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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 (1.5×10^6 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 implicate 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 topic 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 topic 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 microorganism 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 Homeovitae 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 (Oswaldo 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 Paraiba. 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 10^6 microorganisms/ml) were inserted in all wells, except from those corresponding to the sterility control column. Plates were incubated in bacteriological incubator at 37ºC, 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 I – Minimum inhibitory concentration (mg/ml) of the natural products tested against *Candida* strains

Tinctures / Strains	***Salvia officinalis*** **(sage)**	***Anacardium occidentale*** **(cashew)**	***Malva sylvestris*** **(mallow)**
C. albicans (ATCC 40277)	>100.0[a]	100.0[b]	100.0[b]
C. tropicalis (ATCC 13803)	>100.0[a]	>100.0[a]	100.0[b]
C. krusei (ATCC 40147)	100.0[b]	100.0[b]	25.0[c]

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 identified 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, tannin, 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 effectivity 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*.

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