In vitro antifungal activity of Candida culture extracts against Trichophyton rubrum and Trichophyton mentagrophytes

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Research Ethics Committee Approval: Not applicable.

Received on: Oct 11, 2021. Accepted on: Oct 31, 2021. Available online: Nov 15, 2021.

Abstract

Onychomycoses are nail infections caused primarily by dermatophytes fungi, yeasts, and other filamentous fungi, characterized by persistent infections, prolonged therapy, and high recurrence rates. In clinical practice, some of these occurrences present two or more microorganisms, and the interactions among them can change the chemical environment mediated by small diffusible molecules, producing a competitive niche. The aim of this study was to evaluate the antifungal activity of individual extracts of pure cultures of Candida albicans and C. parapsilosis against dermatophytes. To obtain the fungal extracts, cultures were filtered through a 0.2 μm membrane and submitted to liquid-liquid extraction using ethyl acetate. The Minimal Inhibitory Concentration (MIC) of each extract was evaluated by broth microdilution method and checkerboard assay with fluconazole against clinical isolates of Trichophyton rubrum and T. mentagrophytes. The invertebrate model of Galleria mellonella was used to evaluate the toxicity of the extracts. As results, the extracts of C. albicans and C. parapsilosis showed antifungal activity with MICs between 31.2 – 2000 μg/mL. In association with fluconazole, synergistic effect was detected for all combinations. The extracts presented low toxicity in G. mellonella. In the future, isolation and identification of the extract compounds may allow new therapeutic approaches in the control of fungal infections.

Keywords: Onychomycoses; Dermatophytes; Yeast; Antifungal activity.
Introduction

Onychomycoses are nail infections caused by dermatophytes, yeasts, and other non-dermatophytes moulds. They are responsible for 50% of all nail diseases and 30% of cutaneous fungal infections [1]. It is a public health problem, which generates high cost to the health services and psychosocial impact, decreasing work productivity and quality of life to the patients [2, 3]. These nail infections are characterized by high-rate recurrence with a prolonged therapy, with irreversible damage to the nail plate. This event contributes to the emergence of microorganisms resistant to conventional antifungals, which may cause refractory fungal diseases [4, 5]. In addition, the limited antifungal drugs arsenal and the potential side effects, including hepatotoxic damage, contribute to high morbidity rates [6-8].

Dermatophytes are the main etiologic agents of onychomycosis, especially Trichophyton mentagrophytes and T. rubrum species. Moreover, Candida, mostly C. albicans and C. parapsilosis, can also be associated with nail infections, especially fingernails [9]. Interactions between yeasts and dermatophytes in nails diseases may occur in cases of mixed infections or colonization, in which competition or cooperation can alter disease progression. Small diffusible molecules are produced and can mediate these interactions inside nail matrix and affect the survival of these microorganisms in this environment [10]. The mechanisms involved should be explored, once these antifungal molecules can constitute therapeutic alternatives against fungal infections [11-13]. The aim of this work was to evaluate the in vitro antifungal activity of Candida extracts, and their synergistic interaction with fluconazole, against Trichophyton mentagrophytes and Trichophyton rubrum, and to assess their toxicity in vivo model, Galleria mellonella.

Material and Methods

Clinical specimens

Clinical isolates (five of each species) of Candida albicans (CA1–CA5), Candida parapsilosis (CP1–CP5), Trichophyton mentagrophytes (TM1–TM5), and Trichophyton rubrum (TR1–TR5), and Candida albicans ATCC 90028 (CAATCC) and C. parapsilosis ATCC 22019 (CPATCC) from Laboratory of Microbiology of São José do Rio Preto Medical School (FAMERP) were used. The isolates were registered in the Brazilian National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen), under the protocol number AF41CDD.

Preparation of the inoculum and extracts

Each yeast was cultured in Sabouraud Dextrose Agar (SDA, DIFCO®) and incubated at 35 °C for 24 hours. Then, inoculums were prepared in 500 mL Sabouraud Dextrose Broth (DIFCO®, SDB), incubated at 35 °C for 48 hours, and filtered through a 0.2 μm
Millipore® membrane and submitted to liquid-liquid extraction using ethyl acetate (Synth®) as a counter-phase. The procedure was repeated three times, allowing the total extraction of the fungal metabolites from the culture media. The ethyl acetate phase was completely dried using a rotary evaporator and subsequently solubilized in sterile distilled water with 10% dimethyl sulfoxide (Synth®, DMSO).

**In vitro antifungal determination of Candida extracts against dermatophytes isolates**

The antifungal activity of Candida extracts against dermatophytes was evaluated by the broth microdilution technique according to the M38-A2 document protocol, with modifications [14].

The inoculums of dermatophytes were performed after seven days of incubation on potato dextrose agar (Sigma-Aldrich®, PDA) at 28°C. The fungal colonies were covered with 5 mL of distilled water, and the suspensions were made by scraping the surface with the tip of a sterile loop. The mixture of conidia and hyphal fragments was transferred to sterile tubes and the optical density of the suspensions, at 530 nm, adjusted to 70% of transmittance. Each suspension was diluted (1:50) with RPMI to obtain the final inoculum concentration of 0.4 to 5 x 10^4 cells/mL. Aliquots of 100 µL were inoculated into 96 well plates containing 100 µL of specific extract concentration. Controls were included for the fungal growth and sterility control of culture medium, Candida extracts, and solvent. The microplates were incubated at 37 °C for 120h. After incubation, the absorbance of the samples was measured by spectrophotometer (Biospectro, SP22, Curitiba, Brazil). The following formula was applied to calculate the percentage inhibition: \[ I=1-(\text{AbsT}-\text{AbsSC}/\text{AbsGC})\times 100 \] where: \( I \) = percentage inhibition; \( \text{AbsT} \) = absorbance of the inoculum with extract; \( \text{AbsSC} \) = absorbance of sterility control; \( \text{AbsGC} \) = absorbance of growth control. The MIC of extracts was defined as the lowest concentration inhibiting at least 80% of the control growth. An aliquot from each well that showed antifungal activity was plated in Petri dishes containing SDA, to determine the minimum fungicidal concentration (MFC). The MFC was defined as the lowest concentration of the extracts that allowed no visible growth on the solid medium [12]. All tests were carried out in triplicate.

**Antifungal susceptibility testing for fluconazole**

The antifungal susceptibility test for fluconazole (Sigma-Aldrich®) was performed according to the M27-A3 document for yeasts and M38-A2 for dermatophytes, with modifications. Regarding the yeasts, the inoculums were prepared according to the turbidity of a 0.5 McFarland standard (corresponding to 1~5 x 10^6 colony forming units (CFU/mL) in Roswell
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Park Memorial Institute (RPMI) 1640 medium. The suspension was diluted with RPMI 1640 to prepare a final inoculum suspension (1 × 10³ CFU/mL). The test was performed in pre-sterilized, flat-bottom 96-well polystyrene plates. The ATCC strains (C. albicans and C. parapsilosis) were used as control. The dermatophyte inoculums suspensions were prepared as described above. Fluconazole MIC was defined as the lowest concentration inhibiting at least 50% for yeast, and 80% for dermatophytes of the growth, in the relation to the control. According to the CLSI criteria, isolates with fluconazole MIC of ≥ 64 μg/mL were considered as resistant [15].

Checkerboard assay

The Checkerboard testing method was used to evaluate the combinatory effects of the Candida spp. extracts and fluconazole against T. mentagrophytes and T. rubrum by a microdilution technique and a spectrophotometric method. Candida extracts and fluconazole were added to the RPMI-1640 at serially twofold-diluted concentrations. To assess the nature of the in vitro interactions among Candida extracts and fluconazole, it was calculated the fractional inhibitory concentration index (FICI), according to the formula: (Fluconazole MIC of the mix / Fluconazole MIC alone) + (Extract MIC of the mix / Extract MIC alone). The synergistic interaction was classified using the method described by Kumar et al., in which values of FICI ≤ 0.5 indicate synergic interactions; 0.5 < FICI ≤ 1, additive interactions; 1 < FICI ≤ 2, indifferent interactions; and FICI > 2, antagonistic interactions [16].

Toxicity test in Galleria mellonella

The in vivo toxicity of Candida extracts was tested in G. mellonella model. A total of 10 larvae (200 ± 25 mg each) in the sixth-instar of development and absence of cuticle pigmentation were artificially injected with Hamilton micro-syringe 7000.5KH of 10 μL volume. Each larva was injected with 5 μL of Candida extracts at the concentration of 8 mg/mL into de hemocoel through the last right proleg. Additionally, each larva was bathed for two seconds into the Candida extracts at the same concentration to evaluate the superficial toxicity.

The positive control of the experiment was the injection and bath with quaternary ammonium solution. The Candida extracts solvent control was 10% DMSO. After the injection and bath, the larvae (treated and control) were incubated at 28°C, deprived of feed and direct illumination. Throughout the experiment and every 24 h, the larvae were removed from the pre-pupae, in order to delay their metamorphosis. Statistical analyses and graphics were performed by the Log-rank (Mantel-Cox) method in Prism 5 software (GraphPad®) [17].
Results

Minimum Inhibitory Concentration of Candida extracts against dermatophytes

The antifungal activity of Candida extracts is shown in Table 1. Values of MIC were similar for ATCC and clinical strains. In general, the extracts showed antifungal activity within a MIC range of 31.25 - 2000 μg/mL against the dermatophytes isolates tested. The lowest individual MIC values (31.25 μg/mL) were observed for extracts of CP3 and CA6 against TM3, TM4 and TR4.

Figure 1: MIC (A) and MFC (B) (μg/mL) of CP4 extracts against T. rubrum (TR3). SC: Sterility control; FLC: fluconazole; SOL: solvente; EXT: extract; GC: growth control.
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Considering the species in general, according to the geometric means (GM), the best antifungal activity was observed for the C. parapsilosis extracts (GM of 383 μg/mL). The CP4 extract presented the lowest mean against dermatophytes (GM of 134 μg/mL). On the other hand, C. albicans extracts showed less activity (GM of 732 μg/mL). The best activity of Candida albicans extracts was observed by CA5 extract (GM of 406 μg/mL) (Table 2). The Candida extracts against dermatophytes in general showed fungicidal properties (MFC) with a value higher than the MIC (range of 500 - >2000 μg/mL, and GM of 1858 μg/mL) (Table 1).

### Table 1. MICs and MFCs (µg/mL) of Candida extracts against dermatophytes isolates.

|           | Trichophyton mentagrophytes |         |         |         |         |
|-----------|-----------------------------|---------|---------|---------|---------|
|           | MIC    | MFC   | MIC    | MFC   | MIC    | MFC   | MIC    | MFC   |
| CAATCC    | >2000  | >2000 | 1000   | 2000  | 2000   | >2000 | 2000   | 2000  |
| CA2       | >2000  | >2000 | >2000  | >2000 | >2000  | >2000 | >2000  | >2000 |
| CA3       | 1000   | 2000  | 2000   | 2000  | 2000   | 2000  | 2000   | 2000  |
| CA4       | 2000   | >2000 | 2000   | 2000  | 2000   | >2000 | 2000   | >2000 |
| CA5       | >2000  | >2000 | 125    | 2000  | >2000  | 2000  | 125    | 2000  |
| CA6       | 2000   | 2000  | 2000   | 2000  | 2000   | 2000  | 2000   | 2000  |
| CPATCC    | 2000   | >2000 | 2000   | 2000  | 2000   | >2000 | 2000   | >2000 |
| CP2       | 62.5   | 2000  | 2000   | 2000  | 500    | 2000  | 2000   | 2000  |
| CP3       | 125    | >2000 | 250    | 2000  | 1000   | 2000  | 500    | 2000  |
| CP4       | 62.5   | 1000  | 250    | 1000  | 31.25  | 1000  | 250    | 1000  |
| CP5       | 1000   | 2000  | 250    | 1000  | 1000   | 2000  | 1000   | 2000  |
| CP6       | 1000   | >2000 | 500    | >2000 | 2000   | >2000 | 2000   | >2000 |

|           | Trichophyton rubrum         |         |         |         |         |
|-----------|-----------------------------|---------|---------|---------|---------|
|           | MIC    | MFC   | MIC    | MFC   | MIC    | MFC   | MIC    | MFC   |
| CAATCC    | 500    | 2000  | 1000   | 2000  | 62.5   | 2000  | 31.25  | 1000  |
| CA2       | 1000   | 2000  | >2000  | >2000 | 250    | >2000 | 250    | 1000  |
| CA3       | 2000   | 2000  | 2000   | 2000  | 1000   | 2000  | 500    | 1000  |
| CA4       | 2000   | >2000 | 1000   | >2000 | 2000   | 2000  | 500    | 2000  |
| CA5       | 250    | 2000  | >2000  | >2000 | 250    | 1000  | 125    | 1000  |
| CA6       | 500    | 2000  | 2000   | >2000 | 500    | 1000  | 31.25  | 1000  |
| CPATCC    | 125    | >2000 | 250    | 2000  | 500    | 1000  | 500    | 1000  |
| CP2       | 250    | 2000  | 125    | >2000 | 500    | 2000  | 250    | 2000  |
| CP3       | 31.25  | 2000  | 62.5   | 2000  | 125    | 1000  | 500    | 2000  |
| CP4       | 62.5   | 2000  | 1000   | 2000  | 250    | 2000  | 62.5   | 1000  |
| CP5       | 1000   | 2000  | 500    | 1000  | 500    | 1000  | 500    | 1000  |
| CP6       | 2000   | 2000  | 1000   | 1000  | 1000   | 1000  | 1000   | 1000  |
**Table 2.** Geometric means of MICs and MFCs (µg/mL) of *Candida* extracts against dermatophytes isolates.

| Extracts      | Geometric means (µg/mL) | TR | TM | TR/TM |
|---------------|-------------------------|----|----|-------|
|               | MIC         | MFC         | MIC | MFC | MIC         | MFC         |
| CAATCC        | 189         | 1741        | 1516 | 2639 | 536         | 2144        |
| CA2           | 660         | 2297        | 2639 | 4000 | 1320        | 3031        |
| CA3           | 1000        | 1741        | 1149 | 2000 | 1072        | 1866        |
| CA4           | 1000        | 2639        | 1320 | 3031 | 1149        | 2828        |
| CA5           | 287         | 1741        | 574  | 2000 | 406         | 1866        |
| CA6           | 250         | 1189        | 758  | 1741 | 435         | 1439        |
| CPATCC        | 218         | 1516        | 1000 | 2000 | 467         | 1741        |
| CP2           | 287         | 2000        | 500  | 1516 | 379         | 1741        |
| CP3           | 165         | 1741        | 250  | 2000 | 203         | 1866        |
| CP4           | 165         | 1516        | 109  | 758  | 134         | 1072        |
| CP5           | 660         | 1320        | 660  | 2000 | 660         | 1625        |
| CP6           | 1000        | 1149        | 1000 | 3031 | 1000        | 1866        |

**MIC:** Minimal inhibitory concentration; **MFC:** minimum fungicidal concentration

**Antifungal susceptibility testing of clinical isolates for fluconazole**

The MIC values for fluconazole are shown in Table 3. *Candida albicans* and *C. parapsilosis* isolates showed MIC values between 0.25 – 2 µg/mL. Among the dermatophytes, higher values were found for *T. mentagrophytes* (16 – 64 µg/mL), in comparison with *T. rubrum* (4 – 8 µg/mL). According to CLSI interpretive guidelines, isolates of *C. albicans*, *C. parapsilosis* and *T. rubrum* were characterized as susceptible (S) to fluconazole. For *T. mentagrophytes*, isolates were characterized as susceptible dose-dependent or resistant (Table 3).

**Table 3.** MICs (µg/mL) of fluconazole against *Candida* and dermatophytes isolates.

| Isolates | MIC (µg/mL) | Susceptibility |
|----------|-------------|----------------|
| CAATCC   | 1           | S              |
| CA2      | 1           | S              |
| CA3      | 1           | S              |
| CA4      | 0.25        | S              |
| CA5      | 0.25        | S              |
| CA6      | 0.5         | S              |
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|         | MIC      |
|---------|----------|
| CPATCC  | 0.5      |
| CP2     | 1        |
| CP3     | 1        |
| CP4     | 2        |
| CP5     | 1        |
| CP6     | 2        |
| TM1     | 32       |
| TM2     | 64       |
| TM3     | 64       |
| TM4     | 64       |
| TM5     | 16       |
| TR1     | 8        |
| TR2     | 8        |
| TR3     | 4        |
| TR4     | 8        |
| TR5     | 4        |

MIC: Minimal inhibitory concentration; CA: Candida albicans; CP: Candida parapsilosis; TM: Trichophyton mentagrophytes; TR: Trichophyton rubrum; S: Susceptible; SDD: Susceptible dose-dependent; Intermediate; R: Resistant.

Checkerboard assay

The FICI analyses showed synergistic effects between fluconazole and Candida extracts against dermatophytes. The MIC ranges of fluconazole and extracts, when individually tested, were 4 – 64 μg/mL and 31.25 – 2000 μg/mL, respectively (Figure 2).

The results showed decreased in the MIC ranges for the most combinations tested (Table 4). Consequently, a predominantly synergistic effect (FICI ≤ 0.5) could be observed in the combinations evaluated. Considering the results against T. rubrum and T. mentagrophytes together, general GM of FICI was 0.42. The best antifungal synergistic activities were observed for CA3, CA5, and CA4 with GM of FICI of 0.26, 0.28 and 0.3, respectively (Table 5).

Toxicity test in Galleria mellonella

In the experiments with larvae bathed in the Candida extracts, there were no statistical differences among extracts, except when compared to the ammonium quaternary toxicity control (p> 0.05, with 95% confidence) (Figure 3). Regarding the experiment with the Candida extracts injections, the CA5 extract was the only one that show some
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toxicity, being able to kill 40% of the larvae (p= 0.023) (Figure 4). Thus, results show nontoxicity of Candida of the great majority of the extracts at 8 mg/mL in both injection and bath procedure in G. mellonella model.

Figure 2: Checkerboard assay - CA2 extract and fluconazol against T. rubrum (TR2), decrease in the MIC range (IFICI 0.03). FLC: fluconazole; SOL: solvente; EXT: extract; GC: growth control.

Table 5. Geometric means of FICIs of Candida extracts against dermatophytes isolates.

| FICI   | TM  | TR  | TM/TR |
|--------|-----|-----|-------|
| CAATCC | 0.5 | 0.44| 0.46  |
| CA2    | 0.5 | 0.38| 0.43  |
| CA3    | 0.44| 0.15| 0.26  |
| CA4    | 0.38| 0.25| 0.31  |
| CA5    | 0.28| 0.28| 0.28  |
| CA6    | 0.54| 0.17| 0.31  |
| GM     | 0.43| 0.26| 0.33  |

| FICI   | TM  | TR  | TM/TR |
|--------|-----|-----|-------|
| CPATCC | 0.76| 0.76| 0.76  |
| CP2    | 0.6 | 0.24| 0.38  |
| CP3    | 0.87| 0.57| 0.71  |
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|   | CP4 | CP5 | CP6 |   |
|---|-----|-----|-----|---|
| GM | 0.72 | 0.37 | 0.52 |   |
| GGM CA/CP | 0.42 |   |   |   |

FICI: fractional inhibitory concentration index; GM: geometric means; GGM: general geometric means.

**Figure 3.** Representation of the survival (%) of the larvae of Galleria mellonella exposed to the extracts of Candida by bath. Survival was monitored in 24 h intervals over a period of 7 days.

**Figure 4.** Representation of the survival (%) of the larvae of Galleria mellonella exposed to the extracts of Candida by injection. Survival was monitored in 24 h intervals over a period of 7 days.
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Table 4. Values FICI of *Candida* extracts with fluconazole against dermatophytes isolates, showing synergic (≤0.5) interactions.

| TR1   | TR2   | TR3   | TR4   | TR5   |
|-------|-------|-------|-------|-------|
| FLC   | EXT   | FICI  | CE    | FLC   | EXT   | FICI  | CE    | FLC   | EXT   | FICI  | CE    | FLC   | EXT   | FICI  | CE    | FLC   | EXT   | FICI  | CE    |
| CAATCC| 4     | 0.07  | S     | 2     | 0.001 | 0.25  | S     | 2     | 0.0001 | 0.5   | S     | 4     | 0.001 | 0.5   | S     | 2     | 0.001 | 0.5   | S     |
| CA2   | 4     | 0.001 | S     | 1     | 0.03  | 0.12  | S     | 2     | 0.0001 | 0.5   | S     | 4     | 0.001 | 0.5   | S     | 2     | 0.001 | 0.5   | S     |
| CA3   | 2     | 0.0007 | 0.25 | S     | 0.1   | 0.125 | 0.01  | S     | 1     | 0.0007 | 0.25  | S     | 2     | 0.0007 | 0.25  | S     | 2     | 0.0007 | 0.25  | S     |
| CA4   | 4     | 0.0007 | 0.5  | S     | 0.2   | 0.025 | 0.03  | S     | 1     | 0.0007 | 0.25  | S     | 4     | 0.0007 | 0.5   | S     | 2     | 0.0007 | 0.5   | S     |
| CA5   | 4     | 0.0007 | 0.5  | S     | 0.2   | 0.07  | 0.03  | S     | 1     | 0.0003 | 0.5   | S     | 4     | 0.01   | 0.5   | S     | 2     | 0.0007 | 0.5   | S     |
| CA6   | 4     | 0.001 | 0.5   | S     | 0.1   | 0.03  | 0.01  | S     | 1     | 0.001  | 0.25  | S     | 2     | 0.001  | 0.25  | S     | 2     | 0.0007 | 0.5   | S     |
| CPATCC| 4     | 0.0007 | 0.5  | S     | 0.2   | 0.07  | 0.25  | S     | 2     | 0.001  | 0.5   | S     | 8     | 1000   | 2     | I     | 4     | 500   | 2     | I     |
| CP2   | 4     | 0.03  | 0.5   | S     | 0.5   | 0.07  | 0.06  | S     | 2     | 0.001  | 0.5   | S     | 1     | 0.03   | 0.1   | S     | 2     | 0.003  | 0.5   | S     |
| CP3   | 8     | 31.25 | 2     | I     | 2     | 0.07  | 0.25  | S     | 2     | 0.0007 | 0.5   | S     | 4     | 0.0007 | 0.5   | S     | 2     | 0.0007 | 0.5   | S     |
| CP4   | 4     | 0.001 | 0.5   | S     | 2     | 0.003 | 0.25  | S     | 2     | 0.5    | 0.5   | S     | 2     | 0.0007 | 0.25  | S     | 2     | 0.0007 | 0.5   | S     |
| CP5   | 2     | 0.03  | 0.25  | S     | 1     | 0.03  | 0.12  | S     | 2     | 0.001  | 0.5   | S     | 2     | 0.001  | 0.25  | S     | 2     | 0.0007 | 0.5   | S     |
| CP6   | 4     | 0.001 | 0.5   | S     | 0.2   | 0.25  | 0.03  | S     | 2     | 0.001  | 0.5   | S     | 2     | 0.001  | 0.25  | S     | 2     | 0.0001 | 0.5   | S     |

| TR1   | TR2   | TR3   | TR4   | TR5   |
|-------|-------|-------|-------|-------|
| FLC   | EXT   | FICI  | CE    | FLC   | EXT   | FICI  | CE    | FLC   | EXT   | FICI  | CE    | FLC   | EXT   | FICI  | CE    |
| CAATCC| 32    | 2000  | 2     | I     | 32    | 0.001 | 0.5   | S     | 32    | 0.001 | 0.5   | S     | 32    | 0.001 | 0.5   | S     | 32    | 0.001 | 0.5   | S     | 32    | 0.001 | 0.5   | S     |
| CP2   | 16    | 0.001 | 0.5   | S     | 32    | 0.0007 | 0.5  | S     | 0.0007 | 0.5  | S     | 32    | 0.007 | 0.5   | S     | 4     | 0.0007 | 0.5   | S     | 4     | 0.0007 | 0.5   | S     |
| CA6   | 16    | 0.001 | 0.5   | S     | 32    | 0.001 | 0.5   | S     | 16    | 0.0007 | 0.25  | S     | 32    | 0.0007 | 0.5   | S     | 4     | 0.0007 | 0.5   | S     | 4     | 0.0007 | 0.5   | S     |
| CA5   | 16    | 0.0007 | 0.5  | S     | 32    | 0.0001 | 0.5  | S     | 0.0001 | 0.5  | S     | 32    | 0.0001 | 0.5   | S     | 4     | 0.0001 | 0.5   | S     | 4     | 0.0001 | 0.5   | S     |
| CP2   | 16    | 0.0007 | 0.5  | S     | 32    | 0.0007 | 0.5  | S     | 16    | 0.03   | 0.25  | S     | 32    | 0.0007 | 0.5   | S     | 4     | 0.0007 | 0.5   | S     | 4     | 0.0007 | 0.5   | S     |
| CP6   | 16    | 0.001 | 0.5   | S     | 64    | 0.001 | 1     | AD    | 64    | 2000  | 2     | I     | 32    | 0.001 | 0.5   | S     | 4     | 0.001  | 0.5   | S     | 4     | 0.001  | 0.5   | S     |

FLC: fluconazole; EXT: extract; FICI: fractional inhibitory concentration index; CE: combinatorial effect (S: synergism; AD: additive; I: indifferent)
Discussion and Conclusion

This study presents the first analysis of biological interference among species of yeast and dermatophytes from nail origin. During our medical and laboratorial practice, it has been observed cases of two morphological types of fungus in the same sample by direct microscopy, but with positive culture just for yeast. It was hypothesized that the yeast, in nutritional competition in the same ecological niche, could produce metabolites with inhibitory properties against dermatophytes, compromising the culture. This event implicates in diagnostic errors, making cases of onychomycoses difficult to control. Considering this hypothesis, this study assessed the potential of metabolites produced by yeasts as antifungal alternatives against onychomycoses and other fungal infections.

Considering the species included in this study, Candida yeast, although part of the natural human microbiota, have been considered, in the last years, as important agents of onychomycosis [18-20], and Candida albicans and Candida parapsilosis as the predominant species [21]. Among dermatophytes, T. rubrum and T. mentagrophytes remain prevalent in onychomycosis [22].

Considering cases of mixed infection, the chemical environment in the nail can interfere on microbial survival and evolution of the infection [6, 23-25]. The present research showed that yeast culture extracts have antimicrobial properties against dermatophytes, with MIC values ranging from 31.25 to 2000 μg/mL. Several studies have shown antimicrobial products by different species of fungi [26-28]. However, the parameters tested here are different, with extracts from culture of yeasts in direct contact with dermatophytes.

The MIC values of the Candida extracts against the T. mentagrophytes and T. rubrum were similar. We expected a higher activity of the extracts against T. rubrum, since studies indicate that T. mentagrophytes has a higher proteolytic activity in comparison to T. rubrum, which is an important virulence factor that gives it an advantage for its rapid mycelial growth [29-31].

The biological individual characteristics of each strain certainly justify the differences in MIC values. Corroborating with this data, Albuquerque et al. emphasized that microorganisms could adapt and respond to the environment with physiological changes as a mechanism of cellular defense or tolerance. A variety of metabolites are generated by multiple stimuli and complex signaling pathways [32, 33].

The concomitant presence of dermatophytes and yeasts on the nail plate may arise due to the action of
hydrolytic enzymes, produced by some species, facilitating the secondary invasion of others, through a system which may control the expression of genes and cellular processes involved with sporulation, growth and even biofilm formation [34-36].

Several studies have shown antimicrobial molecules isolated from different species of fungi, such as cephalosporins and fusidic acid. The echinocandin family, for example, are an important source of antifungal agents that inhibit the biosynthesis of β-(1,3)-glucan, one of the main components of the fungal cell wall, and was first isolated from Aspergillus nidulans [27, 37].

In this study, the extracts produced in these specific conditions showed antifungal activity against dermatophytes, but there was no identification of the components of the extract. Therefore, it was not possible to know the active principle. Further studies are needed to isolate the compounds and identify the chemical classes in which they are inserted, in association with biological assays of biocidal activity. However, these preliminary results are relevant and may lead to the discovery of new drugs.

The high recurrence rates of onychomycosis and the development of new antifungal drugs are challenges to be faced. Fluconazole is an option for systemic treatment of onychomycosis with broad-spectrum, inhibiting lanosterol 14a-demethylase, with in vitro activity against dermatophytes and Candida species. However, the prolonged use has toxicity effects including headache, nausea, rash, abdominal pain, and elevation of transaminases, rarely associated with liver injury or failure [38]. Fluconazole was tested in this study because is an alternative to terbinafine and itraconazole, with advantages in the therapy (lower risk of treatment discontinuation and less adverse events) [39-41]. Thus, the combination of new antimicrobial agents and known drugs has become an alternative to increase the antifungal spectrum and efficacy of these drugs, with reduction of toxicity [42-44]. The combination of Candida extracts used in this study reduced fluconazole MIC values, indicating a synergistic effect of these compounds, which may allow new approaches in the control of fungal infections.

Due to the similarity to the innate immune system of insects and mammals, G. mellonella larvae have been used to investigate the toxicity of new potential antimicrobial compounds [45]. Using this model of study, the activity of new antifungal compounds can be quickly established, informing the need for synthesis of new derivatives or establishing the relative dosage in mammals [46]. In the present study, the nontoxicity of Candida extracts on G. mellonella model enable further investigation of its use for the control of
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Infectious diseases, such as dermatophytoses.

In conclusion, metabolites released by C. parapsilosis and C. albicans present antifungal activity against T. mentagrophytes and T. rubrum, which may influence the laboratory diagnosis of mixed infections. These results can explain cases of mixed fungal infections where only one of the etiologic agents grows in culture. Furthermore, the isolation and identification of the extracts compounds may allow new therapeutic approaches in the control of fungal infections.

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Conflict of interest: The author declares no conflicts of interest.

Acknowledgements: Authors would like to the financial support of the National Council for the Improvement of Higher Education (CAPES) and the National Council for Scientific and Technological Development (CNPq), for the scholarships granted. We acknowledge the Multi-user Laboratory (LMU) at São Jose do Rio Preto (Famerp).

Funding: National Council for the Improvement of Higher Education (CAPES) – 01

How to cite this article: Lemes TH, Torrezan GS, Polaquin CR, Regasini LO, Almeida BG, Brizzotti-Mazuchi NS, Ribeiro MD, Maschio-Lima T, Caetano MH, Pattini VC, Paziani MH, Marcia von Zeska Kress R, Siqueira JPZ, Almeida MTG. In vitro antifungal activity of Candida culture extracts against Trichophyton rubrum and Trichophyton mentagrophytes. Brazilian Journal of Case Reports. 2021 Oct-Dec;01(4):135-152.