Original Research Article

Antifungal activity of plant-based tinctures on Candida

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Abstract

Objective: To evaluate through determination of minimum inhibitory concentration (MIC) the antifungal activity of Salvia officinalis (sage), Anacardium occidentale (cashew) and Malva sylvestris (mallow) tinctures on Candida albicans (ATCC 40227), C. tropicalis (ATCC 13803) and C. krusei (ATCC 40147). Material and methods: In 96-well microplates, 100 µl of Sabouraud-Dextrose broth doubly concentrated, 100 µl of the tested tinctures and 10 µl of fungal inoculums (1.5 x 10⁶ organisms/ml) were inserted. The products were diluted from initial concentration of 100 mg/ml until 0.78 mg/ml. MIC corresponded to the lowest dilution at which there was no visible fungal growth. Nystatin (100,000 UI/ml) was used as control. Statistical analysis was performed by Kruskal-Wallis and Dunn tests (p < 0.05). Results: S. officinalis tincture did not inhibit the growth of C. albicans and C. tropicalis; MIC was 100 mg/ml for C. krusei. For A. occidentale, MIC was 100 mg/ml for C. albicans and C. krusei, and for C. tropicalis, there was no fungal inhibition. M. sylvestris tincture presented MIC at 25 mg/ml for C. krusei and 100 mg/ml for C. albicans and C. tropicalis. The best antifungal activity was showed by M. sylvestris tincture (p < 0.05). Conclusion: M. sylvestris tincture exhibited antifungal activity against all the tested strains at lower concentrations. S. officinalis tincture inhibited the action of C. krusei and A. occidentale tincture showed activity against C. albicans and C. tropicalis.
Introduction

Oral candidiasis is one of the most common human infections of fungal nature. It has been described as an opportunistic infection, frequently involved with oral microbiota alteration, systemic diseases, and reduction of the host immunity [1, 6]. Among the strains implicated in oral candidiasis development, Candida albicans is the most prevalent and highest-pathogenic microorganism [1, 16]. C. tropicalis, C. krusei, C. parapsilosis and C. guilliermondii are also present in the disease course and together with C. albicans represent more than 80% of the clinical isolates [1, 16].

Stramandinoli et al. [23] (2010) evaluate the main risk factors for oral candidiasis prevalence in hospitalized patients. It was identified that the use of nasogastric tube, prostheses, and poor oral hygiene represented a serious risk to the development of this infection. Several topical and systemic antifungals have been used for candidiasis treatment, according to the patient’s clinical and general state [3].

According to Alves et al. [3] (2009), Nystatin is the drug of choice for topical treatment of C. albicans infections within oral cavity. However, microbial resistance is a growing problem. For the systemic treatment of patients at high risk of developing fungal infections, literature has recommended the use of fluconazole.

Rex et al. [19] (2000) and Khan et al. [8] (2009) affirmed that C. albicans strains have become resistant due to the use of some synthetic antifungal drugs. Aiming to identify substitutes to the traditional drugs, studies on the antimicrobial activity of natural products have been conducted [5, 18]. According to Alves et al. [3] (2009), these products present antimicrobial and anti-inflammatory properties, relatively low cost, and inhibitory activity against resistant microorganisms. Therefore, the search for natural products presenting an efficient antifungal action against resistant microorganisms is a necessary alternative for controlling oral candidiasis [5, 18]. In this context, Maekawa et al. [10] (2007) demonstrated that the use of herbal substances, e.g. chlorophyll extract, may be effective to control C. albicans.

Ethnobotanical researches identified the popular use of Salvia officinalis (sage), Anacardium occidentale (cashew) and Malva sylvestris (mallow) [7, 21], justifying this study execution. Also, the antimicrobial activity of Salvia officinalis (sage), Anacardium occidentale (cashew) and Malva sylvestris (mallow) natural extracts has been described by several studies [3, 11, 12, 13, 17, 18].

On the other hand, there are few reports on these products’ inhibitory concentration. Consequently, it is mandatory to investigate their antifungal activity, aiming to justify and validate their use. Therefore, the aim of this study was to evaluate the in vitro antifungal activity of Salvia officinalis (sage), Anacardium occidentale (cashew) and Malva sylvestris (mallow) tinctures on C. albicans strains.

Material and methods

We conducted a study with an inductive approach, a comparative procedure and direct documentation technique in laboratory [9].

For in vitro antifungal assessment, Salvia officinalis (sage), Anacardium occidentale (cashew) and Malva sylvestris (mallow) tinctures (Farmácia Homeopática Homeovita Ltd., João Pessoa, PB, Brazil) were used. These were obtained at concentration of 20% (200 mg/ml) and density of about 0.9 g/ml.

This study’s reference strains were C. albicans (ATCC 40227), C. tropicalis (ATCC 13803) and C. krusei (ATCC 40147), obtained from the Laboratory of Reference Materials of the National Institute of Quality Control in Health (Osvaldo Cruz Foundation – FIOCRUZ –, Rio de Janeiro, RJ, Brazil). The strains were reactivated in Sabouraud-Dextrose broth (DIFCO, Detroit, Michigan, EUA) at 37ºC, and stored in Sabouraud-Dextrose agar 4% (DIFCO, Detroit, Michigan, EUA) at the Laboratory of Oral Microbiology, Nucleus of Tropical Medicine, Center of Health Sciences, Federal University of Paraíba. To conduct the study, fungal suspensions were prepared in sterile saline (0.85% NaCl), at concentration of 1.5x10^6 microorganisms/ml, equivalent to 10^6 MacFarland scale tube.

The tinctures’ antifungal activity was evaluated by determining the minimum inhibitory concentration (MIC). For this purpose, the technique of microdilution described by Aligiannis et al. [2] (2001) and Castro & Lima [5] (2010) was employed. We use 96-well microplates (Alamar, Diadema, SP, Brazil), disposed in 12 columns (1 to 12) and eight lines (A to H). Each one of these plates was intended to analyze one microorganism. Columns 1, 2, and 3 were used for the antifungal analysis of Salvia officinalis tincture; columns 4, 5, and 6 for Anacardium occidentale tincture; and columns 7, 8, 9 for Malva sylvestris tincture. Column 10 was used for growth control; column 11 for sterility control and column 12 for positive control (Nystatin 100,000 UI/ml).
In each one of the microplates’ well, 100 µl Sabouraud-Dextrose broth doubly concentrated were inserted. Following, 100 µl of the tested tinctures were inserted to obtain the initial concentration of 100 mg/ml in the first line of the microdilution plate [2, 5]. The following concentrations of the tinctures were obtained after the products’ serial dilution in the microdilution plate, from 100 mg/ml (line A) until 0.78 mg/ml (line H), through transferring 100 µl of the content to the next well [2]. In line H’s wells, 100 µl of the content was dispensed to level the total volume of the wells.

Next, 10 µl of the fungal suspension (1.5 x 10⁶ microorganisms/ml) were inserted in all wells, except from those corresponding to the sterility control column. Plates were incubated in bacteriological incubator at 37°C, for 48h. MIC corresponded to the last tinctures concentration in which was not verified the presence of fungal precipitate or culture medium turbidity after the incubation period [2, 5].

Each natural product’s antifungal activity was assessed three times (triplicate) against each C. albicans strain. It was performed three samples for each condition and one reading for each sample (n = 27). This study’s evaluated concentrations were pre-established and it was expected that the same products would exhibit the same MIC, when the triplicate was performed. Therefore, data was statistically analysed by Kruskal-Wallis and Dunn tests, with level of confidence set at 95%.

Results

The methodology employed in this study was validated by the lack of fungal growth for the sterility and positive control (Nystatin 100,000 UI/ml), as well as by the presence of fungal growth for the growth control.

Statistical differences and MIC values of the products tested against Candida strains are shown in Table I.

<table>
<thead>
<tr>
<th>Table I – Minimum inhibitory concentration (mg/ml) of the natural products tested against Candida strains</th>
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<tr>
<td><strong>Tinctures</strong></td>
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<td><strong>Strains</strong></td>
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<td>C. albicans (ATCC 40277)</td>
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<td>C. tropicalis (ATCC 13803)</td>
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<td>C. krusei (ATCC 40147)</td>
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</table>

Different letters in the same line indicate statistically significant differences (p < 0.05) by Kruskal-Wallis and Dunn tests.

At the tested concentrations, we verified that S. officinalis tincture did not exhibited activity against C. albicans and C. tropicalis. A. occidentale tincture did not inhibit the activity of C. tropicalis. Statistically significant difference (p < 0.05) was found for the inhibitory effect of M. sylvestris tincture (against C. tropicalis and C. albicans) and A. occidentale tincture (against C. albicans). M. sylvestris tincture exhibited antifungal activity against all the tested strains at lower concentrations with statistically significant difference (p < 0.05).

Discussion

The reference method for the microdilution technique and determination of the yeast sensitivity to antifungal therapy (M7-A2), described by the National Committee for Clinical Laboratory Standards (NCCLS) [15] (2002), considers the evaluation of synthetic antifungals, the use of the culture medium RPMI-1640 and the standardization of the initial concentration of the tested substances at 64 µg/ml or 16 µg/ml. The technique for determining the MIC employed in this study was based on the protocols described by Aligiannis et al. [2] (2001) and Castro & Lima [5] (2010). These protocols represent the modification of the regulations established by NCCLS [15] (2002).

This present study was developed at different conditions from those established by NCCLS [15] (2002). According to Nascimento et al. [14] (2007), the regulation M7-A2 proposed by NCCLS could not be followed to the letter when performing the antimicrobial evaluation of the plant tinctures. It is considered that the chemical properties of these natural products are different from those presented by the substances, for which the regulation was standardized, i.e., NCCLS Regulation does not meet the specifications of non-synthetic products. Therefore, we reproduced the protocol described by Aligiannis et al. [2] (2001) and by Castro & Lima [5] (2010), in which they employed the Sabouraud-Dextrose broth as culture medium, Nystatin as positive control, and the serial dilution of the tested products. Because this study’s tinctures are hydroalcoholic solutions (hydrophilic), the addition of the emulsifier (Tween 80) in the antifungal evaluation of these products is not necessary.

By comparing the techniques of disk-diffusion and microdilution to evaluate the antifungal activity of natural products, Scorzoni et al. [20] (2007) identified that microdilution was more sensitive for MIC determination. Therefore, to diminish the inconsistency of the obtained results, we employed
the broth microdilution technique, aiming to enable a greater contact between the tested products and the fungal cells. Additionally to these factors, the incorporation of the positive, sterility, and growth control contributes to both the reduction of the methodological bias and the comparison among the tested natural products [5, 20].

The sterility, growth, and positive controls were used to validate the technique employed in this study. The sterility and growth controls proved, respectively, the lack of culture medium contamination and viability of the tested strains. The absence of fungal growth against Nystatin (100,000 UI/ml) demonstrates the samples’ susceptibility against the synthetic antifungal. Due to the differences between the chemical nature of the natural products and of the positive control, it was not possible to compare the minimum inhibitory concentrations obtained against the tested microorganisms.

According to Nascimento et al. [14] (2007), the natural products exhibit greater antimicrobial activity within the formulation of essential oils. This would be justified by the higher concentration of active principles and the lipidic nature of the essential oil. The liposoluble nature of essential oils and its components enables the interaction with the lipidic cellular structures, resulting in the increase of the membranes’ permeability, which can provoke electrolyte imbalance and cellular death [5, 14]. However, the tinctures tested in this study showed a final concentration of 20% due to chemical processes of dilution in ethilic alcohol (hydrosoluble characteristic). Considering the commercial availability of natural extracts for therapeutic use, literature has considered that natural product tinctures exhibit lower antimicrobial activity and active principles’ concentration than essential oils [14].

Pozzatti et al. [18] (2009) evaluated the antifungal activity of S. officinalis against clinical isolates of C. albicans and C. dubliniensis. The authors verified that this natural product did not inhibit the microorganism growth at concentrations lower than 3.2 mg/ml. By evaluating C. albicans sensitivity to S. officinalis extract, Nascimento et al. [13] (2000) did not identify this product’s antifungal activity. The results of this study corroborate the literature findings by demonstrating the lack of antifungal activity of S. officinalis tincture at concentrations lower than 100 mg/ml against C. albicans and C. tropicalis.

Molina et al. [12] (2008) evaluated the antifungal activity of Salvia officinalis glycolic extract by broth dilution method on 20 strains of C. albicans isolated from the oral cavity. The authors identified that the natural product inhibited the fungal activity in 80% of the tested strains [12]. However, this study’s results pointed out that among the microorganisms tested only C. krusei showed growth inhibition against S. officinalis at 100 mg/ml. Therefore, further studies are necessary to refute or confirm the antifungal activity of this natural product.

We did not identify studies on the antifungal activity of A. occidentale on C. albicans. Considering the antibacterial activity of this natural product, literature shows the inhibition of the microorganisms involved in the cariogenic biofilm formation [17] and involved with hospital infections [22]. Pereira et al. [17] (2006) and Silva et al. [22] (2007) considered that polyphenols, tannins, and flavonoids within these products’ composition account for the antimicrobial activity of A. occidentale. Our results demonstrated C. albicans and C. krusei growth inhibition at the concentration of 100 mg/ml. Therefore, there is a need for further studies on the antifungal activity of A. occidentale derivatives to confirm these products’ actions.

The antimicrobial activity of the natural products based on M. sylvestris was researched by several studies [3, 4, 11], highlighting the clinical use of this product in mouthrinse formulations. Matos et al. [11] (2009) evaluated the antifungal activity of mouthrinses based on chlorhexidine gluconate (0.12%), hydrogen peroxide (1.5%), and M. sylvestris tincture on C. albicans strains. Although M. sylvestris tincture presented an antifungal activity against 73% of the strains, the solution exhibited lower effectiveness than chlorhexidine and hydrogen peroxide. Our study found antifungal activity of M. sylvestris tincture at the concentrations of 100 and 25 mg/ml, which corroborates the findings of Alves et al. [3] (2009). When compared to the other products evaluated by our study, it was observed that M. sylvestris tincture exhibited a higher antifungal activity than S. officinalis and A. occidentale tinctures.

The methodology employed in this study demonstrated the antifungal activity of the tested products, highlighting the action of M. sylvestris tincture against the tested strains. Further studies on these products’ antifungal activity through standardized and validated techniques are necessary. These should evaluate the antifungal activity against clinical strains and other standardized strains, employing techniques considering the phytoconstituent isolation, cellular adherence, biofilm formation in vitro, the influence of the human saliva and these products’ toxicology.
Conclusion

Within this study’s conditions, it can be concluded that *M. sylvestris* tincture presented antifungal activity against all tested strains, at lower concentrations. *S. officinalis* tincture inhibited the activity of *C. krusei*, and *A. occidentale* tincture exhibited antifungal activity against *C. krusei* e *C. albicans*.

References


17. Pereira JV, Sampaio FC, Pereira MSV, Melo AFM, Higino JS, Carvalho AAT. In vitro anti microbial activity of an extract from Anacardium occidentale Linn. on Streptococcus mitis, Streptococcus mutans and Streptococcus sanguis. Odontol Clin Cient. 2006 May-Jun;5(2):137-41.


