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Fern extracts potentiate fluconazole activity and inhibit morphological changes in *Candida* species

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ABSTRACT

Objective: To investigate the antifungal activity of the fern species *Lygodium venustum* (*L. venustum*) and *Pityrogramma calomelanos* (*P. calomelanos*) against *Candida albicans* and *Candida tropicalis* strains.

Methods: The microdilution method was used to evaluate the antifungal activity, as well as the modulating effects of ethanolic extracts of these plants in combination with fluconazole. The minimum inhibitory concentration (MIC), minimum fungicide concentration and morphological changes were also determined.

Results: The extract obtained from *L. venustum* presented a MIC > 8 192 μg/mL, while the extract obtained from and *P. calomelanos* presented a MIC = 8 192 μg/mL, indicating that they present weak antifungal activity. However, combination of the extracts with Fluconazole potentiated the antifungal activity of this drug. At different experimental conditions, such as concentration of the extract and type of strain, the extracts inhibited hyphae and pseudohyphae formation, indicating that these fern species can affect the morphology of the fungi.

Conclusions: The extracts obtained from the fern species *L. venustum* and *P. calomelanos* dose not present significant antifungal activity. However, *P. calomelanos* potentiates the activity of fluconazole and both extracts inhibits the morphological changes in *Candida* species, indicating that they have potential pharmacological activity as modulators of fungal biology. Therefore, novel studies are required to characterize the interference of these extracts in the virulence and pathogenicity of *Candida* species as well as the potential of fern species to treat fungal infections.

1. Introduction

Candida species are commensal microorganisms in healthy individuals. However, under certain conditions, such as immunosuppression, these fungi act as opportunistic pathogens, causing various infectious diseases. Thus, the infections caused by *Candida* spp. can range from simple colonization of the mucous membranes to systemic infections, which represent important public health problems due to the high morbidity and mortality rates [1].

Candida albicans (*C. albicans*) is one of the main causative agents of human infections [2]. As in most opportunistic infections, infections caused by this microorganism are favored by failures in the immune response of the host, as well as by the virulence mechanisms and morphological changes of the fungus [3]. *Candida tropicalis* (*C. tropicalis*) is

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an opportunistic pathogen that infects mainly neutropenic patients, causing diseases that are associated with suppression of the bacterial microbiota by uncontrolled antibiotic therapy [4].

Although the infections caused by *C. albicans* are most frequent, other species of the genus, such as *C. tropicalis, Candida glabrata, Candida krusei* and *Candida parapsilosis* have been highlighted as important infectious agents [5]. Therefore, the development of targeted therapies to overcome mechanisms of virulence, as well as to prevent and control fungal infections has gained increasing importance in the field of health research [6,7].

Antifungal agents may present a broad range of pharmacological activities and mechanisms of action by which they exert toxic effects to the microorganisms. Therefore, the discovery of drug targets in the microorganisms is crucial to the development of novel, effective and safe antifungal therapies [8]. In this context, the use of medicinal plants to treat fungal infections has been reported by several studies [9]. In fact, the knowledge of the use of medicinal plants has been handed down from generation to generation in many cultures and places [10], serving as a basis for the treatment of mycoses in traditional medicine [11].

Lygodium venustum (*L. venustum*) is a fern species that is traditionally used as an herbal remedy. The aerial parts of this plant are administrated topically or in the form of teas to treat numerous diseases, including infections and dermatosis [12]. However, the pharmacological activity of this plant against pathogenic microorganisms remains to be characterized [13–15].

The fern species *Pityrogramma calomelanos* (*P. calomelanos*), popularly known as 'feto-branco', 'avenca-branca' or 'avenca-preta', is used both in the decoration of environments and in medicine [16,17]. In folk medicine, this plant is used as astringent, painkiller, chest depurative and emmenagogue. Previous studies demonstrated that it presents antiviral, antihypertensive, antipyretic, antitussive and blood circulation stimulant properties, besides being indicated to treat kidney and bladder disorders [17–19].

Therefore, the aim of this work was to evaluate the antifungal activity of the ethanolic extracts obtained from *L. venustum* and *P. calomelanos* and investigate the modulating effect of these extracts on the morphology of *Candida* species.

2. Materials and methods

2.1. Plant material

The leaves of *L. venustum* and *P. calomelanos* were collected in Crato, Ceará State, Brazil. The plants were identified by Dr. Antonio Álamo Feitosa Saraiva and the samples were deposited in the herbarium (Herbário Caririense Dárdano de Andrade-Lima) of the Regional University of Cariri-URCA, with the following voucher numbers: 5569 and 5570, respectively.

2.2. Extract preparation

Fresh leaves of *P. calomelanos* (950 g) and *L. venustum* (211.18 g) were dried and kept at room temperature. The powder material of each plant was placed to soak in 1 L of 95% ethanol for 72 h at room temperature. Then, the plant materials were filtered and concentrated in a rotary evaporator at 60 °C at 760 mm/Hg pressure, yielding 26.3 of the ethanolic extract of *P. calomelanos* (EEPC) and 12.42 g of the ethanolic extract of *L. venustum* (EELV).

2.3. Microorganisms

The following *Candida* strains were used in the antifungal activity trial: *C. albicans*-CA INCQS 40006 and CA LM 77; *C. tropicalis*-CT INCQS 40042, CT LM 23. These strains were obtained from the Laboratory of Mycology of the Federal University of Paraíba.

2.4. Determination of minimum inhibitory concentration (MIC)

The MIC of the extracts were determined using the microdilution method in 96-well microtiter plates [20,21]. A solution containing 1 350 μL of double concentrated sabouraud dextrose broth and 150 μL of the fungal suspension was prepared in a test tube before distribution to the microtiter plates. Each well was added with 100 μL of this solution and then, 100 μL of the extract solution was added in the first well to be serially diluted [22]. The effects of the extracts were analyzed by observing the turbidity produced by the fungal growth after incubation at 37 °C for 24 h. The MIC was defined as the lowest concentration that visually inhibited the fungal growth in comparison with the control group. Of note, both extracts were used in concentrations ranging from 1 024 to 1 μg/mL.

2.5. Drug modulation test

In this test, the extracts were used at sub-inhibitory concentrations (MIC/16). Briefly, 100 μL of a solution containing sabouraud dextrose broth, fungal inoculum (at 10%) and the extracts was distributed in the microtiter plate. Then 100 μL of a fluconazole solution was added in the first well. This drug was serially diluted as previously described to achieve concentrations ranging from 512.0 to 0.5 μg/mL [23]. The plates were analyzed using a spectrometer (ELISA Termoplate®) and the readings were performed at 630 nm.

2.6. Determination of minimum fungicidal concentration

Petri dishes containing sabouraud dextrose agar were inoculated with 20 μL of solutions removed from each well in which there was no fungal growth. These plates were incubated at 35–37 °C for 24 h. The minimum fungicide concentration was defined as the lowest concentration seeded in sabouraud dextrose agar at which no growth was detected [24].

2.7. Evaluation of effects of L. venustum and P. calomelanos extracts on Candida micromorphology

The micromorphology assay was performed using a wet chamber to observe the morphological changes in *Candida* strains. The fungal strains were cultured in diluted (10×) potato dextrose agar medium [25,26]. The extracts were added to the potato dextrose agar medium using the concentrations determined by the MIC assay (MIC, MIC/2, MIC×2). Briefly, 2 mL of potato dextrose agar were placed in a glass slide with the respective concentration of the extract. After solidification,

the yeasts were seeded using a needle. To this end, 2 parallel lines were designed and covered with sterile glass slides. The wet chamber was prepared by adding 2 mL of distilled water over a piece of sterile filter paper (3 cm × 3 cm) inside a petri dish. Each assay was analyzed using an optical microscopy with a 40× magnification. After the incubation, the presence of *Candida* structures (yeast, hyphae and pseudohyphae) was analyzed. The micro-cultivation was photographed by digital camera with a 5× magnification. This assay was performed in triplicate.

The fungal microculture technique was used to determine the effects of the extracts on the cell morphology of *Candida* strains, using the solid potato agar medium in wet chamber. The extract solutions were added to the potato agar medium culture in the following concentrations-MIC/2, MIC and MIC×2. A group without extract addition was used as growth control.

3. Results

As shown in Figure 1, the extracts obtained from *P. calomelanos* and *L. venustum* presented MICs. The MIC of

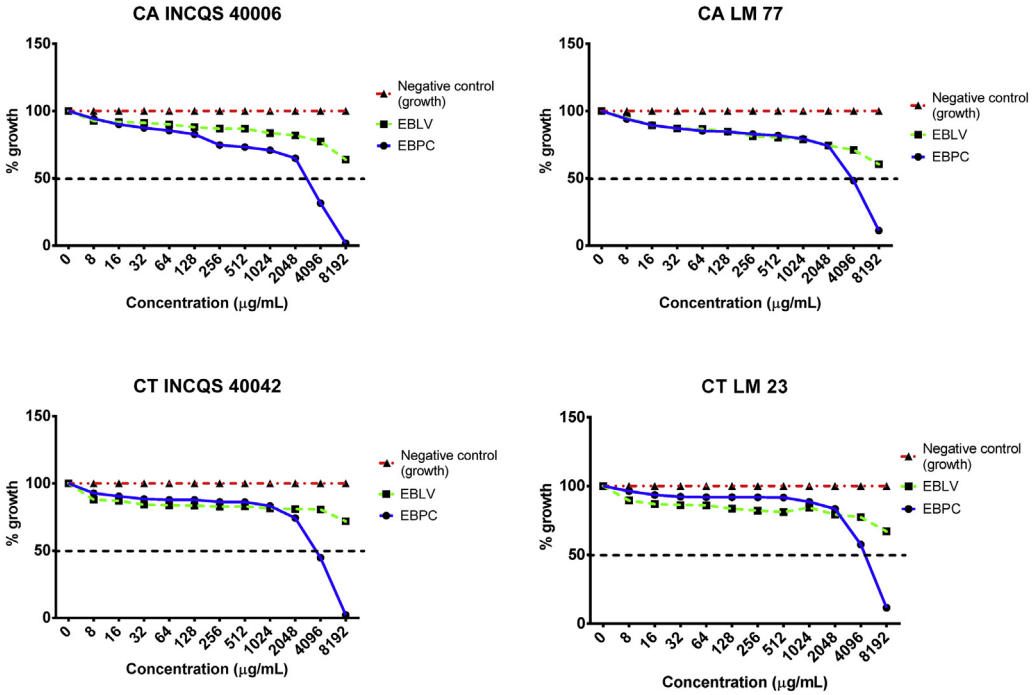

Figure 1. Lethal curves of extracts of *L. venustum* and *P. calomelanos*.
Strains of *C. albicans*: CA INCQS 40006 and CA LM 77; *C. tropicalis*: CT INCQS 40042 and CT LM 23.

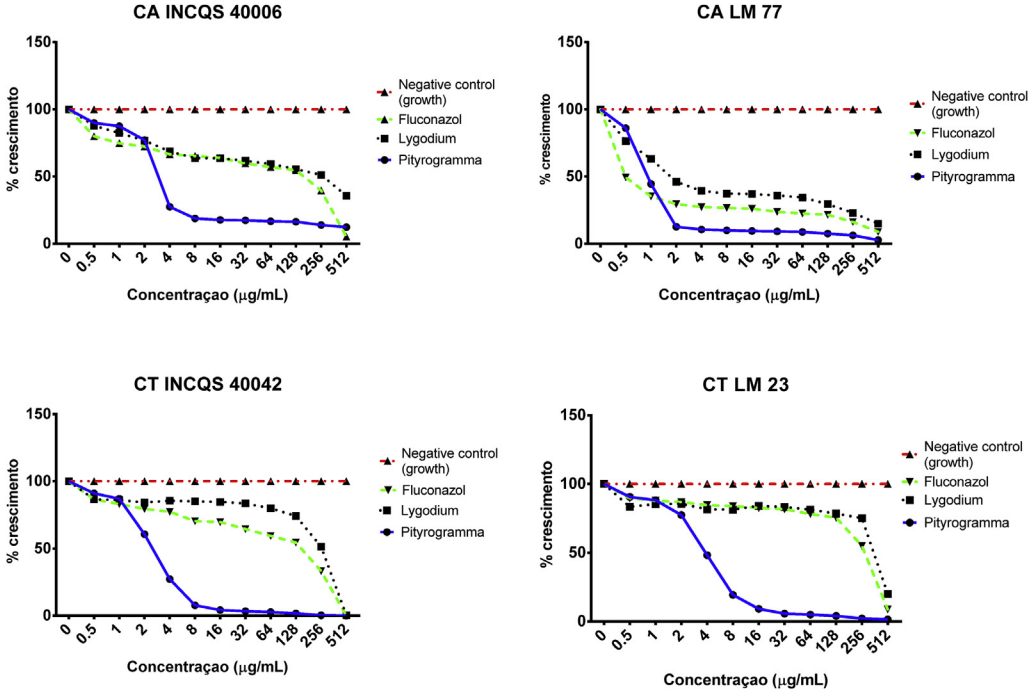

Figure 2. Lethal curves demonstrating modulatory potential of extracts of *L. venustum* and *P. calomelanos* associated with fluconazole.
Strains of *C. albicans*: CA INCQS 40006 and CA LM 77; *C. tropicalis*: CT INCQS 40042 and CT LM 23.

Figure 3. Effect of *L. venustum* against micromorphology of *Candida*.

Figure 4. Effect of *P. calomelanos* against micromorphology of *Candida*.

P. calomelanos was 8 192 μg/mL for both strains, and for *L. venustum* the MIC was ≥ 8 192 μg/mL against all tested strains, indicating that they present weak antifungal activities. However, the combination of the EEPC with fluconazole potentiated the activity of this drug against *Candida* strains, especially against the CT INCQS 40042 and CT LM 23, indicating that this association caused a synergistic antifungal effect (Figure 2). On the other hand, the ethanolic extract obtained from *L. venustum* did not affect the activity of fluconazole against CA INCQS 40006 and CT LM 23, but caused an antagonistic effect against CT INCQS 40042 and CA LM 77, indicating that these plants present different modulating potential of antifungal drug activity.

Figure 3 shows that the EELV at concentrations equivalent to MIC/2, MIC and MIC×2 did not inhibit hyphae or pseudohyphae formation in the CT INCQS 40042 strain. Comparable results were observed using the EEPC at concentrations equivalent to MIC/2 and MIC. However, this extract significantly inhibited the formation of hyphae and pseudohyphae at a concentration equivalent to of MIC×2 (Figure 4), indicating that the effect of this extract on the morphology of this strain is concentration-dependent.

Similarly, the EELV did not affect the morphology of the CA INCQS 40006 strains, which was affected by the EEPC at concentrations equivalent to MIC and MIC× (Figure 4).

On the other hand, the EELV inhibited the formation of hyphae or pseudohyphae in the CT LM 23 strain at concentrations equivalent to MIC/2 and MIC. In turn, the EEPC only affected the morphology of this strain at a concentration equivalent to MIC×2 (Figure 3 and 4). Finally, the morphology of CA LM77 strain was not affected by the EEPP, but was affected by the EELV at a concentration equivalent to MIC×2 concentration (Figure 3 and 4).

Together, these results indicate that both fern species have the potential to modulate morphological changes in *Candida* strains, which depends on the type of strain and concentration of the extract.

4. Discussion

According to Morais-Braga *et al.* [27], *L. venustum* has no clinically relevant antifungal activity, because its extract presented MICs ≥ 1 024 μg/mL against *Candida* strains. Additionally, in their work, these authors demonstrated that the fractions obtained from the extract of this plant did not modulate the activity of antifungal drugs against these microorganisms.

Accordingly, Souza *et al.* [28] demonstrated that *P. calomelanos* presented a MIC ≥ 1 024 μg/mL, therefore, this plant has no significant antifungal activity. However, they demonstrated that the antifungal effect of benzoilmetronidazol was enhanced by the association with the hexanic fraction of *P. calomelanos*, indicating that this plant presents constituents that modulate the activity of antifungal drugs.

The changes in the micromorphology of *Candida* strains constitute a virulence factor that is associated with the pathogenicity of these microorganisms. Accordingly, environmental factors may affect the physiology of these microorganisms, enhancing their infective potential [29].

These morphological changes are called dimorphism, indicating the transition between the aspect of yeast and hyphae [30]. Adherence, polymorphism, phenotypic variability, toxins and extracellular enzyme production are the main virulence factors that give these microorganisms the ability to colonize an organism and cause infections [31]. In this context, hyphae formation not only promotes cellular invasion inside the mucosa, but also prevent the phagocytosis of the fungus by macrophages [32].

The virulence factors of *Candida* species include hyphae switching, germination, recognition of the surface molecules and the production of hydrolytic enzymes against the extracellular matrix [33,34]. Hydrolytic extracellular enzymes play a key role in adherence, invasion and infection of *Candida* species [34,35], which has been demonstrated mainly in *C. albicans* [36,37]. Nevertheless, the effects of natural products on the morphology of *Candida* spp., as well as their interference in the virulence and pathogenicity of these microorganisms remain to be investigated. Of note, this study was the first to report the effects of extracts obtained from ferns on the morphological changes of *Candida* species.

The extracts obtained from the fern species *L. venustum* and *P. calomelanos* did not present significant antifungal activity. However, *P. calomelanos* potentiated the activity of fluconazole and both extracts inhibited the morphological changes in *Candida* species, indicating that they have potential pharmacological activity as modulators of fungal biology. Therefore, novel studies are required to characterize the interference of these extracts in the virulence and pathogenicity of *Candida* species as well as, the potential of fern species to treat fungal infections.

Conflict of interest statement

The authors declare that they have no conflicts of interest to disclose.

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