Journal of Biology & Pharmacy and Agricultural Management
Revista de Biologia & Farmácia e Manejo Agrícola
ISSN: 1983-4209

UEPB

eduepb
Editora da Universidade Estadual da Paraíba

*Short Communication*

# Antimicrobial activity of *Xylosma prockia (Turcz.) Turcz.* leaves against *Candida parapsilosis in vitro*.

**Guilherme Fernando da Silva[1], Queila da Silva Rosa[2], Mariany Lopes da Costa Folly[2], Gustavo José Cota Freitas[3], Daniel de Assis Santos[3], Karen Luise Lang[1,2], Gabriella Freitas Ferreira[1,2]***

[1] Departamento de Farmácia, Universidade Federal de Juiz de Fora, Campus Governador Valadares; Governador Valadares, Brazil.
[2] Programa Multicêntrico de Bioquímica e Biologia Molecular, Universidade Federal de Juiz de Fora, Campus Governador Valadares, Governador Valadares, Brazil.
[3] Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.
*Correspondence: gabriella.freitasferreira@gmail.com

**Abstract:** *Candida parapsilosis* is a prevalent pathogen that contributes to candidemia in Brazil. The therapeutic options available for treating candidiasis are limited due to the high toxicity and high fungal resistance to clinically available drugs. In light of these challenges, there is a growing interest in exploring natural products as a potential source for discovering and developing novel antimicrobial agents. In this study, we aimed to investigate the antifungal activity of the leaves of *Xylosma prockia (Turcz.) Turcz.* against *C. parapsilosis* ATCC 22019 *in vitro*. The minimum inhibitory concentration (MIC) of the ethanolic extract and its fractions was determined using the broth microdilution method. The fraction with the lowest MIC was selected for subsequent experiments, which included a time-kill curve assay, quantification of ergosterol content by spectrophotometry, analysis of lipid peroxidation using the reactive thiobarbituric acid method and the use of fluorescent probes to determine oxidative and nitrosative species. The ethanolic extract, butanol, dichloromethane, and ethyl acetate fractions inhibited fungal growth at concentrations below 100 mg/L. The ethyl acetate fraction inhibited the growth cell at the lowest concentration (16 mg/L). The time-kill curve test showed that this fraction has a fungicidal effect. The mechanism of action does not appear to be related to ergosterol synthesis, as the fraction did not affect this sterol content. Interestingly, the fraction induced an oxidative and nitrosative burst, leading to lipid peroxidation. The results of this study indicate that the ethyl acetate fraction of *X. prockia* leaves has antifungal potential due to oxidative damage.

**Keywords:** candidiasis, medicinal plants, antifungal activity, *Xylosma prockia*

**Editor:** Carlos Henrique Salvino and João Augusto Oshiro Junior

Received: November 27, 2024
Revised: December 28, 2024
Accepted: January 01, 2025
Published: January 02, 2025



## 1. Introduction

Candidiasis is a fungal infection caused by *Candida* spp. These microorganisms usually colonize the human skin and mucous membranes and affect men and women, especially those of childbearing age [1]. In a multicenter study conducted in 16 Brazilian hospitals, microorganisms of the genus *Candida* were found to be the seventh most common causative agent of nosocomial bloodstream infections. It is important to emphasize that 65.7% of the yeast isolates were non-albicans species. In this context, *Candida parapsilosis* stood out, responsible for 24.1% of cases, second only to *Candida albicans* (34.3% of cases) [2].

The pharmacotherapeutic treatment of candidiasis faces two major challenges: the emergence of antifungal resistance and the adverse effects of some drugs. The indis-

criminate use of azole derivatives in mild cases of candidiasis is associated with the emergence of resistant strains. The underlying factors contributing to this phenomenon include incorrect timing of treatment, administration of an inadequate dose and the prevailing clinical condition of the patient [3]. Fewer cases of fungal resistance occur with polyene drugs. However, the clinical use of amphotericin B (AMB) is closely associated with toxic effects such as nephrotoxicity [4]. It is important to mention that AMB is the drug of first choice in the treatment of various systemic fungal infections [4].

The challenges of pharmacotherapy and the high rate of hospital deaths due to candidemia highlight the need for new drugs to manage candidiasis [3,5]. Natural products have emerged as a promising source for discovering and developing of new antimicrobial drugs, as plants are known to produce substances that serve as a defense against external aggressors [6]. *Xylosma prockia (Turcz.) Turcz.* is a species native to Brazil, occurring geographically mainly in the Caatinga, Cerrado, and Atlantic Forest biomes [7]. Our group described the anticryptococcal activity of the extract and fractions from the leaves of *X. prockia.* The investigation focused on the ethyl acetate fraction (EAF), whose main constituents were identified by liquid chromatography–mass spectrometry [8]. Due to the strong anticryptococcal activity of the EAF from the leaves of *X. prockia*, this study aimed to evaluate the activity of this extract and fractions against *C. parapsilosis* ATCC 22019.

## 2. Results

### *2.1. Minimum Inhibitory Concentration (MIC) Test and Time-kill curve*

The MICs of the ethanolic extract (EE) and its fractions are displayed in Figure 1A. Among the fractions evaluated, the butanolic fraction (BF), the ethyl acetate fraction (EAF), and the dichloromethane fraction (DF) were identified as potentially promising, as they exhibited MICs of less than 100 mg/L. Substances with low MIC values demonstrate higher *in vitro* activity against microorganisms than those with high MIC values. Consequently, EAF was selected for further experiments as it showed the ability to inhibit yeast growth at the concentration of 16 mg/L.

A

| Strain | MIC (mg/L) | | | |
|---|---|---|---|---|
| *C. parapsilosis* ATCC 22019 | **EE** | **BF** | **AF** | **DF** |
| | 64 | 32 | > 128 | 64 |
| | **EAF** | **HF** | **AMB** | **FCZ** |
| | 16 | > 128 | 2 | 4 |

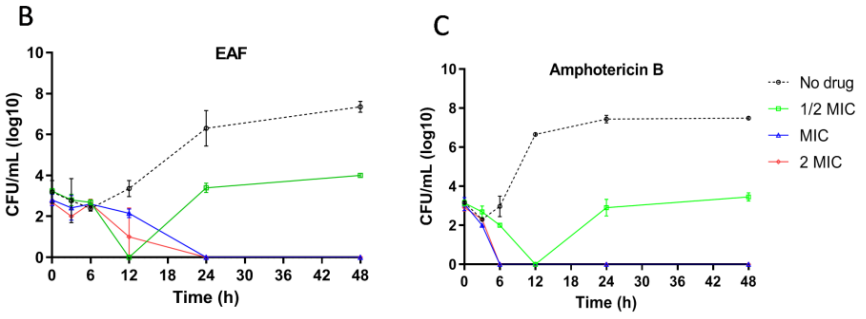


Figure 1: Antifungal activity of *X. prockia* leaves. (A) Minimal inhibitory concentration of extract and fractions. (B) Time-kill curve of *C. parapsilosis* cells treated with EAF (C) and amphotericin B at different concentrations. Results are expressed as mean ± standard deviation (SD). MIC = minimum

inhibitory concentration; EE = ethanolic extract; BF = butanolic fraction, AF = aqueous fraction; DF = dichloromethane fraction; EAF = ethyl acetate fraction; HF = hexane fraction; AMB = amphotericin B; FCZ = fluconazole, CFU: colony forming unit, h: hours.

The time-kill curves of *C. parapsilosis* against EAF and AMB are shown in Figure 1B and Figure 1 C. After 24-hour exposure to EAF (MIC and 2 MIC), no growth was observed. Interestingly, our results show that higher concentrations of EAF achieve inhibition of fungal growth in a shorter time frame. *C. parapsilosis* growth was inhibited after 6 hours of AMB (MIC and 2MIC) treatment. Regrowth was observed after 12 hours of exposure to EAF and AMB at subinhibitory concentrations. These results confirm that EAF is a promising source of compounds with antifungal activity.

### *2.2. EAF did not impair ergosterol levels but caused an oxidative burst*

In the present study, ergosterol quantification data showed that EAF did not reduce ergosterol concentrations (untreated: 0.018mg/L ± 0.001; EAF: 0.017mg/L ± 0.005) (Figure 2A). In contrast, fluconazole (FCZ) resulted in a significant decrease in ergosterol concentrations (untreated: 0.018mg/L ± 0.001; FCZ: 0.008mg/L ± 0.005), which was expected based on the drug's mechanism of action. These results indicate that the antimicrobial mechanism of action of EAF against *C. parapsilosis* is not related to the disruption of ergosterol synthesis.

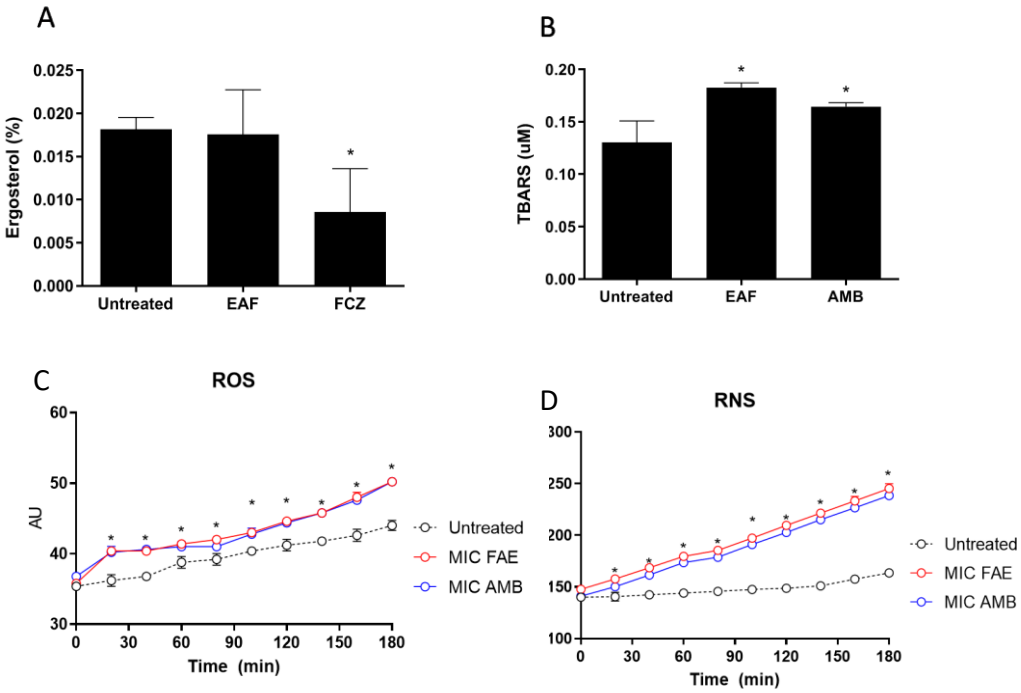


Figure 2: The antifungal effect of EAF on *C. parapsilosis* cells. (A) Quantification of ergosterol (B) and lipid peroxidation in cells of *C. parapsilosis* treated with EAF. Each bar represents the mean + standard deviation. The quantification of ergosterol levels are expressed in %. The TBARS levels are expressed in μM. *: indicates statistical significance ($p<0.05$). Measurement of reactive species produced by *C. parapsolisis* after treatment with (C) EAF and (D) amphotericin B. Results expressed in Arbitrary Units (AU) as a function of time. The symbols represents mean + standard deviation. *: indicate statistical significance between EAF treatment and untreated cells and between AMB treatment and untreated cells ($p<0.05$). AU: arbitrary unit. EAF: ethyl acetate fraction. FCZ: fluconazole. AMB: amphotericin B. MIC: minimum inhibitory concentration. ROS: reactive oxygen species. RNS: reactive nitrogen species. Min: minutes

To understand the effect of EAF on fungal cells, a lipid peroxidation assay was performed. Treatment of fungal cells with EAF and with AMB showed significant lipid peroxidation compared to untreated cells (untreated: 0.130 μM ± 0.020; EAF: 0.182μM ± 0.004; AMB: 0.164μM ± 0.004) (Figure 2B).

Interestingly, a significant increase in reactive oxygen species (ROS) was observed in the cells exposed to EAF after 20 minutes of treatment. The same happened in the cells treated with AMB since the beginning of the treatment (0 min – untreated: 140.00 ± 3.93 AU, EAF: 147.80 ± 2.59 AU; AMB: 141.00 ± 2.91; 20 min – untreated: 140.60 ± 4.16 AU, EAF: 157.8 ± 2.28 AU; AMB: 150.20 ± 2.17) (Figure 2C). On the other hand, compared to untreated cells, EAF led to a significant increase in reactive nitrogen species (RNS) from the start of treatment and AMB after 20 minutes of treatment (Figure2D) (0 min – untreated: 140.00 ± 3.93 AU, EAF: 147.80 ± 2.59 AU; AMB: 141.00 ± 2.91; 20 min – untreated: 140.60 ± 4.16 AU, EAF: 157.8 ± 2.28 AU; AMB: 150.20 ± 2.17). The data suggests that the fungicidal effect of EAF is probably related to lipid peroxidation induced by reactive oxygen and nitrogen species.

## 3. Discussion

Studies evaluating the antifungal activity of *X. prockia* are scarce. To our knowledge, no studies investigating the activity of *X. prockia* against *Candida* species were found in the literature, which makes this study a novelty. Plant extracts with MICs below 100 mg/L have been identified as promising candidates for discovering new active molecules [9]. EAF reached this value because 16 mg/L was necessary to inhibit the growth of the tested strain.

It is important to note that the leaves used in this study were from the same batch as the leaves used in the study that evaluated their anticryptococcal activity [8]. Coincidentally, the EAF also showed the optimal biological activity against *C. parapsilosis*. This fraction was previously characterized. The composition of the fraction can be divided into three different substance groups: caffeoylquinic acids, caffeoylglycosides, and coumaroylglycosides [8].

The time-kill curve test is often used in the literature to evaluate new antimicrobial agents. It provides information about how microorganism tolerance changes over time [10]. A time-kill plot against the tested strain showed that EAF and AMB exhibited fungicidal activity (defined as a kill of ≥log 3) at inhibitory concentrations [11]. For EAF, the time to reach growth reduction tended to shorten with increasing concentration. The concentration-response relationships observed for EAF are comparable to those observed for AMB in this study.

The ergosterol quantification test was performed to determine whether treatment with EAF reduces this sterol content in the fungal cell. It has previously been shown in the literature that azole drugs such as FCZ promote a reduction in the ergosterol content of various yeasts [12]. In the present study, the data showed that EAF did not reduce ergosterol content, while FCZ did. However, in a previously published study, researchers observed that EAF reduced ergosterol content in cryptococcal cells [8]. These results may be explained by morphophysiological differences between these two species (e.g. differences in cell wall composition) [13].

The cells exposed to EAF and AMB showed a significant increase in TBARS levels compared to the untreated cells. The experiment aimed to test whether EAF triggers oxidative peroxidation by using TBARS as a biomarker. One of the secondary mechanisms of action of AMB is lipid oxidation in the fungal cell membrane [14, 15]. The data from this experiment demonstrate the potential of EAF to cause lipid peroxidation in fungal cells, which is a likely mechanism of action of the fraction against *C. parapsilosis*. It is worth noting that Folly et al. [8] also observed an increase in lipid peroxidation induced by EAF in cryptococcal cells.

To analyze the possibility of induction of oxidative and nitrosative stress in the cells of *C. parapsilosis* by EAF, we measured the production of ROS and RNS by fluorescence emission. Under physiological conditions, the superoxide anion is converted into hydrogen peroxide by the enzyme superoxide dismutase. The latter is then converted into water by the action of peroxidases. These enzymes contribute to the antioxidant system of the cell, which neutralizes the reactive species [16]. Oxidative stress is the result of an imbalance in the antioxidant system with the production of reactive species. A previous study has shown that AMB and itraconazole inhibited the enzymes of the antioxidant system that induce the oxidative and nitrosative burst in *C. gattii* yeasts [14]. These results are consistent with other reports highlighting the ability of AMB to induce oxidative stress in cells, as evidenced by the expression of stress-related genes [15].

In our study, we observed that EAF increases the concentrations of ROS and RNS. The data suggest that the fungicidal effect of EAF is probably related to lipid peroxidation induced by reactive oxygen and nitrogen species. Peralta et al. [17] investigated the effect of a natural prenylflavonoid extracted from *Dalea elegans* on the formation of *C. albicans* biofilms. Their data showed that the prenylflavonoid induced an oxidative burst in the tested fungal species, leading to a decrease in biofilms and characterizing a possible mechanism of action.

This study provides an unprecedented description of the antifungal activity of EAF from *X. prockia* leaves. The fraction shows a strong inhibition of yeast growth, a promising feature that it could be a source of antifungal compounds. The analyzes performed in this study showed that oxidative stress appears to play an important role in causing cell death. However, further studies are needed to better understand the fungal activity of the fraction against *C. parapsilosis*.

## 4. Materials and Methods

### *4.1. Obtaining the extract and the plant fractions*

The leaves of *X. prockia* were collected in December 2015 in Governador Valadares (18°51′04″S, 41°56′58″W), Minas Gerais, Brazil. The species was identified by the botanist Dr. Ronaldo Marquete and deposited in the herbarium of the Botanical Garden of Rio de Janeiro, Rio de Janeiro, Brazil (code RB 773293), in August 2018. The research was authorized by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN; No. A66F830). To obtain EE, the plant material was dried in a convection oven and then crushed in a knife mill. The crushed leaves were macerated with 96ºGL ethanol for 5 days, filtered and the process was repeated 3 more times. After extraction, the extract was concentrated in a rotary evaporator at a temperature below 50 °C under reduced pressure. The EE obtained was dissolved in water and fractionated by liquid-liquid separation with solvents in ascending order of polarity: Hexane, dichloromethane, ethyl acetate, and butanol. The resulting fractions were concentrated in a rotary evaporator at a temperature below 50°C under reduced pressure.

### 4.2 *Determination of the minimum inhibitory concentration*

The strain ATCC 22019 (American Type Culture Collection) *C. parapsil*osis was used in this work. The MICs were determined according to the methodology proposed by the Clinical and Laboratory Standard Institute [18] with some modifications. The final concentrations of extract, fractions, and FCZ varied from 0.50 to 256 mg/L. The final concentrations of AMB varied from 0.03 to 16 mg/L. Isolated colonies from a *C. parapsilosis* culture were suspended in a sterile saline solution and adjusted to 75-77% at 530 nm using a spectrophotometer. The fungal suspension was diluted in sterile RPMI-1640 medium buffered with MOPS to reach the final concentration of $1.0 \times 10^3 – 5.0 \times 10^3$ CFU/mL.

The plates were incubated at 35 °C for 48 hours. A positive control (without treatment) and a negative control (medium only) were performed. The last dilution without visible growth for AMB, extract and fractions was considered as MIC. For FCZ, the con-

centration with 50% visible growth was considered the MIC compared to the control. The tests were performed in triplicate on two different days.

*4.3. Determination of the time-kill curve*

The test was performed as described previously, [11] with modifications. Isolated colonies were used to prepare an inoculums in a similar manner to the MIC assay. Dilutions of EAF (½ MIC, MIC, and 2 MIC) were prepared. A positive control (without treatment) and a negative control (medium only) were performed. The 96-well plate was incubated at 35°C and 30 μL were withdrawn after 0, 3, 6, 12, 24, and 48 hours. These aliquots were plated in agar Sabouraud dextrose (ASD) medium and incubated at 35°C for 48 hours. The results were read by visible growth and the number of colonies was noted to quantify CFU/mL. The results were expressed as mean ± standard deviation (SD). The tests were performed in triplicate on two different days.

*4.4. Quantification of ergosterol and lipid peroxidation*

The extraction of intracellular sterols from *C. parapsilosis* was performed as suggested by Arthington-Skaggs et al. [19] and Ferreira et al. [14] with modifications. Fungal cells ($6.0x10^6$ CFU/mL) were treated with FCZ and EAF (MIC) at 35°C for 3 hours. After treatment, the tubes were centrifuged at 3000 rpm for 5 minutes and the cells were washed three times with 1 mL of 0.9% saline. To the tubes containing the fungal pellet, 1.5 mL of a 25% alcoholic KOH solution was added and shaken vigorously for 1 minute. The tubes were incubated for 1 hour at 85°C in a water bath and then cooled to room temperature. The sterols were extracted by adding 1 mL of sterile distilled water and 3 mL of heptane, followed by vigorous shaking for 3 minutes. A UV-visible spectrophotometer measured the organic phase at 230 and 282 nm. The ergosterol content was calculated using a mathematical equation proposed by Arthington-Skaggs et al. [19], which includes: % dihydroergosterol = absorbance at 230 nm / 518, % dihydroergosterol + % ergosterol = absorbance at 280 nm / 290 and % ergosterol = (% dihydroergosterol + ergosterol) – (% dihydroergosterol).

The lipid peroxidation products were measured as TBARS, as previously described with modifications [20]. Fungal cells ($6.0x10^6$ CFU/mL) were treated with AMB and EAF (MIC) at 35°C for 3 hours. After treatment, the tubes were centrifuged at 3000 rpm for 5 minutes and the cells were washed three times with 1 mL of 0.9% saline. The pellet was treated with 1 mL of ice-cold 1% phosphoric acid, and homogenized. 400 μL of the homogenate was mixed with 400 μL of 1% thiobarbituric acid containing 0.1 mM BHT and 50 mM NaOH, followed by 200 μL of 7% phosphoric acid. The solutions were kept cold during manipulation and assay. The tubes were heated in a water bath at 100°C for 1 hour, followed by a 10-minute de-icing bath. Subsequently, 1.5 mL of butanol was added and the samples were shaken vigorously and centrifuged at 3,000 rpm for 5 minutes. The organic layer was separated and read at 532 nm in a spectrophotometer. TBARS concentrations were calculated using the molar extinction coefficient of 156 mM-1 cm-1. The test was performed in triplicate and the results were expressed in μM as mean ± standard deviation (SD).

*4.5 Quantification of the production of reactive oxygen species and nitrogen species*

Cells ($1.0x10^3$ to $5.0x10^3$ CFU/mL) were incubated for 3 hours with EAF (MIC) or AMB (MIC) in RPMI 1640 without phenol red with 10 mM dichlorofluorescein 2',7'-diacetate (for quantification of ROS) or 20 mM dihydrorhodamine 123 (for quantification of peroxynitrite). Fluorescence was measured with excitation and emission wavelengths of 500 nm [21]. The test was performed in triplicate. Results were expressed as arbitrary fluorescence units as mean ± standard deviation (SD).

*4.6. Statistical analysis*

Statistical analyzes were performed using the program GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). Comparisons between the two groups were performed using the Student's test (parametric data) or the Mann-Whitney test (non-parametric data). P-values ≤ 0.05 were assumed for the analysis.

**Funding:**This work was funded by the FAPEMIG under Grant (Project APQ 00265/18 - Call 01/2018 - Universal Demand); FAPEMIG under Grant (FAPEMIG - Project APQ 02648/18 - Call 01/2018 - Universal Demand; UFJF under Grant (BIC – Call 2018/2019).

**Institutional Review Board Statement:** *Not applicable*.

**Informed Consent Statement:***Not applicable*.

**Conflicts of Interest:** "*The authors declare no conflict of interest*."

## References

1 – Rocha, W.R.V.; Nunes, L.E.; Neves, M.L.R.; Ximenes, E.C.P.A.; Albuquerque, M.C.P.A. *Candida* genus - Virulence factores, Epidemiology, Candidiasis and Resistance mechanisms. Res. Soc. Dev.[online] **2021**, 10(4) p. e43910414283. doi: 10.33448/rsd-v10i4.14283**.**

2 – Doi, A.M.; Pignatari, A.C.; Edmond, M.B.; Marra, A.R.; Camargo, L.F.; Siqueira, R.A.; da Mota, V.P.; Colombo, A.L. Epidemiology and Microbiologic Characterization of Nosocomial Candidemia from a Brazilian National Surveillance Program. PLoS One. **2016**, 25;11(1):e0146909. doi: 10.1371/journal.pone.0146909.

3 – Berkow, E.L.; Lockhart, S.R. Fluconazole resistance in *Candida* species: a current perspective. Infect. Drug. Resist., **2017,** 10:237-245. doi: 10.2147/IDR.S118892.

4 – Nivoix, Y.; Ledoux, M.P.; Herbrecht, R. Antifungal Therapy: New and Evolving Therapies. Semin. Respir. Crit. Care Med. **2020**, Feb;41(1):158-174. doi: 10.1055/s-0039-3400291.

5 – Bassetti, M.; Giacobbe, D.;, Vena, A.; Wolff, M. (Diagnosis and Treatment of Candidemia in the Intensive Care Unit. Semin. Respir. Crit. Care Med. [online] **2019**, 40(4):524-539. doi 10.1055/s-0039-1693704.

6 – Newman, D.J.; Cragg, G.M. Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. J. Nat. Prod. **2020**, 27;83(3):770-803. doi: 10.1021/acs.jnatprod.9b01285.

7 – Longhi, S.J.; Brena, D.A.; Gomes, J.F.; Narvaes, I.S.; Berger, A.; Soligo, A. J. Classificação e caracterização de estágios sucessionais em remanescentes de floresta ombrófila mista na flora de São Francisco de Paula. Brasil. Cienc. Florest. **2006**, 16, 113–125.doi: 10.5902/198050981893.

8 – Folly, M.L.C.; Ferreira, G.F.; Salvador, M.R.; Sathler, A.A.; da Silva, G.F.; Santos, J.C.B.; Dos Santos, J.R.A.; Nunes Neto, W.R.; Rodrigues, J.F.S.; Fernandes, E.S.; da Silva, L.C.N.; de Freitas, G.J.C.; Denadai, Â.M.; Rodrigues, I.V.; Mendonça, L.M.; Monteiro, A.S.; Santos, D.A.; Cabrera, G.M.; Siless, G.; Lang, K.L. Evaluationof *in vitro* antifungalactivity of *Xylosma prockia (Turcz.) Turcz. (Salicaceae*) leaves against *Cryptococcus* spp. Front. Microbiol. **2020**, 6;10:3114. doi: 10.3389/fmicb.2019.03114.

9- Ríos, J.L.; Recio, M.C. Medicinal plants and antimicrobial activity. J. Ethnopharmacol. **2005**, 100 (1-2):80-4. doi: 10.1016/j.jep.2005.04.025.

10-Balouiri, M.; Sadiki, M.; Ibnsouda, S.K. Methods for *in vitro* evaluation antimicrobial activity: A review. Journal of Pharmaceutical Analysis, **2016**, 6, 71- 79. doi: 10.1016/j.jpha.2015.11.005.

11-Scorneaux, B.; Angulo, D.; Borroto-Esoda, K.; Ghannoum, M.; Peel, M.; Wring, S. SCY-078 Is Fungicidal against *Candida* Species in Time-Kill Studies. Antimicrob. Agents Chemother. **2017**, 61:10.1128/aac.01961-16. doi: 10.1128/aac.01961-16

12-Shafiei, M.; Peyton, L.; Hashemzadeh, M.; Foroumadi, A. History of the development of antifungal azoles: A review on structures, SAR, and mechanism of action. Bioorg-Chem. **2020** Nov;104:104240. doi: 10.1016/j.bioorg.2020.104240.

13- Garcia-Rubio, R.; de Oliveira, H.C.; Rivera, J.; Trevijano-Contador, N. The FungalCell Wall: *Candida*, *Cryptococcus*, and*Aspergillus*Species. Front Microbiol. 2020, 9;10:2993. doi: 10.3389/fmicb.2019.02993.

14 - Ferreira, G.F.; Baltazar, L.M; Santos, J.R.; Monteiro, A.S.; Fraga, L.A.; Resende-Stoianoff, M.A.; Santos, D.A. The role of oxidative and nitrosative bursts caused by azoles and amphotericin B against the fungal pathogen *Cryptococcus gattii*. J Antimicrob Chemother. **2013**, 68(8):1801-11. doi: 10.1093/jac/dkt114.

15- Mesa-Arango, A.C. Scorzoni, L.; Zaragoza, O. It only takes one to do many jobs: Amphotericin B as antifungal and immunomodulatory drug. Front Microbiol. **2012**, 8;3:286. doi: 10.3389/fmicb.2012.00286.

16 – Afzal, S.; Abdul Manap, A.S.; Attiq, A.; Albokhadaim, I.; Kandeel, M.; Alhojaily, S,M. From imbalance to impairment: the central role of reactive oxygen species in oxidative stress-induced disorders and therapeutic exploration. Front Pharmacol. **2023**Oct 18;14:1269581. doi: 10.3389/fphar.2023.1269581.

17 - Peralta, M.A.; da Silva, M.A.; Ortega, M.G.; Cabrera, J.L.; Paraje, M.G. Antifungal activity of a prenylated flavonoid from *Dalea elegans* against *Candida albicans* biofilms. Phytomedicine. **2015**, 15;22(11):975-80. doi: 10.1016/j.phymed.2015.07.003.

18 **-** Clinical and Laboratory Standards Institute (CLSI). Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A3, 3rd ed. Clinical and Laboratory Standards Institute, Wayne, PA, **2008.**

19- Arthington-Skaggs, B.A.; Jradi, H.; Desai, T.; Morrison, C.J. .Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. J. Clin. Microbiol. **1999,** Oct;37(10):3332-7. doi: 10.1128/JCM.37.10.3332-3337.

20-Alves, J.C.O.; Ferreira, G.F.; Santos, J.R.; Silva, L.C.N.; Rodrigues, J.F.S.; Neto, W.R.N.; Farah. E.I.; Santos, Á.R.C.; Mendes, B.S.; Sousa, L.V.N.F.; Monteiro, A.S.; Dos Santos, V.L.; Santos, D.A.; Perez, A.C.; Romero, T.R.L.; Denadai, Â.M.L.; Guzzo, L.S. Eugenol InducesPhenotypicAlterationsandIncreasestheOxidativeBurst in *Cryptococcus*. Front. Microbiol. **2017**, Dec 7;8:2419. doi: 10.3389/fmicb.2017.02419.

21 - Freitas, G.J.C.; Ribeiro, N.Q.; Gouveia-Eufrasio L.; Emidio, E.C.P.; Guimarães, G.M.; César, I.C.; Paixão, T.A.; Oliveira, J.B.S.; Caza, M.; Kronstad, J.W.; Santos, D.A. Antimalarialsandamphotericin B interactsynergisticallyand are new optionstotreatcryptococco-

sis. Int. J. Antimicrob. Agents. **2023**, Jul;62(1):106807. doi: 10.1016/j.ijantimicag.2023.106807.