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EVALUATION OF ANTIFUNGAL ACTIVITY OF THE HYDROALCOOLIC EXTRACT OF *Pereskia aculeata* MILLER LEAVES IN *Candida* spp

[1]Francisco Glauber Peixoto Ferreira, [1]Gabriela Silva Cruz, [1]Hudson Pimentel Costa [1]Maria Imaculada Lourenço Meirú, [1]Matias Neto Alves Ferreira, [1]Camila Peixoto do Valle, [1]Aluísio Marques da Fonseca, [2]Ana Caroline Rocha de Melo Leite, [2]Érika Helena Salles de Brito and [1,2]Juliana Jales de Hollanda Celestino

[1]Academic Master in Sociobiodiversity and Sustainable Technologies, University of International Integration Lusophone African-Brazilian, Redenção, CE, Brazil; [2]Institute of Health Sciences, University of International Integration Lusophone African-Brazilian, Redenção, CE, Brazil

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****Corresponding author:***

ABSTRACT

The genus *Candida* has about 200 species, of which 17 are of clinical interest in the most diverse environments and body nichesIn this context, medicinal plants have been tested to combat various pathologies, for example, for candidiasis, the *Pereskia* aculeata Miller with different therapeutic effects can be highlighted. The aim of this study was to evaluate the antifungal activity of *Pereskia* aculeata Miller extract in *Candida* albicans, *Candida* tropicalis and *Candid*a albicans ATCC 90028 strains. Therefore, the *P. aculeata* plant was collected from the Biodiversity site located at the Massif of Baturité in the city of Mulungu-CE. The extracts of *P. aculeata* Miller leaves were prepared with 70% alcohol and distilled water. The strains were seeded in 96-well U-well microtiter plates containing RPMI 1640. The strains used in the antifungal test came from different strains of body sites.It was verified in the study that the hydroalcoholic extract of *P. aculeata* demonstrated inhibitory activity against the strains used, where the concentration of the extract that inhibited the growth of the strains varied between 12.5 and 25%. The study demonstrated through the microdilution technique in the broth that there is inhibition of growth in some species of the genus *Candida* using the extract of *P. aculeata.*

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INTRODUCTION

The genus *Candida* has around 200 species, 17 of which are of clinical interest (KARKOWSKA KULETA *et al*. 2009; KURTZMAN *et al*. 2011). These, in turn, constitute in some species the natural microbiota of the healthy individual. However, in the presence of an immune imbalance, this fungus can become pathogenic. Candidiasis is characterized as an opportunistic infection caused by *Candida* spp. *Candida albicans* and non-albicans yeasts, such as *Candida glabrata, Candida tropicalis, Candida parapsilosis, Candida krusei* and *Candida dubliniensis* (QUINDÓS, 2002; SIDRIM; MOREIRA, 1999; WHITE *et al*., 2004 apud AVRELLA; GOULART, 2008), promote localized changes in the mucosa that can spread throughout the body and lead to death (NEGRI *et al*., 2012).

In this sense, *C. albicans* is the most pathogenic species, often related to oral candidiasis. It can be triggered by the use of broad-spectrum antineoplastics, corticosteroids, and antibiotics, as well as decreased immunity, poor oral hygiene, and unbalanced nutrition (LUIZ *et al*., 2008). Another species that deserves attention is *C. tropicalis*, for its extensive power of infection and strong resistance to existing pharmacological measures. This species is capable of harming a variety of tissues, especially the kidneys, in advanced disease (WHIBLEY *et al*., 2015). Regarding *Candida albicans* ATCC90028, it is a standardized species in the laboratory, and its characteristic is the production of biofilm and resistance to triazoles, such as fluconazole, which in most studies this species is used as a control, mainly by related to this factor (TURRAN & DEMIRBILEK, 2018). Thus, its use in studies with natural extracts becomes pertinent. These have been the focus of science as a pharmacological alternative, aiming to

prevent or minimize the effects and resistance observed among antifungals. About medicinal plants, these can be a useful alternative for the treatment of different infections, which has already been done by a considerable portion of the population (MARTINS, 2010). This practice involves several factors, such as lack of access to health services, favoring the use of medicinal plants as the only resource in the treatment of pathologies (LEITE *et al*., 2008; ALBUQUERQUE *et al*., 2010; ROQUE *et al*., 2010). Another point to consider is its cost-effectiveness. Mainly, Brazil, because it has a tropical climate and allows the culture of specific species, presents a higher tendency of consumption and frequency of products of plant origin (PISANO *et al*., 2014). Besides, we can highlight that the use of medicinal plants is more adaptable to the body than synthetic products. Among these plants, we can highlight the genus *Pereskia aculeata* Miller (Figure 1), popularly known as "ora-pro-nobis". It is a plant of shrub climbing structure of the family Cactaceae, being widely used for ornamentation, food product, and mainly for medicinal purposes. In this context, Santos *et al*. (2010) conducted a study with this species to investigate an action on the inflammatory process and recovery of integumentary tissue in burn accidents.

Figure 1. Photos of plant species: *Pereskia aculeata* Miller and "ora-pro-nobis," cultivated at the Biodiversity Valley Site in Mulungu - CE.*SOURCE: Personal Archive*

Other therapeutic effects of *P. aculeata* include treatment of diseases, including diabetes and systemic arterial hypertension (SAH); antitumor, antirheumatic, anti-ulcer, and anti-inflammatory effects; analgesic action on headache and gastric pain; hemorrhoids and atopic dermatitis (GOH, 2010). Moreover, a study by Almeida (2008) showed promising results when observing the antimicrobial action of *P. aculeata* hydroalcoholic extract on bacteria such as *Staphylococcus aureus*, *Bacillus cereus, Salmonella enterica* subsp., *Escherichia coli* and *Enterococcus faecalis*. However, one of the worrying factors regarding the use of plants for medicinal purposes is related to toxicity and severe side effects. Therefore, the Ministry of Health, in the Health Surveillance sector, establishes, based on Ordinance 06/1958, the use of plant species based on scientific studies. Thus, it aims to ensure safety and stability, producing adverse effects of equal or less intensity than its separate components (OLIVEIRA *et al*., 2016). Given the above, this research aimed to evaluate if the hydroalcoholic extract of *Pereskia aculeata* Miller leaves promotes antifungal effect in *Candida* spp.

MATERIALS AND METHODOLOGY

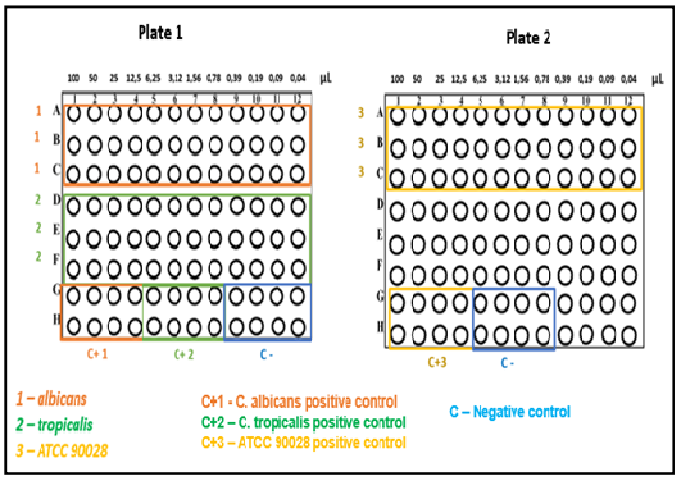
Study Design: It was a laboratory study with a quantitative approach. In quantitative studies, the information collected results in numerical format data, analyzed by statistical procedures (POLIT; BECK, 2011). In descriptive studies, the objective is to determine the distribution of diseases or conditions related to the health of a population according to the time, place, and characteristics of individuals. For this, primary or secondary data may be used (COSTA; POLAK, 2009). As for cross-sectional research, it involves data collection at a certain point and period, being adequate to describe the relationship between phenomena at a fixed location (POLIT; BECK, 2011).

Material collection and preparing the plant: A sample of the *Pereskia aculeata* Miller plant was collected at the Biodiversity Valley Site, located in Massif of Baturité, Mulungu – CE - Brazil. The location is represented by the following coordinates: Longitude: 038° .31'.3928", Latitude: 03° .44'.9775" and Height: 14,587. *Pereskia aculeata* Miller leaves were collected and air stored in a closed-lid container at 34 °C. No preservatives or substances capable of interfering with extraction processing have been added. The leaves of *P. aculeata* were sent to the Plant Physiology Laboratory of the University of International Integration Lusophone African-Brazilian - UNILAB, in the Auroras Campus, and stored in a cold room, with an average temperature of -90 ºC.

Preparation of hydroalcoholic extract of *Pereskia* aculeata Miller leaves: *P. aculeata* leaf extract was obtained according to the modified methodology of Kim et at. (2013). 70% ethanol was added in a beaker containing macerated plant leaves at a ratio of 1:20 (m/v), and the mixture remained under stirring (Solab, model SL-152/10) for 8 hours at room temperature. Subsequently, the filtrate obtained was concentrated on a roto evaporator to remove ethanol and made up to volume with distilled water. Soon after, this mixture was filtered on Whatman # 1 filter paper 5 times. Two procedures were used to obtain the aqueous extracts. In the first, the filtered mixture was added with 1:20 (w/v) distilled water, soon after it was stirred for 1 hour at a temperature ranging between 95 and 100 °C. Then, the obtained mixture was filtered on a filter paper. The extract was stored in vials sealed with film paper and stored in a freezer (-12 °C) until use.

Antifungal Susceptibility Test by Broth Microdilution Method: The Antifungal Susceptibility Test was carried out using the Broth Microdilution method using the species of *Candida albicans*, *Candida tropicalis* and *Cancida albicans* ATCC 90028 characterized as clinical strains, according to Standard M27-A3 of 2008, according to Standard M27-A3 of 2008,which describes the technique recommended by the Clinical Laboratory Standards Institute (CLSI), that is an international institution that develops norms and standards for conducting clinical pathology tests and health care issues (CLSI, 2008; SIDRIM; ROCHA, 2004). For the preparation of the inoculum, the following steps were followed, according to the instructions recommended by CLSI (2008). Initially,

subculture (subculture) of the microorganisms was performed in sterile tubes containing Agar Batata. The incubation temperature was around 35 °C. After 24 hours, the colonies were suspended in 5 mL of 0.145 mol/L sterile saline. The resulting suspension was vortexed for 15 seconds, and the cell density adjusted by spectrophotometer. To this end, sufficient saline was added to obtain the transmittance equivalent to a standard McFarland 0.5 (corresponds to 10^3/mL) scale solution at a wavelength of 530 nm. This procedure provided a standard yeast suspension containing 1 x 10^6 to 5 x 10^6 cells per mL. The working suspension was produced from a 1:20 dilution of the standard suspension with RPMI 1640 liquid medium, resulting in a concentration of 5.0 x 10^2 to 2.5 x 10^3 cells per mL. Extract concentrations were determined according to the modified method of Park *et al.* (1995). The extract was sterilized using a 22 µm milipore micromembrane filter inside the laminar flow hood. Dilution occurred over the twelve wells of the plate in the following percentages: 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, 0.39%, 0.19%, 0.09%, 0.04%, 0.02%,. The hydroalcoholic extract of *P. aculeata* leaves was diluted in the RPMI serial method, the medium used to favor the growth of *Candida* fungus and to facilitate the dilution of certain drugs used in antifungal tests. It allowed the first vertical row of wells from the deep bottom micro-culture plate to contain 100% of the extract (100 µL of the extract). In the 2nd row, this concentration was halved (50 µL) and so on over the 12 wells of the microdilution plate. Other 50 µL correspond to RPMI 1640 Medium, a culture medium used for fungal growth. Thus, each well obtained a final volume of 100 µL.



SOURCE: Personal Archive

Figure 2. Scheme of plaques used in the Antifungal Susceptibility Test with different concentrations of *P. aculeata* hydroalcoholic extract in *C. albicans, C. tropicals* and ATCC 9002 strains

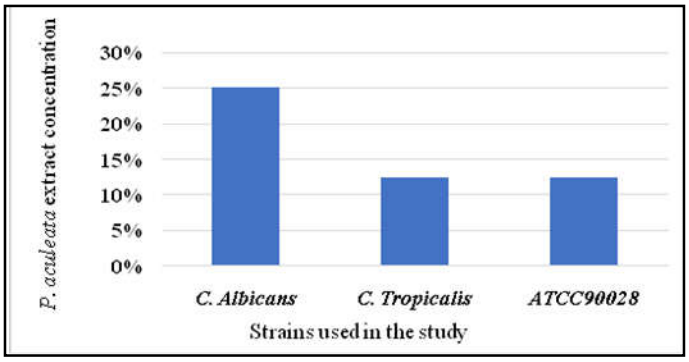
Thus, the first plate obtained presented the following organization: concentrations of the extract in serial dilution in the wells of the vertical rows A, B and C and strain 1 (*C. albicans*); extract concentrations in serial dilution in wells D, E and F and strain 2 (*C. tropicalis*); well G as positive control of strain 1 and well H as positive control of strain 2). A second plate was plotted as follows: Serially diluted extract concentrations in wells A, B and C and strain 3 (*C. albicans* ATCC 90028); well D as a positive control of strain 3; well and with the negative control of the board. Positive control wells contained 100 µL of extract-free sterile medium and 100 µL of the 2X concentrated inoculum suspensions. The negative control wells contained 200 µL (with 100 µL of extract and 100 µL of RPMI) of sterile medium. A negative control was used to effect sterility control. Thus, horizontal row 1 contained the highest concentration of hydroalcoholic extract and 12 the lowest concentration, as outlined in Figure 2. Each well of the microdilution plate was inoculated on the day of the test with 100 µL of the corresponding 2X concentrated suspension of the inoculum. The microdilution plates were incubated at 35 °C, observing visible growth. Results were read after 24 and 48 hours. The microdilution wells received a score (score) according to the yeast growth seen in each well, compared to the positive control wells with the aid of a reading mirror. Thus, each well of the microdilution plate received a numerical value using the following scale: 0 - optically clear; 1 - indefinite growth; 2 - noticeable growth reduction; 3 - a slight decrease in increase; 4- no growth reduction. The MIC value (Minimum Inhibitory Concentration) of the hydroalcoholic extract was defined as the lowest concentration of this substance in which the 0 (optically clear) score was observed, and the MIC value of the related extract was defined as the lowest concentration in which it was observed. Score 2 (noticeable growth reduction). In addition to MIC, we found the Minimum Fungicide Concentration (CFM), represented by the lower concentration of the drug capable of inhibiting 100% yeast growth. Grass staining was performed in those corresponding to the increase in the minimum fungicide concentration test to rule out contamination in the wells of microdilution plates.

Ethical aspects

The project was approved by the Research Ethics Committee of that educational institution, according to CAAE 59953716.5.0000.5576 and opinion number 1.937.092. The subjects' autonomy and non-maleficence and beneficence of the research were guaranteed, as stated in Resolution 466/12 of the National Council. The strains, obtained from the collection of the oral microbiota of UNILAB students and isolation and identification of *Candida* spp. in these samples, were requested by prior authorization duly signed by the technician in charge of the Microbiology Laboratory of the University of International Integration Lusophone African-Brazilian - UNILAB.

RESULTS AND DISCUSSION

After performing the antifungal activity assay by the broth microdilution technique, it was found that all the strains studied were susceptible to specific concentrations of the hydroalcoholic extract of *P. aculeata* Miller established by the serial dilution method. Although the present research used other *Candida* strains, unlike the ones observed here, Vargas (2017) did not show, in his study, and antifungal action of *P. aculeata* Miller hydroalcoholic extract about *Candida albicans* ATCC 10231 and *Candida tropicalis* ATCC 13803. When the MIC of the extract was evaluated about the different strains of *Candida*, the inhibition of well 3 containing *C. albicans* species was observed, which allowed to suggest a MIC of 25% of the extract of *P aculeta* Miller. (Graph 1). In relation to *C. tropicalis* and ATCC 90028 there was an inhibition percentage around 12.5% of the hydroalcoholic extract concentration by serial dilution. That there is a compound related to the inhibiting effect. However, after the Minimum Fungicide Concentration (MFC) test, these strains grew in the tube corresponding to the referent well, suggesting that this concentration was not able to prevent visible growth of the subjunctive.



Graph 1. Antifungal activity of *P. aculeata* hydroalcoholic extract against strains of *Candida albicans*, *Candida tropicalis* and *Candida albicans* ATCC 90028 in the species

Wells 1 and 2 of the cultivation plate corresponding as concentrations of 50 and 25% of the hicroalcoholic extract of the leaves of *Pereskia aculeata* Miller. Gram's staining was performed, so that the possibility of contamination was discarded, resulting in the presence of *Candida albicans* by microscopic morphological analysis. Moreover, this coloring was important to verify if in the well where there was growth, it matched the fungal species used for the test, as shown in Figure 3.

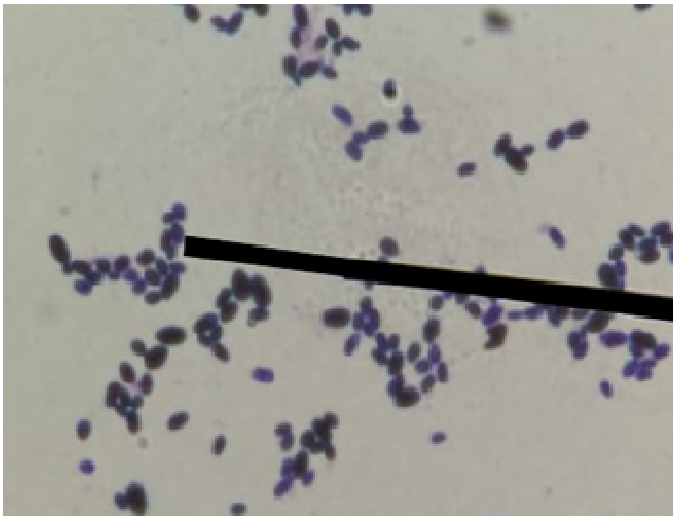


Figure 3. Candida strains, under microscopic morphological analysis, in well 3 at a concentration of 25% of species of *C. albicans* in the Minimum Fungicide Concentration (CFM) test, 400X

Contrary to the data obtained here, Santos *et al*. (2011) did not get promising results when using the crude leaf extract of *P. aculeata* in *Candida albicans* ATCC 18804 and *Candida albicans* ATCC 448858. In the study by Pinto *et al*. (2012), the authors demonstrated the cytotoxic activity of *P. aculeata* against certain types of cancer cells, which may imply its use in specific situations. Regarding the species of *C. tropicalis*, there was no growth until well 3, suggesting a MIC of 12,5% and evaluating that this dilution percentage was able to inhibit such activity also demonstrating that in the previous wells of the plaque of this species there was no presence of the fungus by the Minimum Fungicide Concentration (CFM) test. In research conducted by Turra *et al*. (2007), *Candida albicans* showed no sensitivity to the species of *Pereskia grandifolia*. In *Candida albicans* ATCC 90028 species, growth inhibition occurred in wells 1 and 2, suggesting orresponding to 50% and 25% of the extract. The confirmation was due to the absence of strain in the CFM test. For Kanopka *et al*. (2010), species of *C. albicans* ATCC 90028 has, as resistance mechanism, the presence of biofilm, allowing the loss of antifungal effects, especially Fluconazole. Thus, it can be assumed that this mechanism did not interfere with the antifungal action of *P. aculeata*. Given all of the above, the study suggests that there is some compound in the extract that promotes this inhibition of activity, and further studies are needed to isolate it. Thus, based on these findings, further studies with *P. aculeata* are necessary, as this species has shown promising results when evaluating fungal growth sensitivity and inhibition.

Conclusion

The study confirmed, through the broth microdilution technique, that there is growth inhibition of the genus *Candida,* specifically *C. albicans* and *C. tropicalis*, by the hydroalcoholic extract of *Pereskia aculeata* Miller. Thus, it can be understood that there is a therapeutic possibility of this natural product, and further studies involving the theme should be conducted to clarify questions related to the composition and mechanism of action and minimize risks to future use in clinical practice.

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Conflict of Interest: The authors have declared no conflict of interests.

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