Profiling the *Cymbopogon nardus* Ethanol Extract and Its Antifungal Potential against *Candida* Species with Different Patterns of Resistance

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The essential oil of *Cymbopogon nardus*, citronella, has been extensively studied. However, the chemical and biological properties of the ethanolic extract (EE) of *C. nardus* have not been evaluated. The aim of this study was to characterize the chemical composition of the EE of *C. nardus* and its active fraction (FrD). Moreover, the cytotoxic and antifungal properties of these extracts against *Candida* species with different resistance profiles to conventional drugs were evaluated. The compounds identified in EE were mono-C- and di-C-glycosyl flavones and phenylpropanoid glycosides. Phenylpropanoid glycosides were identified in FrD. EE showed antifungal activity, with minimum inhibitory concentration (MIC) values ranging from 62.5 to 500 µg mL⁻¹. FrD was more effective against *C. glabrata*, as evidenced by the lowest MIC value (15.6 µg mL⁻¹). EE inhibited yeast growth similar to amphotericin-B, as demonstrated by similar time-kill curves. EE inhibited *C. albicans* hyphae formation and mature biofilm of *C. albicans*, *C. krusei* and *C. parapsilosis*. The results of the chemical and biological analyses of EE and its fractions provided novel information and may contribute to control of infections caused by *Candida* species.

**Keywords:** *Cymbopogon nardus*, ethanol extract, flavones, phenylpropanoids, antifungal activity, *Candida* spp.

Introduction

The *Cymbopogon* genus is an important source of compounds with pharmacological properties. *Cymbopogon nardus* (L.) Rendle (Poaceae), commonly known as citronella, is native to Ceylon, and is cultivated in subtropical and tropical regions of Asia, Africa, and America. The essential oil and the ethanolic extract (EE) of citronella leaves have been traditionally used as insect repellents. Moreover, in Thailand, an infusion of citronella leaves is used to treat flatulence, dyspepsia, and abdominal cramps. These positive results were likely due to its bioactive properties, particularly considering that this essential oil contains secondary monoterpene metabolites such as citronellal, citronellol, and geraniol. However, the chemical composition and antifungal activity of EE of *C. nardus* leaves against clinical strains has not been well-characterized.

* C. *citrus* exerts anti-inflammatory, antifungal, antibacterial, and anthelmintic activities, and the essential oil from *C. nardus* can repel *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles dirus* mosquitoes, and exerts antibacterial and antifungal activities.

The antifungal potential of the essential oil of *C. nardus* against species of *Candida* has been studied with satisfactory results. These positive results were likely due to its bioactive properties, particularly considering that this essential oil contains secondary monoterpene metabolites such as citronellal, citronellol, and geraniol. However, the chemical composition and antifungal activity of EE of *C. nardus* leaves against clinical strains has not been well-characterized.

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Some plant extracts have been evaluated against fungi because of the presence of secondary metabolites with antimicrobial properties, such as phenols, flavonoids, terpenes, and alkaloids. A study described the in vitro fungistatic and fungicidal activity of a hydroethanolic extract of C. nardus against Microsporum canis and Trichophyton rubrum isolated from animals and the home environment.

Classical treatments for fungal infections include polyenes, azoles, and echinocandins. However, these treatments induce significant side effects. The side effects associated with synthetic antifungal agents promote aggravation of the disease state, since the side effects are typically related to hepatotoxicity and renal dysfunction. Moreover, the lack of alternative treatment options is problematic in the case of drug resistance. Candida has emerged as important species associated with opportunistic infections, resulting in a significant public health issue. Several predisposing factors including immunodeficiency, antineoplastic therapy, organ transplantation, endocrine dysfunction, and prolonged antibiotic use increase susceptibility to Candida infection.

Candida spp. infections range from superficial infections, such as vulvovaginal candidiasis, esophageal or oropharyngeal candidiasis, and disseminated candidiasis. Candida infections are associated with high morbidity and mortality rates in nosocomial bloodstream infections.

Several virulence factors associated with Candida spp. include morphological transition between yeast and hyphae, ability to defend against the host immune system, adhesion, biofilm formation, and production of harmful enzymes such as hydrolytic proteases, phospholipases, and hemolysin. This study aimed to evaluate for the first time the chemical composition and antifungal activity of C. nardus against standard and clinical strains of Candida species with different biological virulence profiles and antifungal susceptibility.

Experimental

Plant material

C. nardus leaves were collected in the morning (July 2013), in the Garden of Toxic and Medicinal Plants: Profa Dra Célia Cebrian de Araújo Reis (longitude 48.20170°W, latitude 21.81453°S), Universidade Estadual Paulista (Unesp), Araraquara, São Paulo, Brazil. A voucher specimen (HRCB-60752) was deposited at Herbarium Rioclarense of the Institute of Biosciences (Unesp, Rio Claro, São Paulo, Brazil). This work was approved by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under license No. A2B917A and AF35617/CNPJ 48.031.918/0001-24.

Ethanolic extract (EE) preparation

Dried and powered leaves (500 g) were extracted by sonication in ethanol (99%) (Hexis, Jundiaí, São Paulo, Brazil) in four steps (2.0, 1.5, 1.5, and 0.5 L; 20 min per step) with occasional agitation. All extracted solutions were filtered, mixed, concentrated using a rotary evaporator, dried in a fume hood and then in a desiccator with silica gel. The yield of dried EE was 1.35%.

Fractionation of EE by solid phase extraction (SPE)

EE (2.3 g) was loaded onto a glass column containing silica gel (60-200 µm; height: 10 cm, (Merck® KGaA, Darmstadt, Germany). Elution was performed under reduced pressure using hexane:ethyl acetate (9:1, v/v), hexane:ethyl acetate (7:3, v/v), ethyl acetate (100%), ethyl acetate:methanol (9:1, v/v), and methanol (100%), yielding 10 fractions (40 mL each) (two per eluent).

The obtained SPE fractions (Fr) (5.0 mg mL⁻¹; ethyl acetate) were analyzed by thin layer chromatography (TLC) on glass plates with silica gel (Sigma-Aldrich®, Saint Louis, MO, USA; 20 × 20; 0.25 mm). The mobile phases were hexane:ethyl acetate/isopropanol (70:28:2, v/v/v), n-butanol:acetic acid:water (67:30:3, v/v/v), and chloroform:ethyl acetate (60:40, v/v), and 10% aqueous sulfuric acid was used as the spray reagent. Comparison of the Fr chemical profiles by TLC analysis indicated that seven fractions with different profiles could be pooled (FrA, FrB, FrC, FrD, FrE, FrF, and FrG). The fractions (weight; eluent) were as follows: FrA (0.108 g; hexane:ethyl acetate 9:1), FrB (0.067 g; hexane:ethyl acetate 9:1), FrC (0.471 g; hexane:ethyl acetate 7:3), FrD (0.322 g; ethyl acetate), FrE (0.096 g; ethyl acetate:methanol 9:1), FrF (0.078 g; ethyl acetate:methanol 9:1), and FrG (0.446 g; methanol). FrD was selected for chemical analysis since this fraction showed the best anti-Candida activity, mainly against C. glabrata. All solvents used were of analytical grade and purchased commercially (Synth®, Diadema, São Paulo, Brazil).

Ultra-performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOF-MS®) analysis

Prior to analyses, 10.0 mg each of EE and FrD samples were subjected to SPE using Phenomenex® Strata™ C18-E
cartridges (Torrance, CA, USA, 15 × 10 mm; 55 µm). Samples were eluted with 5.0 mL of methanol:water (95:5, v/v). The obtained solutions (5 mL) were dried in a desiccator (silica gel under reduced pressure) and the residues were dissolved in 1.0 mL of methanol, then filtered (0.22 µm, PVDF Merck Millipore®, KGaA, Darmstadt, Germany) prior to analysis.

Chemical analyses of EE and FrD were performed using an Acquity UPLC (Waters Corporation®, Milford, Massachusetts, USA) coupled to a quadrupole/time of flight system (XEVO-QToF, Waters Corporation®, Milford, Massachusetts, USA). The mobile phases were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient program was as follows: (0-15) min, 2-95% B; (15.1-17) min, 100% B; (17.1-19.1) min, 2% B. Separation was performed using a Waters Acquity UPLC BEH C18 column (Milford, MA, USA, 150 × 2.1 mm, 1.7 µm) with a flow rate 0.4 mL min⁻¹. The column oven temperature was maintained at 40 °C. The injection volume was 5 µL. The MS conditions were as follow: negative ionization mode; acquisition range: 110-1180 Da; source temperature: 120 °C; desolvation gas temperature: 350 °C; desolvation gas flow: 500 L h⁻¹; extraction cone voltage: 0.5 V; capillary voltage: 2.6 kV. Leucine enkephalin was used as the lock mass. Instrument control and data acquisition were performed using Masslynx 4.1 (Waters Corporation®, Milford, Massachusetts, USA) software. Acetonitrile, chromatography grade methanol, and ultrapure water (18.2 MΩ cm) were used for analysis.

Fungal strains

Three clinical isolates and one purchased from American Type Culture Collection (ATCC) for each species of Candida spp. were studied: C. albicans (CA ATCC 90028, CA2, CA3, CA4); C. glabrata (CG ATCC 2001, CG2, CG3, CG4); C. tropicalis (CT ATCC 13803, CT2, CT3, CT4), C. parapsilosis complex-C. parapsilosis (CP ATCC 22019, CP1) and C. orthopsilosis (CO ATCC 96141, CO1) and C. krusei (CK ATCC 6258, CK2, CK3, CK4). C. albicans (ATCC 10231) was used for the hyphae formation assay.

The clinical strains were donated to the Microbiology Laboratory of the Medicine School in São José do Rio Preto for the purposes of scientific research through written consent of the donors. The use of these strains was approved by the Human Research Ethics Committee of FAMERP, project identification code 152/2006 (December 6th, 2006), Medicine School in Sao José do Rio Preto (FAMERP).

Determination of minimum inhibitory concentration (MIC)

The antifungal activity of EE was evaluated by determining the MIC using the microplate dilution technique according to the procedures described by Clinical and Laboratory Standards Institute (CLSI),²⁰ with modifications. Roswell Park Memorial Institute (RPMI)1640 medium (Sigma-Aldrich®, Saint Louis, MO, USA) adjusted to pH 7.0 with MOPS (acid 3-[N-morpholino]propanesulfonic acid) buffer (Sigma-Aldrich®, Saint Louis, MO, USA) was added to each well. Solutions of EE (0.1 mL) were added at concentrations ranging from 1000 to 7.8 µg mL⁻¹. A suspension (0.1 mL) containing 2.5 × 10⁴ yeast mL⁻¹ was added to each well. Amphotericin B (Sigma-Aldrich®, Saint Louis, MO, USA) and fluconazole (Sigma-Aldrich®, Saint Louis, MO, USA) were used as antimicrobial positive controls. Controls including culture medium, yeast growth, EE, and solvent were also prepared. The microplates were incubated at 37 °C for 48 h. After incubation, 20 µL of an aqueous 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich®, Saint Louis, MO, USA) were added to each well. Amphotericin B (Sigma-Aldrich®, Saint Louis, MO, USA) and fluconazole (Sigma-Aldrich®, Saint Louis, MO, USA) were used as antimicrobial positive controls. Controls including culture medium, yeast growth, EE, and solvent were also prepared. The microplates were incubated at 37 °C for 2 h.²¹ All experiments were performed in triplicate.

The MIC results of EE strains were used to select the most sensitive strains (one ATCC and one clinical isolate for each species) for evaluation of antifungal activity as previously described.²⁰

Determination of minimum fungicidal concentration (MFC)

The MFC was determined by adding an aliquot from each well that showed antifungal activity to Petri dishes containing Sabouraud Dextrose Agar (SDA) (DIFCO®, Le Pont de Clax, France). These experiments were performed in triplicate. The MFC was defined as the lowest concentration of EE and Fr that resulted in no visible growth on the solid medium.²¹

Inhibition of C. albicans hyphae formation

C. albicans (ATCC 10231) was cultured for 24 h to obtain filamentous yeast. Then, the yeast was suspended at a concentration of 2.5 × 10⁹ cells mL⁻¹ in phosphate-buffered saline (PBS, pH 7.2). Twenty microliters of this suspension were added to microplate wells containing RPMI 1640 medium (Sigma-Aldrich®, Saint Louis, MO, USA) with 10% fetal bovine serum and 1% gentamicin. EE solution was evaluated at concentrations ranging from 1000 to 7.8 µg mL⁻¹. After 12 and 24 h, reductions in hyphal growth were visualized using an inverted light microscope (400×). Amphotericin B (Sigma-Aldrich®, Saint Louis, USA) was used as a positive control. Controls including culture medium, yeast growth, EE, and solvent were also prepared. The microplates were incubated at 37 °C for 2 h.²¹ All experiments were performed in triplicate.
MO, USA) (16 µg mL⁻¹) was used as the positive control. Additional controls included fungal growth, solvent, sterile EE solution, and culture medium.³

**Time-kill assay**

The time-kill assay was carried out according to Santos-Filho et al.²² with modifications. One ATCC strain and one clinical strain of each *Candida* species (CA ATCC 90028, CA3, CK ATCC 6258, CK4, CG ATCC 2001, CG3, CT ATCC 13803, CT3, CP ATCC 22019, CP1, CO ATCC and CO1) was evaluated. Two times the MIC of EE were added to Sabouraud Dextrose broth (DIFCO®, Le Pont de Claix, France), containing 2.5 × 10⁴ colony-forming unit (CFU) mL⁻¹ of *Candida* spp. and incubated at 37 ºC. At different time intervals (0, 1, 2, 4, 8, 12, 24, 36, and 48 h) 100 µL aliquots were removed and diluted 1:100 twice in sterile PBS. Each EE-cell suspension was spread onto SDA (DIFCO®, Le Pont de Claix, France) (incubation 48 h at 37 ºC) for subsequent counting of CFU. As the positive control was used amphotericin B (Sigma-Aldrich®, Saint Louis, MO, USA) and cell suspensions without addition of EE were the negative control.

**Mature biofilm**

The biofilm adhesion method was performed as previously described by Pitangui et al.²³ with modifications. CA ATCC 90028, CA3, CK ATCC 6258, CK4, CP ATCC 22019, and CP1 were evaluated. Inoculum (0.1 mL, 5.0 × 10⁸ yeast mL⁻¹) suspended in saline (0.9%) was added to the microplate wells (96 wells), then incubated at 37 °C for 2 h with stirring at 80 rpm. After the pre-adhesion period, the supernatant was removed and 0.1 mL of RPMI medium (Sigma-Aldrich®, Saint Louis, MO, USA) was added to each microplate well. The plates were incubated for 48 h, with the medium replaced after 24 h. Following incubation, the supernatant was removed, and the wells were washed with 0.1 mL of 0.9% saline. EE solution (0.1 mL) was added to each well at 50 times the MIC. Amphotericin B (Sigma-Aldrich®, Saint Louis, MO, USA) was used as the positive control. Other controls included culture medium, yeast growth, EE solution, and solvent. The microplates incubated for 24 h at 37 °C, then developed with 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[carbonyl (phenylamino)]-2H-tetrazolium hydroxide (XTT®, Sigma-Aldrich®, Saint Louis, MO, USA). The MIC₅₀ was defined as the highest concentration of each fraction that resulted in at least 50% cell viability. All experiments were performed in triplicate. Five percent of dimethyl sulfoxide (DMSO) were used as the control.²⁴

**Results and Discussion**

**UPLC-ESI-QTOF-MSE analysis**

Compounds were identified based on retention time, fragmentation pattern, and accurate mass (chemical formula). Figure 1 shows a total ion chromatogram (TIC) of EE obtained in negative mode. Table 1 summarizes the identities of compounds 1-10 in EE as determined by mass spectrometry (high resolution MS and MS/MS). Data from the acquired spectra were compared with specialized literature data.²⁵-²⁹ Based on these spectral comparisons, the following secondary metabolites were identified: two mono-C- (luteolin and apigenin...
derivatives) and two di-C-glycosyl flavones (luteolin derivatives), and six phenylpropanoid glycosides: three di-O-feruloyl-di-O-acetyl sucrose isomers (e.g., smiglaside A) and three di-O-feruloyl-tri-O-acetyl sucrose isomers (e.g., smiglaside C). The main peak observed in the TIC (t_R = 6.90 min) corresponded to a di-O-feruloyl-tri-O-acetyl sucrose isomer.

The spectra of both mono-C- and di-C-glycosyl flavones (Table 1, compounds 1-4) showed typical sugar moiety fragments resulting from cleavage of the C-hexosyl and C-pentosyl rings (deprotonated ions at m/z [M – H – 60]–, [M – H – 90]–, [M – H – 120]–, and [M – H – 210]–).29,30

Compounds 3 and 4 showed deprotonated molecule signals ([M – H]–) at m/z 447.0922 and 431.0983, respectively. The observed fragmentation patterns were characteristic of C-glycosyl flavones. The MS² data showed fragment ion signals at m/z 357 and 327 for compound 3 and at m/z 341 and 311 for compound 4, which corresponded to loss of 90 and 120 Da from the [M – H]– ions, respectively, which is typical of a hexose substitution in the aglycone moiety. These data supported assignments of luteolin-8-C-glucoside (orientin) for compound 3 and apigenin-8-C-glucoside (vitexin) for compound 4.26,27 Flavonoid C-glycosylation has almost exclusively been found at positions 6 or 829 and according to a previous study26 the relative intensities of the [M – H – 90]– fragment ions were 22 and 100% for orientin and isoorientin (luteolin-6-C-glucoside), respectively, and 8 and 42% for vitexin and isovitexin (apigenin-6-C-glucoside), respectively, supporting identification of orientin and vitexin in EE (Table 1). The fragment ion signal at m/z 285 for compound 3 may correspond to kaempferol (flavonol) or luteolin (flavone) aglycone moieties (Y¹ fragment ion), but data from previous

| Compound | Retention time / min | Empirical formula | [M – H]– observed | [M – H]– calculated | Error / ppm | MS/MS fragment ions (relative abundance / %) | Putative name | Reference |
|----------|---------------------|-------------------|-------------------|---------------------|------------|---------------------------------------------|--------------|----------|
| 1        | 3.46                | C_{25}H_{26}O_{13} | 579.1335          | 579.1350            | 2.6        | 519.1381 (12), 489.1046 (52), 459.0822 (42), 429.0785 (29), 399.0722 (75), 369.0647 (88), 133.0230 (3) | luteolin-6-C-arabinoside-8-C-glucoside | 25,26 |
| 2        | 3.87                | C_{25}H_{26}O_{14} | 549.1209          | 549.1244            | 6.4        | 531.1587 (2), 489.0969 (7), 459.0842 (40), 429.0727 (30), 399.0607 (80), 369.0598 (44), 133.0262 (1) | luteolin-6,8-di-C-arabinoside | 26 |
| 3        | 3.94                | C_{25}H_{26}O_{13} | 447.0922          | 447.0927            | 4.0        | 357.0461 (22), 339.0600 (13), 327.0445 (100), 299.0549 (28), 297.0301 (26), 285.0474 (18), 133.0299 (1) | luteolin-8-C-glucoside (orientin) | 26,27 |
| 4        | 4.25                | C_{25}H_{26}O_{10} | 431.0983          | 431.0978            | 0.2        | 341.0851 (9), 323.0531 (19), 311.0475 (81), 283.0561 (100), 281.0421 (3), 269.0467 (2) | apigenin-8-C-glucoside (vitexin) | 26 |
| 5        | 6.18                | C_{26}H_{26}O_{13} | 777.2248          | 777.2242            | 0.8        | 735.1852 (3), 717.1985 (6), 601.1769 (5), 559.1522 (5), 193.0482 (14), 175.0382 (100) | di-O-feruloyl-di-O-acetyl sucrose | 28 |
| 6        | 6.32                | C_{26}H_{26}O_{13} | 777.2206          | 777.2242            | 4.6        | 735.1752 (3), 717.1892 (7), 601.1716 (6), 559.1675 (8), 193.0491 (12), 175.0380 (100) | di-O-feruloyl-di-O-acetyl sucrose | 28 |
| 7        | 6.63                | C_{26}H_{26}O_{13} | 777.2195          | 777.2242            | 6.0        | 735.2078 (1), 717.1902 (1), 601.1771 (30), 559.1614 (10), 193.012 (14), 175.389 (100) | di-O-feruloyl-di-O-acetyl sucrose | 28 |
| 8        | 6.90                | C_{26}H_{26}O_{10} | 819.2283          | 819.2348            | 7.9        | 777.2292 (8), 601.1830 (8), 513.1558 (3), 193.0468 (6), 175.0377 (100) | di-O-feruloyl-tri-O-acetyl sucrose | 28 |
| 9        | 7.05                | C_{26}H_{26}O_{10} | 819.2291          | 819.2348            | 7.0        | 777.2177 (8), 759.2155 (9), 601.1768 (8), 193.0481 (35), 175.0377 (100) | di-O-feruloyl-tri-O-acetyl sucrose | 28 |
| 10       | 7.21                | C_{26}H_{26}O_{12} | 819.2303          | 819.2348            | 5.5        | 777.1954 (3), 759.2157 (2), 601.1773 (8), 193.0506 (48), 175.0399 (100) | di-O-feruloyl-tri-O-acetyl sucrose | 28 |
study showed that the fragment ion signal at \( m/z \) 133 was characteristic of luteolin aglycone (\( Y^- \) fragment ion). The fragment ion signal at \( m/z \) 269 for compound 4 corresponded to the apigenin aglycone moiety (\( Y^- \) fragment ion). Other major fragment ion signals typical for orientin were observed at \( m/z \) 299 and 297 and at \( m/z \) 283 and 281 vitexin, and were attributed to loss of 148 and 150 Da.

Compound 2 showed deprotonated molecule signals at \( m/z \) 549.1209 [\( M^-H \)] and fragment ion signals in MS\(^2\) spectra at \( m/z \) 489, 459, 429, 399, and 369, which corresponded to losses of 60, 90, 120, 150 (60 + 90), and 180 (60 + 120) Da from the [\( M^-H \)]\(^+\) ion, which were characteristic of a di-C-pentosyl flavone. These data supported identification of this compound as luteolin-6,8-di-C-arabinoside.

Compound 1 showed deprotonated molecule signals at \( m/z \) 579.1335 [\( M^-H \)]\(^+\). The observed fragmentation pattern in the MS\(^2\) spectrum corresponded to a C-hexosyl-C-pentosyl flavone with fragment ion signals at \( m/z \) 519, 489, 459, 429, 399, and 369, corresponding to losses of 60, 90, 120, 150 (60 + 90), 180 (60 + 120), and 210 (90 + 120) Da from the [\( M^-H \)]\(^+\) ion, respectively. These data supported identification of this compound as luteolin-6-C-arabinosyl-8-C-glucoside. Previous studies of di-C-glicoside flavones reported that 6-C-pentosyl-8-C-hexosyl substitution resulted in higher abundance of the [\( M^-H-90 \)] fragment ion relative to the [\( M^-H-120 \)] fragment ion. For example, luteolin-6-C-arabinosyl-8-C-glicoside showed a higher abundance of the [\( M^-H-90 \)] fragment ion at \( m/z \) 489 (52\%) than that of the [\( M^-H-120 \)] fragment ion at \( m/z \) 459 (17\%), while luteolin-8-C-glicosyl-6-C-arabinoside showed a higher abundance of the [\( M^-H-120 \)] fragment ion at \( m/z \) 459 (74\%) than that of the [\( M^-H-90 \)] fragment ion at \( m/z \) 489 (23\%). Therefore, the data from Table 1 suggest a 6-C-pentosyl-8-C-hexosyl substitution pattern for compound 1. As discussed for orientin, the presence of the ion fragment signals at \( m/z \) 133 for compounds 1 and 2 supports luteolin as the aglycone moiety.

Compounds 5-7 showed deprotonated molecule signals [\( M^-H \)]\(^+\) at \( m/z \) 777.2248, 777.2206, and 777.2195, and fragment ion signals at \( m/z \) 601 due to loss of 176 Da, which represented a feruloyl moiety. The fragment ion signals at \( m/z \) 175 and 193 corresponded to a feruloyl moiety and the other fragment ion signals shown in Table 1 supported identification of these compounds as di-O-feruloyl-di-O-acetyl sucrose isomers (e.g., smiglaside A).

Compounds 8-10 showed deprotonated molecule signals [\( M^-H \)]\(^+\) at \( m/z \) 819.2283, 819.2291, and 819.2303, and the fragment ion signals at \( m/z \) 175 and 193 corresponded to a...
feruloyl moiety\textsuperscript{25} which supported identification of these compounds as di-O-feruloyl-tri-O-acetyl sucrose isomers (e.g., smiglaside C)\textsuperscript{28}.

Previous studies have identified phenylpropanoid derivatives (e.g., chlorogenic acid and caffeic acid) and glycosyl flavones (e.g., isoorientin) in the \textit{Cymbopogon} genus. These compounds are structurally and biosynthetically related to the compounds identified in the of EE \textit{C. nardus}. However, no studies have evaluated non-volatile secondary metabolites in \textit{C. nardus}.\textsuperscript{30}

FrD was the most active EE fraction against \textit{Candida} strains. TIC data of FrD were compared with data from a previous study\textsuperscript{28} and identification of the compounds in FrD was performed identically during evaluation of EE of \textit{C. nardus}. The identified secondary metabolites included the same six phenylpropanoid glycosides found in EE: three di-O-feruloyl-di-O-acetyl sucrose isomers (e.g., smiglaside A) and three di-O-feruloyl-tri-O-acetyl sucrose isomers (e.g., smiglaside C). Fractionation resulted in collection of each of these compounds in FrD and the main peak observed in the TIC ($t_R = 6.90$ min) corresponded to a di-O-feruloyl-tri-O-acetyl sucrose isomer, as observed in EE.

MIC and MFC determination of EE

The data showed that EE exhibited antifungal activity with MIC values ranging from 62.5 to $500 \mu g \text{ mL}^{-1}$, including in isolates resistant to fluconazole and amphotericin-B (Table S1, Supplementary Information (SI) section). The lowest MIC value (62.5 $\mu g \text{ mL}^{-1}$) in response to treatment with EE was observed for \textit{C. glabrata} clinical isolates. EE showed a fungistatic profile against all tested isolates with MFC $> 500 \mu g \text{ mL}^{-1}$. The solvent and growth controls produced satisfactory results.

These analyses showed that EE was active against all strains, except CK-ATCC and CO1. These results are very important, since EE was able to inhibit different species of \textit{Candida}, including those resistant to fluconazole, the main antifungal agent used in medical practice. In addition, the results of MFC analysis showed that EE did not induce cell death, but only promoted growth inhibition. These results may be related to fungistatic mechanisms of action.

The antimicrobial potential exerted by plant extracts from the genus \textit{Cymbopogon} has been observed previously. The study performed by Oloyede\textsuperscript{32} evaluated the performance of the aqueous extract of the leaves of \textit{C. citratus} against \textit{Escherichia coli}, \textit{Staphylococcus aureus}, \textit{Bacillus cereus}, and \textit{Salmonella typhi}, and showed excellent results.

The antimicrobial activity of \textit{C. nardus} is reportedly due to properties of its essential oils. Previous studies demonstrated the antifungal potential of the essential oil of \textit{C. nardus} against \textit{Candida} species.\textsuperscript{7,33,34} However, the antifungal properties and chemical composition of the EE of \textit{C. nardus} have not been evaluated. Thus, this study was the first to evaluate these parameters, which may be of interest in the pharmaceutical and medical fields.

We highlighted the results obtained from testing of the \textit{C. glabrata} species. The MIC values for this species were the lowest compared to those for the other strains evaluated in this study, and all strains of this species were resistant to fluconazole. \textit{C. glabrata} is considered the second-most pathogenic yeast that affects humans, after only \textit{C. albicans}.\textsuperscript{35} This species is directly involved in invasive fungal infections ranging from local to blood infections. In the case of systemic infections, treatment is challenging due to a dearth of therapeutic options.\textsuperscript{19}

The antimicrobial performance of products derived from medicinal plants may be explained by the presence of secondary metabolites. Previous studies have demonstrated the antimicrobial activity of secondary metabolites against different types of microorganisms.\textsuperscript{11} The major classes of secondary metabolites are phenolic compounds, phenolic acids, quinones, saponins, flavonoids, tannins, phenazine, coumarins, lignans, neolignans, alkaloids, and terpenoids.\textsuperscript{36}

Chemical analysis of EE and FrD in this study showed the presence of C- and di-C-glycosylated flavones, and glycosylated phenylpropanoid derivatives, which directly exert antifungal activity. The antifungal activity of flavonoids in plant species has been studied extensively. Furthermore, glycosylated phenylpropanoid derivatives have been shown to inhibit the growth of several species of \textit{Candida}.\textsuperscript{37}

MIC and MFC determination of the Fr

Only FrC and FrD exhibited antifungal activity against the majority of the \textit{Candida} strains. FrD showed the lowest MIC value (15.6 $\mu g \text{ mL}^{-1}$) against \textit{C. glabrata} ATCC (Table S2, SI section). FrA, FrB, FrE, FrF, and FrG showed no antifungal activity, with MFC $> 500 \mu g \text{ mL}^{-1}$.

Comparison between EE and each of the fractions of EE demonstrated that FrD was the most active against \textit{C. krusei}, \textit{C. glabrata}, \textit{C. tropicalis}, and \textit{C. orthopsilosis}. These results indicated that the phenylpropanoid glycosides identified in this study were the most likely substances responsible for EE antifungal activity, since they were concentrated in FrD after fractionation, and FrD exhibited greater anti-\textit{Candida} activity than EE. The MIC of FrD against CG-ATCC (15.6 $\mu g \text{ mL}^{-1}$) was lower than that of EE (250 $\mu g \text{ mL}^{-1}$). Moreover, FrD exhibited greater activity against the \textit{C. glabrata} clinical isolate (31.2 $\mu g \text{ mL}^{-1}$) than EE (62.5 $\mu g \text{ mL}^{-1}$).
Previous studies\textsuperscript{37-40} have shown that phenylpropanoids glycoside exert pronounced antioxidant activity, antimicrobial activity, cytotoxic activity against some tumor cell lines, and strong anticandidal activity. A previous study\textsuperscript{37} demonstrated that the antifungal activity of phenylpropanoid glycosides such as verbascoside and isoverbascoside (MIC = 1.5 µg mL$^{-1}$) against several Candida strains was similar to the conventional antifungals, miconazole and amphotericin B (MIC = 0.5 µg mL$^{-1}$).

The mechanisms of action of natural products vary. The cytoplasmic membrane is the most common site of action of secondary metabolites, with action on this structure resulting in extravasation of cellular contents and fungal death. The interaction with genetic material and protein synthesis is also a predisposing factor for promotion of therapeutic actions of natural products. Interaction of genetic material with secondary metabolites promotes changes in deoxyribonucleic acid (DNA), resulting in ineffective transcription, leading to aberrant cellular function and cell death.\textsuperscript{36}

Phenylpropanoid glycosides may act through formation of intramolecular interactions (for example, hydrogen bonding) which disrupts the physicochemical properties of the fungal cell, including membrane permeability, water solubility, and lipophilicity.\textsuperscript{41}

Inhibition of C. albicans hyphae formation

EE was able to inhibit the transition of C. albicans from yeast to the hyphal form. Microscopic observation of EE-treated fungal cells demonstrated the absence of filamentous cells at concentrations ranging from 250 to 1000 µg mL$^{-1}$ after 12 and 24 h (Figure 2).

These results are relevant to the pharmaceutical and medical fields because hyphae forming ability is the main risk factor during infections.\textsuperscript{42} No previous studies have shown that EE of C. nardus can inhibit hyphae formation in C. albicans.

Several studies evaluating natural products observed prevention of hyphal development and proliferation of C. albicans. Chevalier et al.\textsuperscript{43} evaluated the capacity of the aqueous extract of Solidago virgaurea to inhibit C. albicans (ATCC 10231) hyphae formation and showed that this extract inhibited hyphal proliferation.

Vediyappan et al.\textsuperscript{44} showed that an extract of Gymnema sylvestre (50 µg mL$^{-1}$) inhibited C. albicans hyphae formation within 24 h of contact. Araújo et al.\textsuperscript{45} showed that the methanolic extract of scapes of S. nitens inhibited C. albicans NCPF 3153 hyphae formation at concentrations of 500, 250, and 125 µg mL$^{-1}$ within 12 to 24 h.

The observation that EE inhibited hyphae formation suggested that this extract may act through control of yeast morphology, resulting in decreased proliferation, thus facilitating the activity of the active components present in the extract.

Time-kill assay

The effects of EE on Candida growth are shown in Figures 3-8. The results confirmed the fungistatic mechanism observed during evaluation of MFC, since treatment with EE led to reduced numbers of colonies compared to that with control treatments. Furthermore, EE showed activity similar to that of amphotericin-B against all tested strains.

EE inhibited growth of CG ATCC 2001, CG3, CP1, and CO ATCC 96141 to a greater extent at 48 h (final time) than amphotericin B. These results are important because they further demonstrated the inhibitory capacity of EE against different Candida species, especially the C. glabrata strains, which were fluconazole-resistant (MIC > 64 µg mL$^{-1}$). The data found in this work corroborate with study developed by Toledo et al.\textsuperscript{7} whereby the essential oil of C. nardus showed similar growth against the same of Candida species.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure_2.png}
\caption{Inhibitory effect of EE of C. nardus on the transition of C. albicans from yeast to the hyphal form (photomicrographs by inverted light microscopic under 400x magnification).}
\end{figure}
Figure 3. Time-kill curves of *C. albicans* ATCC 90028 and CA3 following exposure to the EE of *C. nardus* and amphotericin-B. Control represents the untreated *Candida* cell. Note: time zero value = $2.5 \times 10^3$ CFU mL$^{-1}$.

Figure 4. Time-kill curves of *C. krusei* ATCC 6258 and CK4 following exposure to the EE of *C. nardus* and amphotericin-B. Control represents the untreated *Candida* cell. Note: time zero value = $2.5 \times 10^3$ CFU mL$^{-1}$.

Figure 5. Time-kill curves of *C. glabrata* ATCC 2001 and CG3 following exposure to the EE of *C. nardus* and amphotericin-B. Control represents the untreated *Candida* cell. Note: time zero value = $2.5 \times 10^3$ CFU mL$^{-1}$. 
Figure 6. Time-kill curves of *C. tropicalis* ATCC 13801 and CT3 following exposure to the EE of *C. nardus* and amphotericin-B. Control represents the untreated *Candida* cell. Note: time zero value = $2.5 \times 10^3$ CFU mL$^{-1}$.

Figure 7. Time-kill curves of *C. parapsilosis* ATCC 22019 and CP1 following exposure to the EE of *C. nardus* and amphotericin-B. Control represents the untreated *Candida* cell. Note: time zero value = $2.5 \times 10^3$ CFU mL$^{-1}$.

Figure 8. Time-kill curves of *C. orthopsilosis* ATCC 96141 and CO1 following exposure to the EE of *C. nardus* and amphotericin-B. Control represents the untreated *Candida* cell. Note: time zero value = $2.5 \times 10^3$ CFU mL$^{-1}$.
Effect of EE on mature biofilms of Candida species

The results showed that EE inhibited C. albicans, C. krusei, and C. parapsilosis mature biofilms. The concentrations (fifty times the MIC value) of EE able to eradicate mature biofilms of ATCC strains of C. albicans, C. krusei and C. parapsilosis were 25 mg mL\(^{-1}\). For clinical strains of C. krusei (CK4) and C. parapsilosis (CP1) the inhibition concentration was 12.5 mg mL\(^{-1}\) of EE. EE showed no biofilm inhibition (> 6.25 mg mL\(^{-1}\)) against strain of C. albicans (CA3). As this was the first report evaluating EE of C. nardus, these results are of great potential importance in the scientific field.

Biofilms represent a significant impact on public health, especially during establishment of chronic fungal diseases.46 Biofilm formation is an important virulence factor associated with the Candida species, and treatments that prevent biofilms are limited because biofilms have a complex structure composed of polysaccharide extracellular matrix, which limits targeting of antifungal agents into biofilms. Furthermore, extensive communication among cells resulting in production of virulence-related molecules and the presence of a high fungal burden contribute the lack of efficacy of antifungal drugs.45

Natural products have been shown to exhibit anti-biofilm potential. A study by Sangetha et al.47 demonstrated that the methanolic extract of leaves of Cassia spectabilis inhibited C. albicans biofilm formation at 6.25 mg mL\(^{-1}\). However, this extract did not effectively eliminate mature biofilms.

A study performed by Ramos et al.48 showed that the methanolic extract of Syngonanthus nitens was ineffective against mature C. krusei biofilms. To overcome this issue, the authors employed a nanoparticle drug delivery system. Based on these findings, the 50 × MIC value observed in our study suggested a satisfactory inhibition profile.

Cytotoxic evaluation

The IC\(_{50}\) values of EE and FrD are summarized in Table 2. Both EE and FrD exhibited higher IC\(_{50}\) values against HepG2 cells than MRC-5 cells. The differences in responses between the cells could be related to the greater metabolic capacity of HepG2 cells, which mimic the metabolic status of human liver cells. These cells have the ability to retain the activities of various phase I and phase II enzymes which play important roles in elimination and detoxification of these classes of compounds in viva.49

| Cell line | IC\(_{50}\) (µg mL\(^{-1}\)) FrD | Control b |
|-----------|-----------------|-----------|
| HepG-2    | 348.9 ± 12.1    | 135.7 ± 1.9 | > 1000 |
| MRC-5     | 181.3 ± 11.6    | 69.4 ± 4.9  | > 1000 |

*IC\(_{50}\)* concentration required to inhibit 50% of cell growth (average ± standard error (SE)); b dimethylsulfoxide. EE: ethanol extract; FrD: fraction D.

Conclusions

In conclusion, EE from leaves of C. nardus contained compounds that exerted significant antifungal activity. The identified secondary metabolites in EE were phenolic compounds, including C- and di-C-glycosylated flavones, and glycosylated phenylpropanoid derivatives. These metabolites were abundant in FrD, and likely explained the antifungal potency of this fraction. Biological assays showed that EE exhibited activity against several strains of Candida species, including those resistant to fluconazole. Furthermore, EE was able to inhibit the main virulence factors associated with Candida species such as biofilms and C. albicans hyphae formation. In previous study, the essential oil of C. nardus showed superior activity than EE against the main virulence factors of Candida species. However, the EE exhibited lower MIC values than essential oil against planktonic Candida cells.

Table 2. Cytotoxic activity (IC\(_{50}\)) of EE and FrD against MRC-5 and HepG2 cell lines

Supplementary Information

Supplementary data (tables) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Luciani Gaspar de Toledo was responsible for conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, validation, writing-original draft, writing-review and editing; Matheus Aparecido dos Santos Ramos for conceptualization, data curation, investigation, methodology; Elza Maria Castilho for project administration, supervision; Fernando Rogério
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