IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Human Journals
Research Article
January 2023 Vol.:23, Issue:3

Evaluation of the Antifungal Activity of the Hexanic Phase of *Rhaphiodon echinus* Nees Mart. Schauer (Lamiaceae) Against Strains of *Candida* Spp.

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Submitted: 25 December 2022
Accepted: 31 December 2022
Published: 30 January 2023

Keywords: Phytotherapy, Microbiology, Dentistry, Oral candidiasis.

ABSTRACT

Oral candidiasis is the most common fungal infection found in the human oral cavity, with its etiology related to yeast species of the genus *Candida* spp. With the indiscriminate use of synthetic antifungal agents, the emergence of resistant fungal isolates has occurred; thus, natural products with antimicrobial activity have been highlighted. Thus, this study aimed to evaluate the antifungal activity of the hexanic extract of *Rhaphiodon echinus Nees* Mart. Schauer (Lamiaceae), popularly known as "betonica", endemic to the caatinga, on strains of *C. albicans, C. tropicalis,* and *C. krusei*. Emulsions of the extract at different concentrations were prepared at the time of the assays and through dilutions in distilled water. Sabouraud dextrose agar medium was used for the maintenance of the strains, and Sabouraud dextrose broth for the in vitro assays. The Minimum Inhibitory Concentration (MIC) of *Rhaphiodon echinus*hexanic extract was the lowest concentration able to visually inhibit fungal growth when compared to control growth, and was determined by the microdilution broth technique, in sterile, capped 96-well plates; aseptically closed and incubated at 35°C for 24-48 hours for reading. The analyses and tests were performed in duplicate. Thus, the results obtained showed that the hexane extract of *Rhaphiodon echinus* leaves presented MIC_{50} of 1024 µg/mL against *Candida albicans* and *Candida krusei* strains and 512 µg/mL against *Candida tropicalis* strains. It was concluded that the hexane extract of *Rhaphiodon echinus* leaves can be considered a promising resource for the treatment of oral candidiasis, presenting moderate to strong antifungal activity.

INTRODUCTION

The oral microbiota is influenced by both external factors (smoking, alcoholism, antibiotic therapy, hospital admissions, nutrition, and oral hygiene) and intrinsic to the patient, such as age and immune status; it is possible that the presence of a particular fungal isolate, such as *Candida*, *Aspergillus*, *Fusarium*, and *Cryptococcus*, is the first step towards an opportunistic infection in the host [1].

Oral candidiasis is the most common fungal infection found in the human oral cavity, with its etiology related to the yeast species of the genus *Candida* spp., a component of the normal oral microbiota, *Candida albicans* being considered the most pathogenic species, however, other species have also been associated with this pathology, such as *C. dubliniensis, C. parapsilosis, C. glabrata, C. krusei* and *C. tropicalis* [2].

The yeasts of the *Candida* genus, from the Saccharomycetaceae family, are polymorphs, with incomplete sexual cycles, including approximately 150 species, of which about 20 have been described as etiologic agents of candidiasis, and may be opportunistic or pathogenic [3]. They are hyaline yeeduriform fungi, with the formation of blastoconidia, pseudohyphae, and, in some cases, true hyphae; in Sabouraud dextrose agar cultures, in a macroscopic view, colonies usually present a cream or whitish color, and their texture may be smooth or wrinkled, shiny or dry [4-6].

After the discovery of antifungal drugs of the azoles class (ketoconazole, econazole, sulconazole, miconazole, clotrimazole, and fluconazole), there was a decrease in side effects in the treatment of fungal infections, however, due to *Candida* resistance to these antifungal drugs, this treatment has been failing over time [7]. Thus, with the advent of multidrug-resistant strains and unwanted effects presented by synthetic drugs, the search for natural products with antimicrobial activity has been highlighted [8].

The plants used in popular medicine have been studied due to their therapeutic properties; several studies show that plant species have promising compounds in the production of new drugs, especially species from the Northeast of Brazil [9]. Currently, research has been developed with extracts or essential oils of medicinal plants, demonstrating a high potential in pathogen control, which control is given, among others, by the antifungal action [10].

Thus, it is possible to highlight an extract little reported in the literature, from the species *Rhaphiodon echinus*, of the botanical family Lamiaceae, popularly known as "betonica" or "benthic", endemic of the caatinga, adapted to the region of the backlands and northeastern semi-arid [11]. In the existing literature, the reactions of the phytochemical components of the species *R. echinus* were more explored with its essential oil and aqueous extract, demonstrating antioxidant, antibacterial and antifungal effects [11, 12]. Thus, there is a need to analyze the hexanic extract of this plant, and the objective of this study was to evaluate the antifungal activity of the hexanic phase of *Rhaphiodon echinus* Nees Mart. Schauer (Lamiaceae) against strains of *Candida* spp.

METHODOLOGY

Test Substances

For the in vitro assays, the hexanic extract of *Rhaphiodon echinus* leaves, provided by Prof. Dr. Gabriela Lemos de Azevedo Maia, from Universidade Federal do Vale do São Francisco (UNIVASF), was used. The hexanic extract was stored in an amber glass flask and kept under refrigeration. The emulsions of the extract at different concentrations were prepared at the time of the assays. In a sterile test tube, 60,000 µg of hexanic extract, 0.15 mL of dimethyl sulfoxide (DMSO), 0.06 mL of Tween 80 (INLAB/Brazilian Industry), and enough for 3 mL of sterile distilled water were added. The mixture was stirred for 5 minutes, obtaining an emulsion with a concentration of 20,000 µg/mL extract, 5% DMSO, and 2% Tween 80. And through dilutions in distilled water, the desired concentrations of the hexanic extract were obtained.

Microorganisms

Four strains of *Candida albicans* (ATCC 76645, LM 106, LM 108 and LM 111), four strains of *Candida tropicalis* (ATCC 13803, LM 14, LM 31 and LM 36) and four strains of *Candida krusei*(LM 08, LM 13, LM 656, LM 978), previously isolated and identified, were maintained on Sabouraud dextrose agar (SDA) at 4°C. For the assays, 24-hour replicates were used on SDA incubated at 35°C. In the antimicrobial activity study, a fungal inoculum of approximately 1,5 x 10^6 CFU/mL was standardized according to the turbidity of the 0.5 McFarland scale tube [13, 14].

Culture media

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

Determination of the Minimum Inhibitory Concentration (MIC)

The MIC of the hexanic extract of *Rhaphiodon echinus* was determined by the broth microdilution technique [13, 14]. Sterile, capped 96-hole plates were used, and 100 µL of double-concentrated Sabouraud dextrose broth liquid medium was added to each hole in the plate. Then, 100 µL of the hexanic 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. And by serial dilution in the ratio of two, the concentrations of 1024, 512, 256, 128, 64, 32, 16, 8, and 4 µg/mL were obtained, so that the first row of the plate contained the highest concentration and the last one, the lowest concentration. Finally, 10 µL of the inoculum of approximately 1,5 x 10^6 CFU/mL of the fungal species was added to the wells, where each column of the plate referred to a fungal strain, specifically. Three rows of plates were reserved to represent the positive control, negative control, and sterility control, respectively. The plates were aseptically closed and incubated at 35°C for 24-48 hours to be read. The MIC for the hexanic extract was defined as the lowest concentration able to visually inhibit fungal growth seen in the holes when compared to control growth. The experiments will be performed in duplicate.

RESULTS AND DISCUSSION

The Minimum Inhibitory Concentration (MIC) is referred to as the lowest concentration of a test substance that visibly inhibits microbial growth. The MIC_{50} is said to be the lowest concentration capable of inhibiting 50% of the strains during the experiment. After the antimicrobial analysis of the hexanic extract of the *Rhaphiodon echinus* leaves against the tested strains, it was obtained MIC values ranging from 1024 to 512 µg/mL.

Thus, the results obtained in the present study exhibit that the hexanic extract of *Rhaphiodon echinus* leaves showed MIC_{50} of 1024 µg/mL against *Candida albicans* strains (Table 1), 512

µg/mL against *Candida tropicalis* strains (Table 2) and 1024 µg/mL against *Candida krusei* strains (Table 3).

Table 1. Minimum Inhibitory Concentration (MIC in µg/mL) of the hexanic extract of *Rhaphiodon echinus* against *C. albicans* strains.

	ATCC 76645	LM 106	LM 108	LM 111
1024 µg/mL	+	+	+	+
512 µg/mL	+	-	-	-
256 µg/mL	-	-	-	-
128 µg/mL	-	-	-	-
64 µg/mL	-	-	-	-
32 µg/mL	-	-	-	-
Negative control	-	-	-	-
Positive control	+	+	+	+

(-) = Did not inhibit, (+) = Inhibited

Table 2. Minimum Inhibitory Concentration (MIC in µg/mL) of the hexanic extract of *Rhaphiodon echinus* against strains of *C. tropicalis*.

	ATCC 13803	LM 14	LM 31	LM 36
1024 µg/mL	+	-	+	+
512 µg/mL	-	-	+	+
256 µg/mL	-	-	-	-
128 µg/mL	-	-	-	-
64 µg/mL	-	-	-	-
32 µg/mL	-	-	-	-
Negative control	-	-	-	-
Positive control	+	+	+	+

(-) = Did not inhibit, (+) = Inhibited.

Table 3. Minimum Inhibitory Concentration (MIC in µg/mL) of the hexanic extract of *Rhaphiodon echinus* against *C. krusei* strains.

	LM 08	LM 13	LM 656	LM 978
1024 µg/mL	+	+	+	+
512 µg/mL	-	-	+	-
256 µg/mL	-	-	-	-
128 µg/mL	-	-	-	-
64 µg/mL	-	-	-	-
32 µg/mL	-	-	-	-
Negative control	-	-	-	-
Positive control	+	+	+	+

(-) = Did not inhibit, (+) = Inhibited.

According to Sartorattoet *al*.[15] (2004), products with MIC between 50 and 500 µg/mL demonstrate strong antifungal activity, between 600 and 1500 µg/mL moderate antifungal activity, and above 1500 µg/mL weak antifungal activity. Thus, according to the MIC_{50} results obtained in this research, the hexanic extract of *Rhaphiodon echinus* leaves showed antifungal activity ranging from moderate to strong, being moderate against *Candida albicans* and *Candida krusei* strains, and strong against *Candida tropicalis* strains.

There is a scarcity of studies on the isolation and purification of molecules with *Rhaphiodon echinus*, although the infusion of *Rhaphiodon echinus* leaves is used in Brazilian folk medicine for the treatment of inflammation, coughs, and infectious diseases [16]. Despite this, according to a literature survey conducted by Cruz Neto *et al*.[11] (2021) on the medicinal use of *Rhaphiodon echinus* Schauer (Lamiaceae), and its biological and pharmacological activities, studies have shown that plant products of *Rhaphiodon echinus* showed antimicrobial, anti-inflammatory and analgesic effects. However, no antifungal activity studies have been conducted with the hexanic extract of *Rhaphiodon echinus* leaves.

Pio *et al*.[12] (2019), when conducting a phytochemical and pharmacological investigation using aerial parts of *Rhaphiodon echinus* (Ness & Mart.) Schauer, revealed the prevalence of flavonoids, tannins, lignans, and saponins in the freeze-dried product and ethyl acetate extract, as

well as terpenic compounds in the hexanic and chloroform extract. In the same study, tests showed that the hexanic extract has concentration-dependent spasmolytic activity.

Ferreira *et al*.[4] (2019), when analyzing the antifungal activity of ethanolic and aqueous extracts of *Rhaphiodon echinus* on *Candida albicans* strains (ATCC 76645, LM 106, LM 108 and LM 111), with in vitro assays using the broth microdilution technique to obtain the MIC, observed that the MIC_{50} of both the aqueous and ethanolic extracts of *Rhaphiodon echinus* against *Candida albicans* strains was 256 µg/mL, thus having the same effect when compared, showing a strong antifungal capacity. Thus, corroborates with the present study, which demonstrated moderate to strong antifungal activity of *Rhaphiodon echinus*hexanic extract on the same *Candida albicans* strains.

In a study conducted by Costa *et al*.[17] (2017), which investigated the chemical activity, toxicological and increased antimicrobial activity of antifungal and antibacterial drugs by the plant *Rhaphiodon echinus* (Nees& Mart.) Schauer, it was observed that the association between aqueous extract of *R. echinus* and antifungal drugs such as nystatin showed antagonistic effect against *Candida albicans* and *Candida tropicalis* when compared to the control; while the ethanolic extract of the same plant potentiated the effect of nystatin.

Phytotherapy brings the possibility of expanding these therapeutic options, constituting an important source of innovation in health, and can further strengthen innovation, production, and exploitation of the rich Brazilian biodiversity; the herbal medicine industry, besides being accessible to most patients, can represent an excellent alternative to ensure access to safe, effective, and quality medicines [18].

CONCLUSION

The hexanic extract of *Rhaphiodon echinus* leaves against *Candida albicans*, *Candida tropicalis*, and *Candida krusei* strains showed moderate to strong antifungal activity and can be considered a promising resource for the treatment of oral candidiasis. However, further studies are essential to emphasize its efficacy against different types of microorganisms and, subsequently, in vivo research to verify its behavior in the human organism.

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