glabrata e Candida tropicalis*). A associação da nistatina ao extrato aquoso de *Schinus terebinthifolius* Raddi resultou no halo de inibição de 14,25 mm e 16,50 mm respectivamente, sendo que as comparações destes resultados são estatisticamente significantes ($p < 0,001$).

Conclusão O extrato aquoso de *Schinus terebinthifolius* Raddi não demonstrou propriedade antifúngica in vitro frente às cepas testadas, e a associação da nistatina ao extrato aquoso de *Schinus terebinthifolius* Raddi causou a diminuição do halo de inibição quando comparado à nistatina isolada.

Palavras-Chave
- anacardiaceae
- candida
- nistatina
- plantas medicinais
- medicamentos fitoterápicos

Introduction

Vulvovaginal candidiasis (VVC) is caused by opportunistic yeasts that may cause infections of the mucosa, for they inhabit the gastrointestinal and genitourinary tracts in the human species. These microorganisms are commensal inhabitants, but can become pathogenic in certain conditions that alter the vaginal environment.[1] It is estimated that ~ 75% of women will present with an episode of infection during their lifetime, around 40% will experience a second episode, and ~ 5–8% will present with episodes of recurrence, defined usually as the occurrence of four or more episodes of VVC over a 12-month period.[2,3]

Studies show that 80 to 90% of the mycoflora is comprised of *Candida* (*C.*) *albicans*, but there are other species, such as *C. glabrata* (9 to 15% of cases) and *C. tropicalis* (up to 15% of cases).[4] Of the VVC cases, 85 to 95% are caused by *C. albicans*, whereas 10 to 20% are caused by *C. glabrata*, *C. tropicalis* and *C. krusei*.[5]

Infection of the vaginal mucosa by yeast can become systemic, and symptoms range from mild to severe fever, and may evolve to a rapid septic shock. These more severe cases are more common in immunosuppressed patients, and are often associated with high mortality.[6]

Before treating any supposedly fungal infection, it is advisable to identify the etiological agent, for most antifungal drugs are inactive against bacteria, and have a narrow antifungal spectrum.[7]

One of the drugs indicated for treating VVC caused by the *C. albicans* species is nystatin, which is produced by fermentation with the bacterium *Streptomyces noursei*, and has a higher affinity for ergosterol, the major component of fungal cell membranes, than it has for the cholesterol present in human cell membranes. This affinity produces pores in the cell membrane of the fungus, allowing the outflow of cytosolic components and leading to death.[8,9] According to Sheppard and Lampiris,[10] nystatin has been shown to be active against most *Candida* species, and is generally used for suppressing local infections; nevertheless, treatment is only effective if it is administered for a long enough period of time.

Although there are new drug options for treating candidiasis, there are recent studies evaluating the action of nystatin, and even establishing a synergy of nystatin with other substances.[11–13]

Schinus terebinthifolius Raddi (*S. terebinthifolius* Raddi), popularly known as beach Brazilian pepper tree ('aroeira da praia'), "tame" Brazilian pepper tree ('aroeira mansa'), red Brazilian pepper tree ('aroeira vermelha'), pink pepper, or 'cambuí', is a species native to South America, belonging to the Anacardiaceae family.[14,15] In Brazil it is found in an area spanning from the state of Rio Grande do Norte, in the north, all the way down to the state of Rio Grande do Sul, in the south.[16] In France, the fruits are known as *poivre rose*, and are widely used in French cuisine.[17]

S. terebinthifolius Raddi is on the list of 71 medicinal plants made by the Brazilian Ministry of Health as being of interest to the Brazilian Unified Health Care System's National List of Medicinal Plants of Interest (SUS – RENISUS, in the Portuguese acronym). On this list are medicinal plants that have the potential to generate products because of their use based on popular knowledge and because there is evidence permitting their indication for use in the primary health care setting.[18]

In February 2006, the Brazilian National Health Council unanimously approved the document that supports the National Policy on Integrative and Complementary Practices (PNPIC, in the Portuguese acronym) in the SUS. In its final document, the PNPIC defines "complex medical systems" and "therapeutic resources" inserted by the World Health Organization (WHO) within the field of traditional and complementary/alternative medicine, one of its aspects including medicinal plants and phytomedicines.[19–21]

In October 2011, the Phytotherapeutic Form of the Brazilian Pharmacopoeia was published, and in it were included pieces of information on various plants, among which

S. terebinthifolius Raddi, spanning its agronomical, safety, effectiveness and control aspects.[22]

In the year 2012, through ordinance no. 1102, of 12 May 2010, the Brazilian Ministry of Health launched the National List of Phytomedicines (RENAFITO, in the Portuguese acronym), in which *S. terebinthifolius* Raddi was included for funding and availability purposes, in the SUS.[23]

Gilbert and Favoreto[24] describe five plant derivatives of medicinal use: the extract of the inner bark (bark devoid of external suberous layers) of the stem, the extract of the leaves, the essential oil of the leaves, the extract of the fruit, and the essential oil of the fruit.

In 1999, Laboratório Hebron launched the Brazilian peppertree (*S. terebinthifolius* Raddi) vaginal gel in the market under the name Kronel®, indicated for the treatment of cervicitis, vaginitis, and cervicovaginitis.[25,26]

When analyzed from a clinical point of view, the existence of a phytotherapeutic vaginal gel (Kronel®), produced from the aqueous extract of the bark of *S. terebinthifolius*, has exhibited antifungal properties in vivo against strains of the *Candida* genus and even improved the vaginal flora.[27]

In researching the different studies in the literature, we can see that many of them showed antifungal properties against *S. terebinthifolius* Raddi,[14,28–33] whereas other studies state the opposite.[34–36] Hence, our study aimed to assess the antifungal susceptibility profile of the aqueous extract of the bark of *S. terebinthifolius* Raddi against strains of *Candida* spp and the association between nystatin and the aqueous extract of *S. terebinthifolius* Raddi.

Methods

To assess the antifungal susceptibility profile, we used the disk diffusion method. We tested 50 strains of the *Candida* genus: from patients of Hospital Santa Casa de Misericórdia de São Paulo; from the mycology collection at the institution's laboratory of microbiology; and a standard strain of each species: *C. albicans* (ATCC 10231, lot: 443–346–3, expiration date: 12/2015), *C. krusei* (ATCC 14243, lot: 809–41–8, expiration date: 01/2016), *C. glabrata* (ATCC MYA-2950, lot: 122–19–1, expiration date: 05/2016) and *C. tropicalis* (ATCC 13803 lot: 450–66–3, expiration date: 12/2016).

In order to be able to associate the statistical significance of the tests with the meaning of the results, it is important that an appropriate sampling plan is drawn up, and that depends on a basic knowledge of the study statistics and a deep knowledge of the problem being investigated. While planning, the calculation of the sample size (n) was based on the Analysis of Variance (ANOVA). By adopting a significance level of 5% (α) and power of 80% (1 - β), with a standard deviation (SD) of 5 units and difference of 2 units, we found $n = 52$ samples.[37]

In order to perform the tests, we used the aqueous extract of the bark of the plant *S. terebinthifolius* Raddi, which was prepared from the standardized dried extract of *S. terebinthifolius* Raddi 0.4% (lot: 200115.121 and expiration date: 01/2016), provided by Laboratório Hebron, the supply of which was free of interest or sponsorship. For every 4.5 g of the dried extract, 6 mL of purified water were added, thereby resulting in the concentration of the aqueous extract, similar in appearance to a suspension, which is then incorporated into the vaginal gel, Kronel®.[25]

Strains of the *Candida* genus were initially identified by using the chromogenic method and stored in a freezer at -4°C. The day before the tests were performed, the strains were reconstituted with tryptic soy broth (TSB), and incubated for 24 hours, with the researchers paying special attention to the presence of turbidity, which is representative of the multiplication and viability of the strains (log-growth phase). Tests were started 24 hours after colony growth was observed.

To determine the antifungal property of the extract of *S. terebinthifolius* Raddi in vitro, we used three groups of drugs against the strains of the four *Candida* spp, namely: the aqueous extract of *S. terebinthifolius* Raddi; nystatin 100 IU (an antifungal drug); and the aqueous extract of *S. terebinthifolius* Raddi associated with the antifungal drug.

In Petri dishes previously prepared with Sabouraud Dextrose Agar (SDA), a culture medium, supplied by Probac, was seeded with fungal strains (previously prepared in saline), using alginate swabs at a concentration of 1.5×10^8 CFU/mL, corresponding to 0.5 units in the McFarland nephelometer scale. Subsequently, sterile absorbent filter paper disks were placed onto the surface of the solidified SDA medium; the disks had been previously: immersed in the aqueous extract of *S. terebinthifolius* Raddi in a 15-µL volume (maximum capacity); impregnated with nystatin (10µL); or impregnated with nystatin (10µL) plus a 15-µL volume of the aqueous extract of *S. terebinthifolius* Raddi.

The plates in duplicate were incubated at 35 $\pm$ 2°C for 24 and 48 hours in order to determine the antifungal susceptibility. The measurements of fungal growth inhibition halos were taken with a manual caliper at 24 and 48 hours, and the data were recorded and tabulated in Excel spreadsheets. The readings obtained were reconfirmed by two professionals in the field, to ensure good laboratory practices and results.

For the statistical analysis, the species of the *Candida* genus were grouped into *C. albicans* (CA) and non-*C. albicans* (NCA).

The results underwent statistical analysis with the aid of the SPSS software version 13.0, and those with $p < 0.05$ were considered significant. The following non-parametric tests were used: Mann-Whitney and Friedman.

Results

Altogether, 26 samples of CA and 28 samples of NCA were analyzed, totaling 54. Microbial growth or inhibition are checked by halo formation, which is determined in millimeters (mm).

The diameter of the inhibition halo formed by nystatin (positive control) was considered sensitive when larger or equal to 10 mm, and resistant when smaller than 10 mm.[38–40]

Laboratory tests were performed at 24 and 48 hours, and no significant difference was observed in the diameter of halos formed in any sample. We therefore chose to work with the 24-hour samples.

Table 1 Comparison of the different CA and NCA strains at 24 hours relative to samples of aqueous extract of *S. terebinthifolius* Raddi, nystatin, and the association of nystatin + the aqueous extract of *S. terebinthifolius* Raddi. Inhibition halo measurements (median: minimum – maximum) and significance as per Mann-Whitney Test

Samples (24 hours)	*Candida albicans*	*Non-Candida albicans*	p^*
S. terebinthifolius	0.00	0.00	1.000
Nystatin	18.50 (17.00–22.00)	19.50 (14.00–21.00)	0.185
Nystatin + *S. terebinthifolius*	14.25 (17.00–22.00)	16.50 (0.00–19.00)	0.052

p^* = Mann-Whitney test.
Note: Values equal to 0.00 indicate that there was no inhibition halo observed.

Table 2 Comparison of the different samples: the aqueous extract of *S. terebinthifolius* Raddi, nystatin, and the association of nystatin + the aqueous extract of *S. terebinthifolius* Raddi relative to the AC and NCA strains. Inhibition halo measurements (median: minimum – maximum) and significance as per Friedman Test

Candida	*S. terebinthifolius*	Nystatin	Nystatin + *S. terebinthifolius*	p^*
CA	0.00	18.50 (17.00–22,00)	14.25 (6.00–18.50)	< 0.001
NCA	0.00	19.50 (14.00–21.00)	16.50 (0.00–19.00)	< 0.001

Abbreviations: CA, *Candida albicans*; NCA, non-*Candida albicans*. p^* = Friedman test.
Note: Values equal to 0.00 indicate that there was observed no inhibition halo.

There was no statistically significant difference ($p > 0.05$) in the comparison between CA and NCA strains on the extract of *S. terebinthifolius* Raddi, nystatin, and the association of nystatin + aqueous extract of *S. terebinthifolius* Raddi at 24 hours, as shown in ►**Table 1**.

►**Table 2** shows the comparisons at 24 hours of the different samples of the aqueous extract of *S. terebinthifolius* Raddi, nystatin, and the association of nystatin + aqueous extract of *S. terebinthifolius* Raddi relative to the CA and NCA strains; the results were significant ($p < 0.05$).

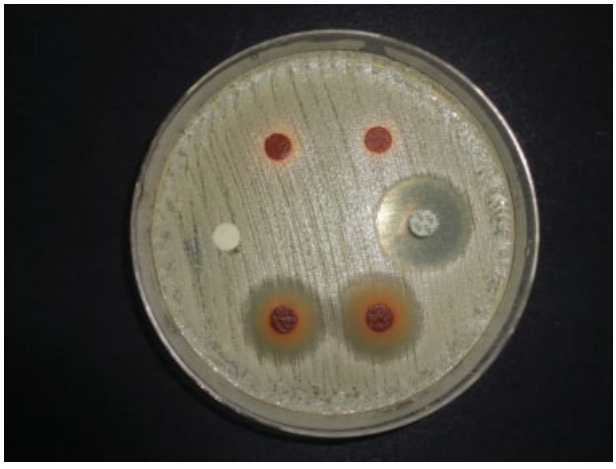

Fig. 1 Picture of the experiment: Sabouraud agar plate seeded with *C. albicans*, incubated at 37 ± 2°C for 24 hours; samples performed in duplicate. Disk no. 1: blank disc without product, study control; disks no. 2 and no. 3: impregnated with *S. terebinthifolius* Raddi, no inhibition halo observed; disk no. 4: with 100 IU nystatin and inhibition halo observed, experiment control; and nystatin disks no. 5 and no. 6: impregnated with *S. terebinthifolius* Raddi, showing smaller inhibition halo. *Source:* Faculdade de Ciências Médicas da Santa Casa de São Paulo, 10/06/2015.

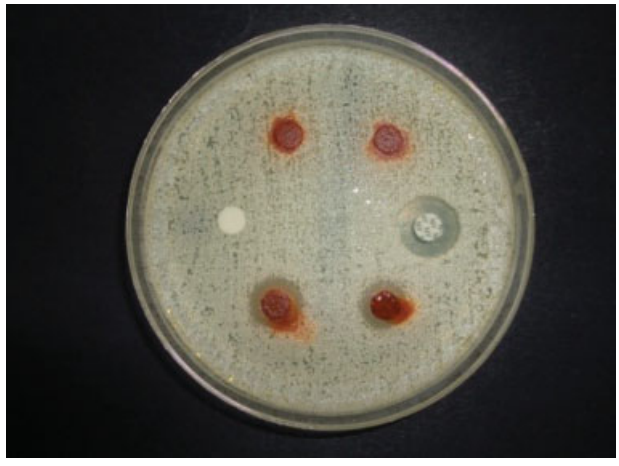

Fig. 2 Picture of the experiment: Sabouraud agar plate seeded with *C. krusei*, incubated at 37 ± 2°C for 24 hours; samples performed in duplicate. Disk no. 1: blank disc without product, study control; disks no. 2 and no. 3: impregnated with *S. terebinthifolius* Raddi, no inhibition halo observed; disk no. 4: with 100 IU nystatin and inhibition halo observed, experiment control; and nystatin disks no. 5 and no. 6: impregnated with *S. terebinthifolius* Raddi, showing smaller inhibition halo. *Source:* Faculdade de Ciências Médicas da Santa Casa de São Paulo, 10/06/2015.

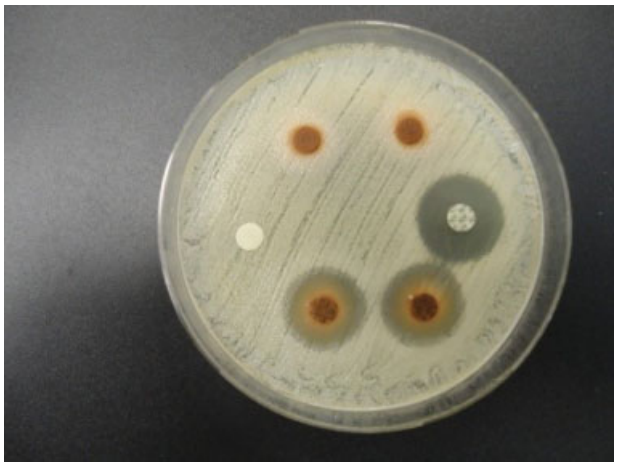

Fig. 3 Picture of the experiment: Sabouraud agar plate seeded with *C. glabrata*, incubated at 37 ± 2°C for 24 hours; samples performed in duplicate. Disk no. 1: blank disc without product, study control; disks no. 2 and no. 3: impregnated with *S. terebinthifolius* Raddi, no inhibition halo observed; disk no. 4: with 100 IU nystatin and inhibition halo observed, experiment control; and nystatin disks no. 5 and no. 6: impregnated with *S. terebinthifolius* Raddi, showing smaller inhibition halo. *Source:* Faculdade de Ciências Médicas da Santa Casa de São Paulo, 10/06/2015.

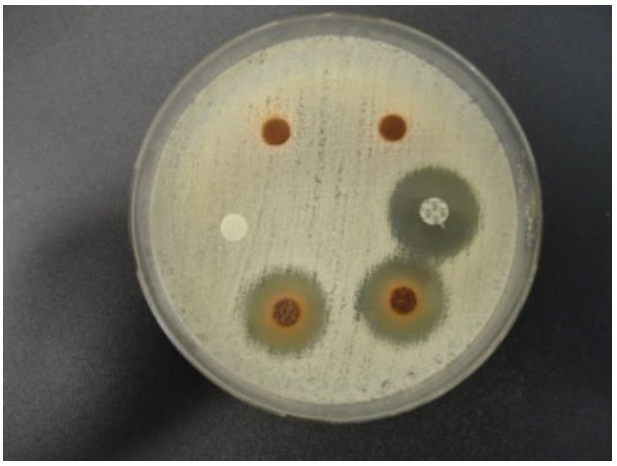

Fig. 4 Picture of the experiment: Sabouraud agar plate seeded with *C. tropicalis*, incubated at 37 ± 2°C for 24 hours; samples performed in duplicate. Disk no. 1: blank disc without product, study control; disks no. 2 and no. 3: impregnated with *S. terebinthifolius* Raddi, no inhibition halo observed; disk no. 4, with 100 IU nystatin and inhibition halo observed, experiment control; and nystatin disks no. 5 and no. 6, impregnated with *S. terebinthifolius* Raddi, showing smaller inhibition halo. *Source:* Faculdade de Ciências Médicas da Santa Casa de São Paulo, 10/06/2015.

►**Figs. 1–4** represent the four samples of the four *Candida* spp tested.

Discussion

Brazil has the greatest botanical diversity in the world, with an estimated 350–550 thousand species, of which 55 thousand are cataloged; the therapeutic knowledge, which stems from the popular use of medicinal plants, is passed down from generation to generation. The amount of information on medicinal plants has grown only 8% annually over the past 20 years, despite the great diversity of our flora.[41] It is believed that at least half of the plants contain active ingredients, that is, substances responsible for the therapeutic effect, although investments for these searches are reduced.[42]

Brazil's National Health Surveillance Agency (ANVISA, in the Portuguese acronym) has regulated phytomedicines in Brazil as being conventional medicines, but has required the pharmaceutical industry to meet quality, safety and efficacy criteria by means of conducting ethnopharmacological surveys and gathering technoscientific documentation on preclinical and clinical pharmacological and toxicological studies[43]

Many studies have shown different pharmacological properties of *S. terebinthifolius* Raddi, such as: anti-inflammatory,[44] antioxidant,[45] antitumoral,[46] antibacterial[26,29,34,36,47] and antifungal.[14,30,33] This study aimed to assess the antifungal susceptibility profile of *S. terebinthifolius* Raddi against strains of *Candida*.

Even though the standards established by the National Committee for Clinical Laboratory Standards[38,39] advise that the inhibition halos readings at 48 hours are required only when insufficient fungal growth after 24 hours of incubation is observed, we initially chose to work with these two time intervals. Since all of the analyses showed that there were no significant differences between the two incubation times, we chose to study the results obtained at 24 hours.

According to Ostrosky et al,[48] in the disk diffusion method, the growth inhibition halo is measured starting from the circumference of the disk to the edge, where microorganism growth is observed, and it may be classified as: sensitive, moderately sensitive, and resistant. This is the method we used in our study. On the other hand, the broth dilution method considers the relationship between the growth proportion of the microorganisms challenged in the liquid medium and test substance concentration, allowing quantitative results, not being influenced by the speed of growth of the microorganisms .[31–33]

Vulvovaginal candidiasis caused by the CA species affects 85 to 95% of women; *C. glabrata* affects 10 to 20%; and *C. tropicalis* and *C. krusei* may also be associated with this infection.[5] In our study, in the same treatment, whether using the aqueous extract of *S. terebinthifolius* Raddi, nystatin, or the association of nystatin + the aqueous extract of *S. terebinthifolius* Raddi, we observed no significant difference in the inhibition response across the species of the *Candida* genus (CA and NCA).

The results of the test with the isolated aqueous extract of *S. terebinthifolius* Raddi proved not to be effective against the strains of *Candida* tested, since no inhibition halo was observed.

The association of nystatin + the aqueous extract of *S. terebinthifolius* Raddi proved to be less effective when compared with isolated nystatin. An acceptable explanation for this decrease in the halo is the antagonistic action between them. According to Dias et al,[8] Spampinato and

Leonardi,[9] and Perea and Patterson,[49] nystatin, a polyene antifungal agent, acts by altering the membrane function of the fungal cell due to its high affinity for ergosterol, the main component of the cell membranes of fungi, causing the production of aqueous pores in the cell membrane, changing its permeability, which facilitates the outflow of cytosolic components and leads to death. Some as of yet still unknown compound, deriving from the secondary metabolism of *S. terebinthifolius* Raddi, is likely to interfere with nystatin's mechanism of action, thus resulting in the reduction of its pharmacological activity.

When we searched various databases by using the key words: *Schinus terebinthifolius* Raddi, *nystatin* and the species of *Candida* tested, we found studies that assessed the antifungal property of this plant and showed positive results. The extracts used in these studies were exclusively prepared to be used in the respective tests.[14,30–33] We, in turn, have not prepared the extract, but chose to use an already standardized product instead, which we believed would also have an antifungal property; nevertheless, our results did not support that. However, had we also prepared an exclusive extract for our tests, we might have also obtained positive results.

According to Dos Santos et al,[50] in the literature there is no systematization permitting a correlation between the parts of the plant used and the type of extract tested and the reported antimicrobial property. In addition, the leaves seem to be the main part of the plant studied and/or that with a higher antimicrobial property than its bark and fruits, but many studies use different parts of the plants in various extracts.[14,28–33,35]

Considering the antifungal potential of this species, further studies with other plant parts and/or different extraction methods are expected to yield positive results for the development of new phytotherapeutic products.

Up to the present day, we have not yet found in the literature reports describing the use of the aqueous extract of the stem bark of this plant in antifungal property assessments; thus, we hope that our results may contribute to new research in the field of fungal microbiology, since isolated nystatin still has a good activity against the strains of *Candida* sp tested. Still, we have not found, to date, studies that have evaluated the association of nystatin with *S. terebinthifolius* Raddi or any other medicinal plant – and this may just as well constitute a new research line aimed at finding a plant capable of providing a synergistic effect for the treatment of VVC.

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