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Comparison of Antifungal Activity of Aqueous and Ethanolic Extracts of *Rhaphiodon echinus* (Lamiaceae) against *Candida krusei* Clinical Strains

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ABSTRACT

The main species of clinical interest causing candidiasis, especially oral, are: *Candida albicans, Candida tropicalis, Candida parapsilosis, Candida glabrata, and Candida krusei*. The excessive and indiscriminate use of these drugs leads to the emergence of resistant yeasts, especially in immunosuppressed patients, susceptible to frequent infections. Thus, there is a need for the development of new therapeutical alternatives of greater effectiveness, among the possibilities, the use of herbal medicines based on medicinal plants appears as an alternative treatment. An example of a plant species present in the Brazilian Northeast and reported in the scientific literature regarding its pharmacological properties is the *Rhaphiodon echinus* plant. This work aims to evaluate the antifungal activity of the aqueous and ethanolic extract of *Rhaphiodon echinus (*Lamiaceae) against strains of *Candida krusei*. For the determination of MIC (minimum inhibitory concentration) of the extracts, the broth microdilution technique was performed. The following strains of *Candida krusei* were used: ATCC 76645, LM 106, LM 108 and LM 111. A viability control and a positive control with nystatin were performed. The results obtained showed inhibition of *Candida krusei* strains under the MIC of 512 µg / mL for the ethanolic extract and 256 µg / mL for the aqueous extract. It is concluded that the aqueous and ethanolic extract of *Rhaphiodon echinus* is effective for the strains of *Candida krusei*. Experimental tests associated with antifungal products already marketed are necessary.

INTRODUCTION:

The resident microbiota of the mouth is highly diversified, with more than 700 species of microorganisms identified, many of which have not yet been formally described. In this vast and complex microbial ecology of the human oral cavity, there are at least twenty genera and approximately ninety species of yeasts isolated and classified[1,2].

The genus *Candida* is composed of hyaline yeast fungi, with the formation of blastoconidia, pseudohyphae and, occasionally, true hyphae. Macroscopically, in cultures of Sabouraud dextrose agar, the colonies are generally cream or whitish in color and the texture may be smooth or wrinkled, shiny or dry[3,4]. The main species of clinical interest causing candidiasis, especially oral, are: *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata* and *Candida krusei* [5,6,7].

In relation to the treatment of oral candidiasis, a large quantity of drugs obtained through organic synthesis has been used in the treatment of mycotic infections, such as antiseptics based on iodine tincture, gentian violet, salicylic and benzoic acid, sulphamidic derivatives, dyes, quinones and polyphenic antifungals (nystatin, amphotericin). However, fungal infections are difficult to treat, a fact related to the high resistance of *Candida* to the action of some conventional antifungals[8,9].

The excessive and indiscriminate use of these drugs leads to the emergence of resistant yeasts, especially in immunosuppressed patients, susceptible to frequent infections. Thus, there is a need for the development of new therapeutical alternatives of greater effectiveness, among the possibilities, the use of herbal medicines based on medicinal plants appears as an alternative treatment. They differ by having a higher molecular diversity to synthetic, providing new findings with research in biological activities that can promote the prevention and treatment of diseases[10].

In this way, medicinal plants constitute an important source of new biologically active compounds and are extensively studied in order to find more effective and less toxic compounds[11,12,13]. Brazilian plant species are usually used as antifungal and it is clear that Brazil, due to its plant diversity, is a country known worldwide for the variety of products with medicinal properties, widely used in its various regions[14].

An example of a plant species present in the Brazilian Northeast and reported in the scientific literature regarding its pharmacological properties is the plant *Rhaphiodon echinus*, a Brazilian plant, belonging to the family Lamiaceae and popularly known as "Betônica" and "flor-de-urubu". In folk medicine, its leaves are used for various therapeutic purposes such as treatment of a cough, inflammation in the oral cavity and infections in the genitourinary tract[15,16].

Based on the information about the therapeutic potential of medicinal plants and the importance of combating yeast infections of the genus *Candida*, this study aims to evaluate the possible antifungal activity of the aqueous and ethanolic extract of *Rhaphiodon echinus (*Lamiaceae*).*

MATERIALS AND METHODS:

***In-vitro* assay**

1.1 Substance test

For the tests, the aqueous and ethanolic extracts of the aerial parts of *Raphiodon echinus* were used, which were assigned by the team of Profª. Drª. Gabriela Lemos de Azevedo Maia, of the Federal Univesity of Vale, do São Francisco (UNIVASF).

The extracts were preserved in an amber glass bottle and kept under refrigeration. The emulsions of the extracts at the different concentrations were prepared at the time of the tests. used 0,5% DMSO and 0,02% Tween 80. By dilutions in distilled water or in the culture medium itself, the desired concentrations of both extracts were obtained.

1.2 Fungal Species:

Four clinical strains of *Candida krusei* (LM 08, LM 13, LM 656 and LM 978), previously isolated, were identified and kindly provided by the Mycology Laboratory of the Department of Pharmaceutical Sciences, Health Sciences Center, Federal University of Paraíba, Brazil. the direction of Profª. Dr. Edeltrudes de Oliveira Lima.

All strains were maintained on Sabouraud dextrose agar (SDA) at a temperature of 4 ° C and used for assays raises 24 hours in ASD incubated at 35 ° C. In the study of antifungal activity

was used a fungal inoculum of approximately 10^6 CFU / mL standardized according to the turbidity of the tube 0.5 of the McFarland scale [17,18].

1.3 Culture mediums

Sabouraud dextrose agar - ASD (Difco Lab., USA) medium for maintenance of the microorganisms, and Sabouraud dextrose broth - CSD (Difco Lab., USA) were used for the *in-vitro* assays prepared according to the manufacturer's instructions.

1.4 Antifungal drug:

Was used as a standard antifungal (positive control), Nystatin powder (Pharma Nostra, Rio de Janeiro). The solutions were prepared at the time of the tests to reach the desired concentrations.

1.5 Determination of Minimum Inhibitory Concentration (MIC):

The minimum inhibitory concentrations of the aqueous and ethanolic extracts of Raphiodon echinus were determined by the broth microdilution technique [17,18]. 96 holes sterile plates with a lid were used. In each well of the plate, 100 µL of the liquid medium was added to the double concentrated Sabouraud dextrose broth.

Then, 100 µL of the extract emulsion at the initial concentration of 2048 µg / mL (also doubly concentrated), was dispensed into the wells of the first row of the plate. The concentrations of 1024, 512, 256, 128, 64, 32, 16, 8 and 4 µg / mL were obtained by means of a two-fold serial dilution so that in the first row of the plate is the highest concentration, and in the latter, the lowest concentration. Finally, 10 µl of the inoculum of the fungal species were added to the wells, where each plate column refers to a fungal strain, specifically.

In parallel, the same assay was performed with the antifungal Nystatin. A micro-culture control was performed by placing 100 µL of the same doubly concentrated CSD, 100 µL of distilled water and 10 µL of the inoculum of each species into the wells.

To verify the absence of interference in the results by the solvents used in the preparation of the emulsion, in the case of DMSO (dimethylsulfoxide) and Tween 80, a control was made in which were placed in the wells 100 µL of the doubly concentrated broth, 50 µL of DMSO, 50 µL of Tween 80 and 10 µL of the fungal suspension. A sterility control of the medium was

also performed, where 200 µL of the CSD was placed in an orifice without suspension of the fungi.

The plates were aseptically closed and incubated at 35 ° C for 24-48 hours to be read. The MIC for the extracts and antifungal was defined as the lowest concentration capable of visually inhibiting the fungal growth observed in the orifices when compared to the controlled growth. The experiments were performed in duplicate.

RESULTS AND DISCUSSION:

Minimum Inhibitory Concentration (MIC) is referred to as the lowest concentration of a test substance capable of inhibiting microbial growth in a visible manner. MIC50 is said to be the lowest concentration capable of inhibiting 50% of the strains during the experiment;

Following the premise, it is observed that the MIC_{50} of the aqueous extract for *Candida krusei* is 256 µg / mL. MIC_{50} of the ethanolic extract of *Rhaphiodon echinus* against *Candida krusei* strains is 512 µg / mL. The data obtained are presented in the tables below.

Table 1. Minimum Inhibitory Concentration (MIC) in µg / mL of the aqueous extract of *Rhaphiodon echinus* against strains of *Candida krusei.*

FUNGAL STRAINS	MIC
LM 08	512 µg/mL
LM 13	-
LM 656	256 µg/mL
LM 978	256 µg/mL
Positive control	+
Negative control	-

(-) = There was no apparent inhibition of the strain

(+) = visible inhibition of the strain

Table 2. Minimal Inhibitory Concentration (MIC) in µg / mL of the ethanolic extract of *Rhaphiodon echinus* against strains of *Candida krusei*.

FUNGAL STRAINS	MIC
LM 08	512 µg/mL
LM 13	512 µg/mL
LM 656	512 µg/mL
LM 978	512 µg/mL
Positive control	+
Negative control	-

(-) = There was no apparent inhibition of the strain

(+) = visible inhibition of the strain

Comparing the results obtained after the experiments, it is observed that the aqueous extract is more effective against the strains of *Candida* since its MIC_{50} is lower than the MIC_{50} of the ethanolic extract. According to Sartoratto et al. (2004), the antifungal potential is said to be strong when the MIC reaches values below 500 µg / mL, it is said to be moderate between 600 - 1500 µg / mL and considered weak when the MIC values exceed 1500 µg / mL. The aqueous and ethanolic extract of *Rhaphiodon echinus* demonstrated strong antifungal activity on the strains of *Candida krusei* since they obtained MIC_{50} lower than 600 µg / mL[19].

Salari et al. (2016) evaluated the antifungal activity of the methanolic extract of *Salvia rhytides Benth.* (Lamiaceae) against isolates of several species of *Candida*, where the value of inhibition of growth showed that the isolates of *Candida krusei* are among the most susceptible to the extract [20].

Studies by Ibrahim et al. (2017) showed susceptibility of strains of *Candida krusei* and *Candida glabrata* to the volatile oil of Mentha australis, belonging to the family Lamiaceae; Violante et al. (2012) evaluated the effect of *Hyptis crenata*, Lamiaceae from Brazilian cerrado, under *Candida* species, where a strong inhibitory effect against *Candida krusei* was also observed[21,22].

Costa et al. (2017) verified the antifungal potential of *R. echinus* extracts against strains of different *Candida* species and observed the modulatory effect of these extracts when associated with synthetic antifungals[23].

CONCLUSION:

It is concluded that the aqueous and ethanolic extracts of *Rhaphiodon echinus* are effective against the strains of *C. krusei*, therefore, further studies are necessary to uncover the mechanism of action of the antifungal activity of these natural products.

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