

Pimenta pseudocaryophyllus inhibits virulence factors and promotes metabolic changes in *Candida* yeast

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ABSTRACT

Introduction: This is the first study to examine the *in vitro* susceptibility and the expression of virulence factors in *Candida* species in the presence of *Pimenta pseudocaryophyllus* (Gomes) L.R. Landrum (Myrtaceae), a Brazilian plant known as *pau-cravo*. Additionally, the mechanisms of action of the crude ethanol extract and the ethyl acetate and aqueous fractions of this plant were investigated. **Methods:** The *in vitro* susceptibility of *Candida* was tested using the broth microdilution method, whereas an XTT reduction assay was used for biofilms. Adherence was determined by counting the number of yeast cells that adhered to 100 oral epithelial cells, and hyphal formation was verified in the hyphal induction medium M199. Flow cytometry with propidium iodide and FUN-1 was performed to assess the mechanism of action. **Results:** The results revealed that the crude ethanol extract and the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus* inhibited the growth of *Candida* isolates at a minimal inhibitory concentration (MIC) ranging from 64 to 256 µg/mL, whereas the 50% sessile minimal inhibitory concentration (SMIC₅₀) ranged from 512 to >1,024 µg/mL. Adherence and hyphal formation were significantly reduced in the presence of the crude ethanol extract and both fractions. Although cell membrane injury was detected, the predominant mechanism of action appeared to be the alteration of yeast metabolism, as demonstrated by flow cytometry. **Conclusions:** Our results indicated that antifungal activity reduced the expression of virulence factors in yeast via the alteration of yeast metabolism, suggesting that the crude extract of *P. pseudocaryophyllus* and its fractions may contain novel antifungal agents.

Keywords: *Pimenta pseudocaryophyllus*. *Candida* spp. Antifungal activity. Virulence factors. Metabolic changes.

INTRODUCTION

Fungal infections have increased significantly in recent years due to an increase in the number of immunocompromised individuals. *Candida* species are responsible for the majority of yeast infections in humans¹. Resistance in yeast and many side effects associated with antifungal agents have been reported^{2,3}, and thus, alternative therapeutics lacking these limitations are under investigation. In particular, medicinal plants are being explored for novel treatments. *Pimenta pseudocaryophyllus* (Gomes) L.R. Landrum (Myrtaceae), popularly known as *pau-cravo*, *craveiro-do-mato*, *louro-cravo* or *chá-de-bugre*, is found in the Brazilian Atlantic Forest and Cerrado regions, especially in *cerradão*; it is a tree with a height of approximately four meters or a small bush in some regions, and it displays antifungal

activity *in vitro*. According to Paula et al.^{4,5}, the crude extract of this plant exerts antiedematogenic and antinociceptive effects on mice.

Biofilm formation, adherence to epithelial cells and filamentation are virulence mechanisms associated with yeast species of the *Candida* genus⁶. The ability to inhibit or reduce the expression of virulence factors is a desirable characteristic of an antifungal agent⁷.

In the present study, we examined the *in vitro* susceptibility, biofilms, adherence to epithelial cells and hyphal formation of *Candida* species in the presence of the crude extract and the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus*. Furthermore, we investigated the basic mechanisms of action of these extracts against these yeast species.

METHODS

Plant material and preparation of the *Pimenta pseudocaryophyllus* extract and fractions

The leaves of *P. pseudocaryophyllus* were collected in São Gonçalo do Abaeté in the State of Minas Gerais, Brazil at 18°20'58.4" south, 45°55'23.4" west at an altitude of 864m. The plant was identified by Carolyn Elinore Barnes Proença,

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and the voucher specimen was deposited in the herbarium of the *Universidade Federal de Goiás* (UFG) under number 27159. The crude ethanol extract and fractions obtained from the leaves of *P. pseudocaryophyllus* (citral chemotype) were prepared according to Paula et al⁵. Four fractions were obtained: hexane, dichloromethane, ethyl acetate, and aqueous fractions. Previous experiments had demonstrated that the crude ethanol extract and the ethyl acetate and aqueous fractions provide the best results based on *in vitro* susceptibility tests against *Candida* yeast species. Therefore, these extracts were selected for the present study. Fluconazole (Pfizer Pharmaceutical Group, New York, NY) was used in all assays.

Chemical components of the extract

High-performance liquid chromatography (HPLC) was performed to characterize and quantify the primary constituents of the crude extract. Quercitrin (quercetin 3-O- α -L-rhamnopyranoside; Sigma), catechin (Sigma), and gallic acid (Sigma) were used as reference standards.

Organisms

Bioassays were performed on 12 *Candida* isolates obtained from blood and nail samples and stored at -70°C in yeast extract potato dextrose (YEPD) agar (Difco®) containing 10% glycerol in the Laboratory of Mycology at the Institute of Tropical Diseases and Public Health of the Federal University of Goiás. These isolates were obtained from a previous study that was approved by the Bioethics Committee of the Hospital das Clínicas in Goiânia, Goiás. *Candida parapsilosis* ATCC 22019 was used as the standard strain.

Antimicrobial activity

Antifungal susceptibility tests using the crude extract and the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus* leaves were performed using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) document M27-A3⁸. The crude extract and the fractions were dissolved in 0.1% dimethyl sulfoxide (DMSO) (0.1mL DMSO in of 9.9mL Roswell Park Memorial Institute [RPMI] medium), which exerted a negligible effect on the growth kinetics of the yeast species⁹. Then, the extract and the fractions were diluted in RPMI medium buffered to pH 7.0 using morpholinepropanesulfonic acid (MOPS). The final concentrations of these samples ranged from 2.0 to 1,024 $\mu\text{g}/\text{mL}$.

The inoculum of *Candida* isolates was suspended in sterile saline (0.85%) that was adjusted to a cell density of 0.5 McFarland standards using a spectrophotometer at a wavelength of 530nm. This suspension was diluted 1:50, followed by a 1:20 dilution in RPMI-1640 medium to obtain a final concentration from 1×10^3 to 5×10^3 cells/mL. Aliquots of 100 μL of the fungal suspension and 100 μL of plant extract were inoculated in the wells of a microtiter plate. To determine the minimal inhibitory concentration (MIC), the plates were incubated at 35°C and examined after 72h. Each assay was performed in duplicate. The MICs for the *P. pseudocaryophyllus* crude extract and fractions were defined as the lowest concentrations at which there was an absence of fungal growth. The MIC of fluconazole was defined

as the lowest concentration that achieved 80% growth inhibition compared to the growth of the drug control.

The minimal fungicidal concentrations (MFCs) of the *P. pseudocaryophyllus* crude extract and its fractions were determined as described by Torres-Rodriguez et al¹⁰. Briefly, 10 μL of the samples were collected from optically clear wells and seeded on Sabouraud dextrose agar. The plates were incubated at 35°C for 72h, and the MFC was defined as the lowest concentration at which no fungal colony growth was detected.

In vitro susceptibility of *Candida* biofilms

The biofilm was formed in pre-sterilized polystyrene 96-well microtiter plates as described by Pierce et al.¹¹. Briefly, 100 μL of the standardized suspension (RPMI-1640 broth) containing 10^6 cells/mL (counted using a hemocytometer) of *C. albicans* (n=6) or *C. parapsilosis* (n=6) isolates were placed in the selected wells of the microtiter plates and incubated for 48h at 37°C . The crude extract and the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus*, which varied from 1,024 to 1 $\mu\text{g}/\text{mL}$ (according to two-fold serial dilutions), were added to the formed biofilms and incubated for 48h at 35°C . The 50% and 80% sessile minimal inhibitory concentrations (SMIC50 and SMIC80, respectively) relative to the control were determined using a 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay and were measured spectrophotometrically using a microtiter plate reader at 550nm.

Yeast adherence

The adherence of *Candida* isolates was conducted according to Kimura and Pearsall¹², with some modifications: 0.5mL of the yeast suspension (10 isolates) containing 2.5×10^7 yeast/mL with or without the crude extract or the ethyl acetate or aqueous plant fraction at a concentration of 1/4, 1/2 or 1 x MIC (as previously determined) was mixed with 0.5mL of a pooled oral epithelial cell suspension collected from healthy adult volunteers. Adherence was determined by counting the number of yeast cells that adhered to 100 epithelial cells via microscopy¹³.

Hyphal induction

The hyphal induction assay was performed according to a previously published method by Ha and White¹⁴. *Candida albicans* isolates (n=5) grown in YEPD medium for 48h were subcultured in 5mL of yeast nitrogen without amino acids supplemented with ammonium sulfate and dextrose (YAD) growth medium with or without the crude ethanol extract or the ethyl acetate or aqueous fraction at a concentration of 1/4, 1/2 or 1 x MIC. The cells in the YAD culture medium were transferred to 5mL of the hyphal induction medium M199 (containing L-glutamine and Earle's salts, without bicarbonate; Gibco) with or without the crude ethanol extract or the ethyl acetate or the aqueous fraction at the same concentrations described above. This medium was incubated at 37°C for 3h with shaking (240rpm), and 250 cells (yeast + hyphae) were counted using a hemocytometer.

Mechanism of action

The mechanisms of action of the crude ethanol extract and the ethyl acetate and aqueous fractions against *C. parapsilosis* (ATCC 22019) and two clinical isolates of *C. albicans* were analyzed via flow cytometry (Accuri C6 cytometer, Becton Dickinson) according to the approach developed by Vale-Silva et al.¹⁵ and Pina-Vaz and Rodrigues¹⁶. Two markers were used: propidium iodide (PI), which is a marker of membrane integrity due to its ability to permeate only damaged membranes, and 2-chloro-4-(2,3-dihydro-3-methyl-[benzo-1,3-thiazol-2-yl]methylidene)-1-phenylquinolinium iodide (FUN-1), which is an indicator of yeast metabolic activity. The inoculum of 400µL containing 2x10⁶ cells/mL was added to 100µL of the crude ethanol extract or either fraction at a concentration of 1/4, 1/2, 1, 2 or 4 x MIC. Amphotericin B (2mg/mL) was used as a control. Controls included the following: an autofluorescence control, consisting of untreated yeast, and a labeled cell control, consisting of yeast that were treated with markers but not exposed to the crude ethanol extract or either fraction.

Data analysis

All the assays were performed in triplicate. Microsoft® Excel 2007 was used to analyze the data, and statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS®) for Windows® version 16.0. The influence of the crude ethanol extract and the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus* on *Candida* adhesion and hyphal formation were evaluated using the Mann-Whitney U-test. P values <0.05 were considered to be significant.

RESULTS

Comparison of the retention times (RT), which was based on the HPLC profiles, between the reference standards and the samples revealed the presence of catechin and quercetin in the crude ethanol extract of *P. pseudocaryophyllus*. These substances were identified in the extracts and fractions obtained from the leaves of the citral chemotype of *P. pseudocaryophyllus*.

The *in vitro* antifungal susceptibility of the crude extract of *P. pseudocaryophyllus* leaves, as well as the ethyl acetate and aqueous fractions, inhibited the growth of *Candida* at a

MIC range of 64 to 256µg/mL (**Table 1**) and at a MFC range of 256 to >1,024µg/mL. The aqueous fraction inhibited the growth of 41% of the *Candida* isolates at a MIC of 64µg/mL and a MFC of 256µg/mL.

Of the 12 *Candida* isolates, biofilm formation was only detected in 10 isolates (6 *C. albicans* and 4 *C. parapsilosis*). *In vitro* susceptibility assays demonstrated high SMIC values for the crude ethanol extract and the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus*, as well as fluconazole, against the yeast in the biofilms. The SMIC₅₀ values ranged from 512 to >1,024µg/mL and the SMIC₈₀ values were greater than 1.02µg/mL for all of the *Candida* isolates evaluated.

The crude extract and the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus*, at their respective MIC, significantly reduced the number of *Candida* yeast that adhered to oral epithelial cells compared to the control. The aqueous fraction was the most active and displayed a level of performance similar to that of fluconazole in terms of the MIC. The number of yeast cells that adhered at 1/2 x MIC of the aqueous fraction was similar to that in the presence of 1 MIC of the crude extract. The means and standard deviations (SDs) of adherence are shown in **Table 2**.

The crude extract and fractions of *P. pseudocaryophyllus*, at their respective MIC, significantly reduced hyphal formation compared to the control. At a concentration of 1/2 x MIC, the crude extract and the aqueous fraction also caused a significant reduction in hyphal formation (**Table 2**).

Flow cytometric analysis demonstrated that the crude extract and the ethyl acetate and aqueous fractions affected the metabolism of *C. albicans* and *C. parapsilosis*, while the crude extract exhibited an additional capacity to injure the membranes of these cells. The metabolism of the yeast treated with these components was impaired in a dose-dependent manner compared to the control yeast, and this effect was intensified at higher concentrations. **Figure 1** shows the alterations in *Candida* metabolism in the presence of various concentrations of the aqueous fraction of *P. pseudocaryophyllus*. The crude extract and the ethyl acetate fraction induced lesions in the plasmalemma of *Candida* spp. at approximately 1 x MIC (**Figure 2**) and 2 x MIC, respectively. The aqueous fraction did not damage the yeast membranes at any of the examined concentrations.

TABLE 1 - Inhibitory activity of *Pimenta pseudocaryophyllus* against 12 yeast isolates of the genus *Candida*.

Plant	MIC (µg/mL)			
	>256	256	128	64
Crude extract	2	8	2	-
Hexane fraction	12	-	-	-
Dichloromethane fraction	12	-	-	-
Ethyl acetate fraction	-	11	1	-
Aqueous fraction	-	5	2	5
Essential oil	11	1	-	-

MIC: minimal inhibitory concentration.

TABLE 2 - The number of *Candida* isolates (n=10) that adhered to 100 epithelial cells and the number of hyphae in isolates of *Candida albicans* (n=5) in the presence of the crude extract or the ethyl acetate or aqueous fraction of *Pimenta pseudocaryophyllus* compared to the control.

	Adherence mean \pm SD	P	Hyphae mean \pm SD	P
Control	503.05 \pm 75.80		200.00 \pm 6.12	
Crude extract				
1 MIC	118.00 \pm 16.53	<0.001*	134.60 \pm 33.76	0.008*
1/2 x MIC	387.75 \pm 58.47	0.002*	174.60 \pm 13.18	0.016*
1/4 x MIC	445.05 \pm 69.49	0.165	186.40 \pm 9.74	0.056
Ethyl acetate fraction				
1 MIC	204.60 \pm 29.96	<0.001*	185.40 \pm 9.66	0.032*
1/2 x MIC	478.10 \pm 72.52	0.436	192.80 \pm 5.89	0.095
1/4 x MIC	498.10 \pm 76.68	0.739	196.00 \pm 4.18	0.222
Aqueous fraction				
1 MIC	34.00 \pm 4.68	<0.001*	124.80 \pm 19.89	0.008*
1/2 x MIC	122.15 \pm 15.56	<0.001*	171.80 \pm 16.18	0.016*
1/4 x MIC	498.10 \pm 76.68	0.315	195.00 \pm 9.35	0.421

*Statistically significant as evaluated by the Mann-Whitney U Test. **MIC**: minimal inhibitory concentration.

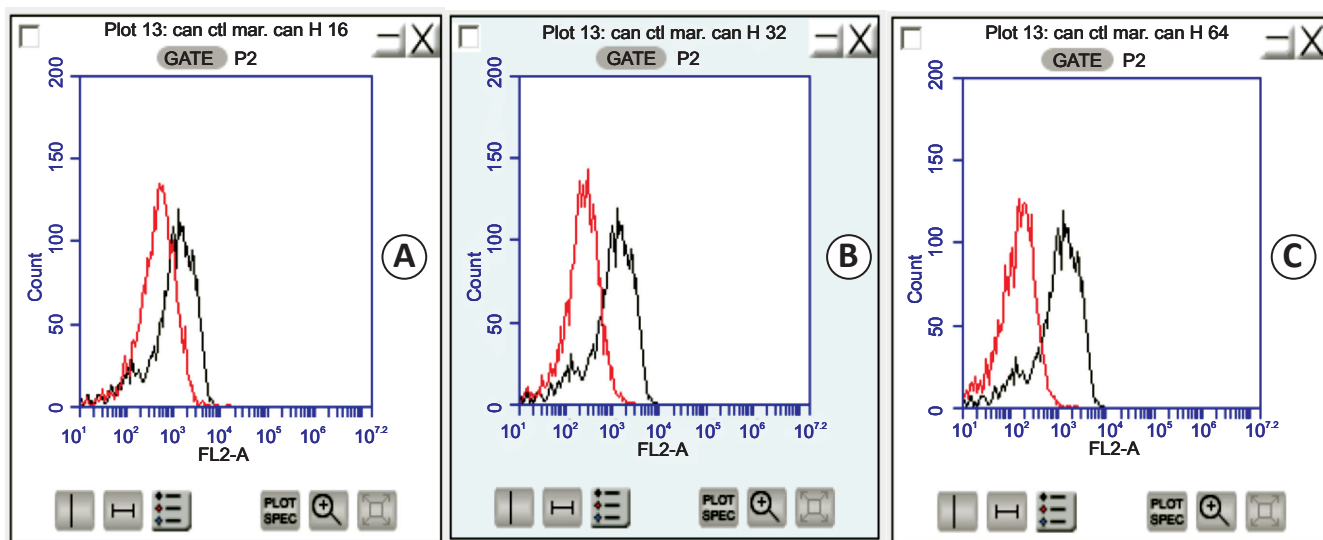


FIGURE 1 - Histograms showing the fluorescence in relative units (orange fluorescence: FL2 channel, FL2-A log). Metabolism in yeast (*Candida parapsilosis* ATTC 22019) treated with the aqueous fraction (AF) of *Pimenta pseudocaryophyllus* (in red) at 1/4 x MIC (A), 1/2 x MIC (B) and 1 x MIC (C) compared to the control (in black). MIC: minimal inhibitory concentration.

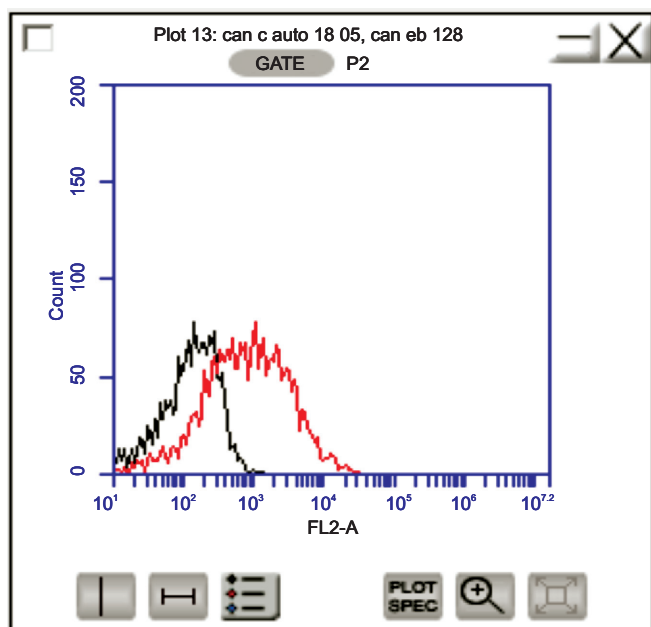


FIGURE 2 - Histogram showing the red fluorescence in relative units (red fluorescence: FL3 channel, FL3-A log) of *Candida parapsilosis* (ATCC 22019) stained with propidium iodide. Control consisting of stained untreated cells (in black). Stained cells treated with the crude extract of the leaves of *Pimenta pseudocaryophyllus* (in red).

DISCUSSION

Medicinal plants are emerging as sources of new antifungal drugs that display greater efficiency, wider availability and lower toxicity^{17,18}. The growth inhibition of yeast caused by *P. pseudocaryophyllus* and the antiedematogenic and antinociceptive effects observed in mice in previous investigations by our group^{4,5} have increased interest in this plant. According to Scorzoni et al¹⁹, plant compounds displaying antifungal activity at an MIC of 250 µg/mL are considered to be useful for therapeutic purposes. Paula et al.⁵ determined that the MIC of the crude extract and the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus* was <256 µg/mL for isolates of *Candida* spp. and was ≤128 µg/mL for isolates of *Cryptococcus neoformans* complex species. In the current study, the MIC of the aqueous fraction was 64 µg/mL for 42% of the examined isolates.

The antifungal activity of *P. pseudocaryophyllus* may be attributed to flavonoids, which are constituents of the crude ethanol extract of this plant and some of its fractions. Narayana et al.²⁰ have reported the biological activity of flavonoids, specifically quercetin, against yeast. Furthermore, the antifungal activity of catechin has been demonstrated against *C. albicans* and *T. mentagrophytes*. Masatomo and Kazuko²¹ demonstrated the inhibitory activity of catechins against *C. albicans*, and Toyoshima et al.²² examined the mechanism underlying the effects of the green tea of catechin on *T. mentagrophytes* and

suggested that catechin attacked the cell membrane and caused the lysis of conidia and hyphae.

Antifungal agents that act by reducing the expression of yeast virulence factors are expected to be more efficient. According to Zuzarte et al.²³, inhibition of filamentation appears to be sufficient to treat candidiasis. In the present study, the *P. pseudocaryophyllus* crude extract and aqueous fraction (which displayed performance similar to that of the fluconazole control) reduced hyphal formation and decreased adherence to buccal epithelial cells, significantly affecting these *Candida* virulence mechanisms. Similar results obtained by Zuzarte et al.²³ and Vale-Silva et al.²⁴ verified that hyphal formation in *Candida* was reduced in the presence of essential oils from *Thymus x viciosoi* and *Lavandula multifida* L. Our results indicate that further studies of *P. pseudocaryophyllus* should be conducted to assess its therapeutic potential for the treatment of *Candida* infections.

Elucidating the mechanism of action of each antifungal agent is an important step toward their appropriate use and may help to reduce toxicity. In the present study, the examined fractions of *P. pseudocaryophyllus* altered the metabolism of the target yeasts, as shown in **Figure 1**. The results obtained by Evensen and Braun²⁵ indicated that polyphenols from *Camellia sinensis* inhibited biofilm formation by *C. albicans* via the alteration of yeast protein metabolism. In addition to changes in metabolism, we observed injury to the fungal cell membrane in response to the *P. pseudocaryophyllus* crude extract and ethyl acetate fraction. It is difficult to explain why the aqueous fraction was not able to injure the yeast membranes. However, we suggest that this finding may be due to the absence of polar compounds from the aqueous fraction, causing it to act preferentially on fungal metabolism but preventing it from damaging the cell membrane. High concentrations (2 x MIC) of the ethyl acetate fraction were necessary to damage the cell membrane; however, at a concentration equivalent to 1 x MIC, the crude extract caused similar damage. These results indicate that the crude extract likely contains constituents that act synergistically. Although both mechanisms of action have been observed in the crude ethanol extract as well as the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus*, the metabolic effects on yeast appear to predominate. The altered metabolism and plasma membrane injury of yeast observed in this study indicated that the constituents of *P. pseudocaryophyllus* might inhibit yeast development and most likely induce the destruction of the microorganism.

These results demonstrated that the antifungal activity of the crude extract and the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus* of the citral chemotype inhibited *Candida* virulence mechanisms, including adherence and hyphal induction (**Table 2**). Flavonoids, such as quercetin and catechin, found in *P. pseudocaryophyllus* are considered to be antimicrobials and explain this antifungal activity. In conclusion, our results indicate that the crude extract and the ethyl acetate and aqueous fractions of *P. pseudocaryophyllus* display antifungal activity, induce metabolic changes, cause membrane injury and reduce virulence factor expression in yeast, suggesting that these components may lead to the development of novel antifungal agents.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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REFERENCES

- Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol Rev* 2012; 36:288-305.
- Pappas PG, Kauffman CA, Andes D, Benjamin Jr DK, Calandra TF, Edwards Jr JE, et al. Clinical Practice Guidelines for the management of Candidiasis (2009) update by the Infectious Diseases Society of America. *Clin Infect Dis* 2009; 48:503-535.
- Arnold TM, Dotson E, Sarosi GA, Hage CA. Traditional and emerging antifungal therapies. *Proc Am Thorac Soc* 2010; 7:222-228.
- Paula JAM, Paula JR, Bara MTF, Rezende MH, Ferreira HD. Pharmacognostic study about *Pimenta pseudocaryophyllus* (Gomes) L.R. Landrum leaves - Myrtaceae. *Rev Bras Farmacogn* 2008; 18: 265-278.
- Paula JAM, Silva MRR, Costa MP, Diniz DGA, Sá FAS, Alves SF, et al. Phytochemical analysis and antimicrobial, antinociceptive, and anti-inflammatory of two chemotypes of *Pimenta pseudocaryophyllus* (Myrtaceae). *Evid Based Complement Alternat Med* 2012; 2012:1-15.
- Calderone RA, Fronzi WA. Virulence factors of *Candida albicans*. *Trends Microbiol* 2001; 9:327-335.
- Espinel-Ingroff A. Novel antifungal agents, targets or therapeutic strategies for the treatment of invasive fungal diseases: a review of the literature (2005-2009). *Rev Iberoam Micol* 2009; 26:15-22.
- Clinical and Laboratory Standards Institute (CLSI). Reference method for broth dilution antifungal susceptibility testing of yeasts, 3rd ed. Approved standard. CLSI M27-A3 (28). Wayne, PA: CLSI; 2008.
- Rodríguez-Tudela JL, Cuenca-Estrella M, Diaz-Guerra TM, Mellado E. Standardization of Antifungal Susceptibility Variables for a Semiautomated Methodology. *J Clin Microbiol* 2001; 39:2513-2517.
- Torres-Rodríguez JM, Alvarado-Ramírez E, Murciano F, Sellart M. MICs and minimum fungicidal concentrations of posaconazole, voriconazole and fluconazole for *Cryptococcus neoformans* and *Cryptococcus gattii*. *J Antimicrob Chemother* 2008; 62:205-210.
- Pierce CG, Uppuluri P, Tristan AR, Wormley Jr FL, Mowat E, Ramag G, et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nature Protocols* 2008; 3:1494-1500.
- Kimura LH, Pearsall NH. Adherence of *Candida albicans* to human buccal epithelial cells. *Infect Immun* 1978; 21:64-68.
- Biasoli SM, Tosello ME, Magaró HM. Adherence of *Candida* strains isolated from the human gastrointestinal tract. *Mycoses* 2002; 45:465-469.
- Ha KC, White TC. Effects of azole antifungal drugs on the transition from yeast cells to hyphae in susceptible and resistant isolates of the pathogenic yeast *Candida albicans*. *Antimicrob Agents Chemother* 1999; 43:763-768.
- Vale-Silva LA, Buchta V, Vokurková D, Pour M. Investigation of the mechanism of action of 3-(4-bromophenyl)-5-acyloxymethyl-2,5-dihydrofuran-2-one against *Candida albicans* by flow cytometry. *Bioorg Med Chem Lett* 2006; 16:2492-2495.
- Pina-Vaz C, Rodrigues AG. Evaluation of Antifungal Susceptibility using flow cytometry. Cap. 21. In: Sharon A, editor. *Molecular and Cell Biology Methods for Fungi, methods in molecular biology*. 2010; 638:281-289.
- Singh G, Kumar P, Joshi SC. Treatment of dermatophytosis by a new antifungal agent 'apigenin'. *Mycoses* 2014; 57:497-506.
- Danielli LJ, Reis M, Bianchini M, Camargo GS, Bordignon SAL, Guerreiro IK, et al. Antidermatophytic activity of volatile oil and nanoemulsion of *Stenachaenium megapotamicum* (Spreng.) Baker. *Ind Crops and Prods* 2013; 50:23-28.
- Scorzoni L, Benaduci T, Almeida AMF, Silva DHS, Bolzani VS, Gianinni MJSM. The use of standard methodology for determination of antifungal activity of natural products against medical yeasts *Candida* sp and *Cryptococcus* sp. *Braz J Microbiol* 2007; 38:391-397.
- Narayana KR, Reddy MS, Chaluvadi MR, Krish DR. Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. *Indian J Pharmacol* 2001; 33:2-16.
- Masatomo H, Kazuko T. Multiple effects of green tea catechin on the antifungal activity of antimycotics against *Candida albicans*. *JAC* 2004; 53:225-229.
- Toyoshima Y, Okubo S, Hara Y, Shimamura T. Effect of catechin on the ultrastructure of *Trichophyton mentagrophytes*. *Kansenshogaku Zasshi* 1993; 68:295-303.
- Zuzarte M, Vale-Silva LA, Gonçalves MJ, Cavaleiro C, Vaz S, Canhoto J, et al. Antifungal activity of phenolic-rich *Lavandula multifida* L. essential oil. *Eur J Clin Microbiol Infect Dis* 2012; 31:1359-1366.
- Vale-Silva LA, Gonçalves MJ, Cavaleiro C, Salgueiro L, Pinto E. Antifungal Activity of the Essential Oil of *Thymus x viciosoi* against *Candida*, *Cryptococcus*, *Aspergillus* and Dermatophyte species. *Planta Med* 2010; 76:882-888.
- Evensen NA, Braun PC. The effects of tea polyphenols on *Candida albicans*: inhibition of biofilm formation and proteasome inactivation. *Can J Microbiol* 2009; 55:1033-1039.