

## *Talinum paniculatum*: a plant with antifungal potential mitigates fluconazole-induced oxidative damage-mediated growth inhibition of *Candida albicans*

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### SUMMARY

**Aims:** This study investigated the bioactivity of the crude leaf extract (CLE) and fractions hexane (HX) and ethyl acetate (EtOAc) from *Talinum paniculatum* alone and in association with fluconazole (FLC) against reference strain and clinical isolates of FLC-resistant *Candida albicans*. Furthermore, the antioxidant capability, chemical composition of this plant, and the effect's underlying mechanisms were evaluated. **Methods:** The antifungal activity was evaluated using checkerboard assay to establish the minimum inhibitory (MIC) and minimum microbicidal concentrations (MMC). During FLC and plant products challenges, the reactive oxygen species (ROS) generation (hydroxyl radicals [HO•]) were detected in *C. albicans* cells using the membrane-permeable fluorescent probes APF and HPF. High-performance liquid chromatography (HPLC) profile, quantitative analysis of antioxidant compounds, and free radical scavenging activity (DPPH assay) tests were performed. **Results:** The CLE and fractions presented outstanding antifungal activity and selectivity against *C. albicans* cells but had no synergistic effects with FLC. The MIC values for CLE and its fractions against *C. albicans* reference strain were in the order of HX (31.25 µg ml<sup>-1</sup>) < EtOAc (62.5 µg ml<sup>-1</sup>) < CLE (500 µg ml<sup>-1</sup>), and

against FLC-resistant *C. albicans* HX ( $125 \mu\text{g ml}^{-1}$ ) = EtOAc < CLE ( $500 \mu\text{g ml}^{-1}$ ). CLE and its fractions had more potent antifungal activities than FLC against the clinical isolates. Moreover, fungicidal effects for these plant products were demonstrated against FLC-resistant *C. albicans*, which further confirmed an antifungal potential. Conversely, during association, plant products were shown to cause an increase in FLC MIC anywhere from 2- to 16-fold. FLC exposure led to an increase in the steady-state levels of ROS ( $\text{HO}^\bullet$ ) in *C. albicans* cells. Next, we found that the increases in FLC MICs were owing to action of antioxidants containing-CLE and its fractions in preventing FLC-induced ROS-mediated growth inhibition of *C. albicans*. **Conclusion:** *T. paniculatum* can be a source of bioactive compounds with antifungal potential. However, because of the common use of its edible leaf, caution is advised during therapy with FLC (since it can decrease FLC susceptibility).

*Key words:* Antimicrobial resistance, *Candida albicans*, susceptibility, reactive oxygen species, *Talinum paniculatum*, fluconazole.

## RESUMEN

*Talinum paniculatum*: una planta con potencial antifúngico atenúa la inhibición del crecimiento de *Candida albicans* mediada por el daño oxidativo inducido por fluconazol

**Objetivos:** este estudio investigó la bioactividad del extracto de hoja en bruto (EHB) y las fracciones hexano (HX) y acetato de etilo (AcOEt) de *Talinum paniculatum* solo y en asociación con fluconazol (FLC) contra cepas de referencia y aislados clínicos de *Candida albicans* resistente a FLC. Además, evaluó la capacidad antioxidante, la composición química de esta planta y los mecanismos subyacentes del efecto fungicida. **Métodos:** la actividad antifúngica se evaluó mediante microdilución en caldo para establecer las concentraciones inhibitorias mínimas (CIM) y microbicidas mínimas (CMM). Durante el tratamiento con FLC y productos vegetales se detectó la generación de especies reactivas de oxígeno (ERO) (radicales hidroxilo [ $\text{HO}^\bullet$ ]) en células de *C. albicans* utilizando las sondas fluorescentes permeables a la membrana APF y HPF. El perfil de cromatografía líquida de alta resolución (CLAR), el análisis cuantitativo de compuestos antioxidantes y el ensayo DPPH fueron evaluados. **Resultados:** el EHB y las fracciones presentaron una excelente actividad antifúngica y selectividad contra las células de *C. albicans*, pero no tuvieron efectos sinérgicos con FLC. Los valores de CIM para EHB y sus fracciones contra la cepa referencia de *C. albicans* fueron del orden de: HX ( $31,25 \mu\text{g ml}^{-1}$ ) < AcOEt ( $62,5 \mu\text{g ml}^{-1}$ ) < EHB ( $500 \mu\text{g ml}^{-1}$ ), y contra *C. albicans* resistente a FLC: HX ( $125 \mu\text{g ml}^{-1}$ ) = AcOEt < EHB ( $500 \mu\text{g ml}^{-1}$ ). EHB y sus fracciones fueron más potentes antifúngicos que

FLC contra los aislados clínicos. Además, estos productos vegetales tienen efectos fungicidas contra *C. albicans* resistentes a FLC, esto confirmó el potencial antifúngico. Por el contrario, durante la asociación se demostró que los productos vegetales causan un aumento en la CIM de FLC de 2 a 16 veces. La exposición a FLC aumentó los niveles de ERO (HO•) en las células de *C. albicans*. Los aumentos en las CIM de FLC se debieron a la acción de los antioxidantes presentes en EHB y sus fracciones para prevenir la inhibición del crecimiento mediada por ERO inducida por FLC en *C. albicans*. Conclusión: *T. paniculatum* puede ser una fuente de compuestos bioactivos con potencial antifúngico. Sin embargo, debido al uso común de su hoja comestible, se recomienda usarla con precaución durante la terapia con FLC (ya que puede disminuir la susceptibilidad a FLC).

*Palabras clave:* Resistencia a los antimicrobianos, *Candida albicans*, especies reactivas de oxígeno, *Talinum paniculatum*, fluconazole.

## INTRODUCTION

Over the last few decades, the relentless increase of antimicrobial resistance and multi-drug resistance (AMR/MDR) in microorganisms has been observed in low-, middle-, and high-income countries and has resulted in mortality rates in patients who have hospital- or community-associated infections (HAI/CAI) reaching numbers comparable to the pre-antimicrobial era [1-4]. The ever-increasing incidence of AMR/MDR combined with a weak pipeline of new antimicrobial agents launching on the market has created a great threat to the successful management of infectious diseases as well as a major global public health problem [5-7].

Since the early 1980s, AMR has risen to alarming levels in *Candida albicans* (i.e., elevated MICs to azoles and cross-resistance to related antifungal agents) –a frequent pathogen of immunologically compromised individuals, but an even more common commensal fungus from the microbiome of healthy humans–, and the severity of candidiasis caused by antimicrobial-resistant *C. albicans* strains has increased over the years due to clinical interactions, such as the use of corticosteroids, immunosuppressive agents, radiotherapy, and anti-tumoral chemotherapy [1, 2, 8]. Furthermore, the spread of resistance, emergence of highly virulent and pathogenic strains of *C. albicans* that commensally colonize 30-70% of healthy individuals, and formation of biofilms underlie chronic and recurrent infections accordingly have increased the ability of this microorganism to cause disease, further aggravating the issue of AMR/MDR. These factors are, at least partially, responsible for the high invasive fungal infections (IFIs) and mortality rates from *C. albicans* infections [2].

Approximately 400 000 life-threatening infections caused by *C. albicans* are reported per year worldwide. In the United States, from the estimated 46 000 healthcare-associated *C. albicans* infections reported each year, 3400 are caused by Fluconazole-resistant *C. albicans*, with approximately 220 deaths. In this context, regarding level of concern, Fluconazole-resistant *C. albicans* is today recognized by the centers for disease control and prevention (CDC) as a “serious threat” to people’s health, the second major threat into one of three threat categories: urgent, serious, and concerning. In Brazil, the real data are still underestimated, but studies have showed a considerable prevalence of Fluconazole-resistant *C. albicans* infections, and besides the natural process of AMR/MDR, the inadequate antimicrobial use (most from food production) is the single most important factor leading to antibiotic resistance in this country [9-12].

The serious threat of AMR/MDR in *C. albicans* strains represents a current public health problem and poses a burgeoning need for new antifungal agents that can tackle AMR/MDR as well as novel antifungal agent deployment strategies are imperative and urgent, to address the issue of AMR/MDR [6,9-13]. Moreover, new antifungal mechanisms are a field to be explored against the broad antifungal resistance seen in epidemiology. Among the approaches to cope with AMR, plants have been used for medicinal purposes long before recorded history [14]. *Talinum paniculatum* (Jacq.) Gaertner (*Talinaceae* family) is commonly known in Brazil as “Erva-gorda” and “Língua-de-vaca”, or Jewels of Opar and Ginseng Java worldwide. Nowadays, this plant is widely spread throughout the world and all Brazilian territories. Its edible leaves are succulent subshrub and make an excellent addition to salads, among other culinary purposes (a non-conventional edible leaf) [15].

In folk medicine, *T. paniculatum* is used to treat several pathological conditions including headaches, ulcers, and diarrhea; as an emollient for fighting gastrointestinal disorders; and used topically to combat a broad spectrum of wounds and skin infections. Furthermore, it is used to ease digestion, moisten the lungs, as an aphrodisiac, and to promote breast milk production [15, 16]. Studies have demonstrated the phytochemical constitution [17] and several bioactivities of this plant and its metabolites, including antibacterial and antifungal [18], estrogenic [19], antifertility [20], antinociceptive [21], and the induction of uterine contractility [16].

Over the past decade, intense investigation revealed that the action of antibiotics with specific targets into microbial cells can be accompanied by oxidative stress (i.e., mainly via an increase in respiratory chain-dependent reactive oxygen/nitrogen species [ROS/RNS] production), which is considered a common mechanism of antibiotic-mediated cell death (referred to as “unified mechanism of killing”), as those seen during the killing of *C. albicans*, *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium*

*tuberculosis*, and *Pseudomonas aeruginosa* [22-25]. Recently, a great number of antibiotics have been demonstrated to stimulate the production of ROS in microbial cells [22-25]. More specifically, studies conducted by Kobayashi *et al.* [26]; Silva *et al.* [23]; and Mahl *et al.* [22] have shown a participation of ROS in antifungal mechanism of fluconazole (FLC)—the first-line antifungal treatment agent against *C. albicans* infections—and other azoles, resulting in oxidative DNA damage-mediated cell death [9].

Additionally, in recent years, studies have shown that the interaction between plant product and antibiotic can increase the minimum inhibitory concentration (MIC) of some antibiotics, so that their actions could be hampered by antioxidants containing-plant products [27, 28]. This effect could be from a previous decrease in oxidants; thus, compromising the mechanisms of antibiotic-induced ROS/RNS-mediated cell death, such as ROS-induced guanine pool oxidation that leads to double-strand DNA breaks and cell death. This might also have consequences *in vivo*, reducing the pharmacokinetics, efficacy, and even safety of antibiotics and leading to microbial resistance, causing high medical costs during treatment and higher risk of death for patients [27, 28].

In that way, reductive stress caused by antioxidants has been investigate in the non-communicable diseases, for example, cardiovascular disease and cancer (i.e., via down regulation of Nrf2 pathways), and communicable diseases, for example, infectious diseases (i.e., via a decrease in oxidants generated in phagocytic cells) [29, 30]. However, the influence of reductive stress on the susceptibility of microorganisms to antibiotics remains poorly explored. Moreover, to the best of the authors' knowledge, there are no reports about the influence of *T. poaniculatum* on the susceptibility of *C. albicans* to FLC. The elucidation of possible interactions between antioxidants-rich foods and antibiotic is important because of many nutrition products and medicines –including the leaves from *T. paniculatum* that are often used as a green leafy vegetable for human consumption– may interact with antibiotics during medical treatment and modify their action hence having a great impact on clinical practice and patient outcomes.

Based upon the above, we evaluated the antifungal activity of the crude leaf extract (CLE), fractions hexane (HX), and ethyl acetate (EtOAc) from *T. Paniculatum*, alone or in association with the azole antifungal FLC, against reference and FLC-resistant *C. albicans* strains. We also investigated the influence of the CLE, HX, and EtOAc from *T. paniculatum* during the action of FLC against these strains with focus on oxidative/reductive stress. Furthermore, chemical constituents and antioxidant activity of these plant products were checked.

## METHODOLOGY

### Chemicals

Fluconazole (FLC); Roswell Park Memorial Institute (RPMI)-1640 *medium*; 3-(N-morpholine) propanesulfonic acid (MOPS); Ascorbic acid; Benzoic acid ( $\geq 99.5\%$ ); Chlorogenic acid ( $\geq 95.0\%$ ); Caffeic acid ( $\geq 98.0\%$  HPLC); Ferulic acid ( $\geq 98.0\%$ ); 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) were purchased from Sigma-Aldrich, St. Louis, MO, USA. 2-[6-(4'-Hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (HPF); and 2-[6-(4'-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (3'-p-(aminophenyl) fluorescein, APF) were acquired from Life Technologies. Nutriente Agar (Difco); Sabouraud Dextrose Agar (SDA, BD Difco™); Agar/broth Brain Heart Infusion (BHI, HiMedia Laboratories); 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetra-zolium bromide (MTT Ultrapure, USB Corp., Cleveland, OH); Dimethyl sulfoxide (DMSO, Neon Comercial); Tryptone Soy Agar (TSA, Oxoid Ltd, London, UK); Sodium chloride 0.9% (145 mM NaCl); Phosphate-buffered saline (PBS, pH 7.4): NaCl (120 mM),  $\text{KH}_2\text{PO}_4$  (1.7 mM),  $\text{Na}_2\text{HPO}_4$  (8.3 mM) e KCl (5 mM). Others used chemicals and solvents were of analytical grade. The reagents were sterilized in an autoclave (whenever possible) or by filtration (Millipore Corporation, hydrophilic Durapore PVDF, 0.22  $\mu\text{m}$ ,  $\emptyset$  47 mm).

### Ethics statement

This study did not involve any endangered or protected species and no specific permits were required for the described studies. Botanical material from *T. paniculatum* was collected in an area, with access permitted to researchers.

*Candida albicans* from human subjects were obtained in a previous approved study (FOP/UNICAMP Institution Review Board approved protocol #082/2014) after obtaining an informed consent, in compliance with the relevant laws, institutional guidelines, and ethical standards of the Declaration of Helsinki.

### Plant material

#### *Provenience*

The leaves from *T. paniculatum* were collected in the mornings of June, near the town of Fama, MG, Brazil (Geographic coordinates obtained from GPS observations: 21° 24' 53.4" S; 45° 52' 15.8" W). The climate of this region is classified as humid temperate, with a hot summer and a dry winter (type Cwa in the Köppen classification). A voucher specimen was deposited in the UALF Herbarium at the Federal University of Alfenas (UNIFAL-MG) after proper taxonomic identification (sample #2338).

**Crude leaf extract (CLE) and fractions (EtOAc and HX) from *T. paniculatum***

The dried leaf powder (200 g) from *T. paniculatum* was percolated (with an alcohol to water ratio of 7:3) at 1.0 mL/min/kg. After fluid extraction, the fluid was placed in a rotary evaporator under reduced pressure and at a temperature of 45 °C to completely remove the alcohol. The extract was then lyophilized to completely remove water and obtain the dry extract, hereafter referred to as CLE (17.0% yield). To obtain the fractions, the dried extract (CLE) was subjected to a liquid-liquid partition with hexane (1:1, v/v [4×]) or ethyl acetate (1:1, v/v [6×]), yielding hexane (HX) or ethyl acetate (EtOAc) fractions, and a final residue termed the aqueous fraction (this last not used in this study) [18]. At time of their use, the CLE or fractions were solubilized in DMSO (at the concentrations used for solubilization in this study, DMSO does not display antifungal, cytotoxic, antioxidant, or other activities here evaluated) and adjusted at used concentrations.

**Antifungal activity**

**Strains**

*C. albicans* (ATCC® 90028 and ATCC 10231) used in this study were from the American Type Culture Collection (ATCC, Manassas, VA). Antibiotic-resistant *C. albicans* were isolated from clinical source (patients with orofacial cleft), as shown in table 1 and identified as previously reported [31]. AMR in the isolates were interpreted for FLC against *C. albicans* according to document M27A3 (CLSI, 2008) of the Clinical and Laboratory Standards Institute (CLSI), where FLC MIC ≤ 16 µg ml<sup>-1</sup>= sensitive, MIC 16–32 µg ml<sup>-1</sup>=intermediate, and MIC ≥ 64 µg ml<sup>-1</sup>= resistant [32].

**Table 1.** Used microorganisms, clinical sources, identification, and code.

Microorganism	Code	Source	Identification methods	Primers (PCR)*
<i>C. albicans</i>	Sample 1	Clinical isolates**	PCR***/MEE	OK3 (forward 5' - ATG TAT TCA TTA ATC AAA TCA - 3'); OK4 (reverse 5' - ATT TAA AAA ACA ACG GAC AT - 3')
<i>C. albicans</i>	Sample 2	Clinical isolates	PCR/MEE Microsatellite loci****	
<b>Microsatellite loci****</b>			<b>Primers F (forward) e R (reverse)</b>	
<i>CAI</i> (noncoding region) (CAA) <sub>32</sub>			5' ATG CCA TTG AGT GGA ATT GG (F) 5' AGT GGC TTG TGT TGG GTT TT (R)	
<i>CDC3</i> (cell division cycle protein) (AGTA) <sub>8</sub>			5' CAG ATG ATT TTT TGT ATG AGA AGA A (F) 5' CAG TCA CAA GAT TAA AAT GTT CAA G (R)	

(Continued)

**Table 1.** Used microorganisms, clinical sources, identification, and code.

Microorganism	Code	Source	Identification methods	Primers (PCR)*
<i>CPH1</i> (Ste 12-like transcription factor) (CAA) <sub>5</sub> (CAG) <sub>1</sub> (CAA) <sub>3</sub> ... (CAA) <sub>5</sub> (CAG) <sub>5</sub> (CAA) <sub>3</sub>				5' GCC ATG GGA TAT CAA AGC (F) 5' CTT GGT AAT GCC ACC GCC (R)
<i>ERK1</i> (extracellular-signal-regulated kinase) (CAGGCT) <sub>a</sub> (CAAGCT) <sub>b</sub> ...(CAA) <sub>c</sub> ...(GCCGCA) d...(CTT) <sub>e</sub>				5' CGA CCA CGT CAT CAA TAC AAA TCG (F) 5' CGT TGA ATG AAA CTT GAC GAG GGG (R)
<i>KRE6</i> (1,6-glucan synthesis) (AAT) <sub>n</sub>				5' CAA GCT TAT AGT GGC TAC TA (F) 5' - CCA ACA CTG ATA CAT CTC G (R)
<i>LOC4</i> (anonymous locus) (GAA) <sub>6</sub>				5' GTA ATG ATT ACG GCA ATG AC (F) 5' AGA ACG ACG TGT ACT ATT GG (R)
<i>MNT2</i> (mannosyltransferase) (CTT) <sub>5</sub>				5' GCC AAT ACT GGA AAC TGT GCC (F) 5' CGG GCT AAA GTG ACA AAT GTG GC (R)
<i>ZNF1</i> (zinc finger transcription factor) (CAA) <sub>8-11</sub>				5' CCA TTA CAG CTG AAC CAG CGA GGG (F) 5' CGC TAG GTA ACC TAC AGA TTG TGG C (R)

MEE: Multilocus Enzyme Electrophoresis; PCR: Polymerase Chain Reaction; \*These species-specific primers for *C. albicans* were used to amplify a DNA fragment of approximately 1,644 bp (PHR1 gene); \*\*Origin: from children with cleft lip and palate; \*\*\*Conditions: Amplifications were conducted using an initial program for DNA denaturation at 95 °C for 5 minutes, followed by 30 cycles at 95 °C for 20 seconds (denaturation), 50 °C for 1 minute (annealing), and 72 °C for 1½ minute (extension). The final cycle was conducted at 72 °C for 10 minutes for the final extension; \*\*\*\*(i) CAI: initial program for DNA denaturation at 95 °C for 5 minutes, followed by 30 cycles at 94 °C for ½ minute (denaturation), 60 °C for ½ minute (annealing), and 72 °C for 1 minute (extension). The final cycle was conducted at 72 °C for 7 minutes for the final extension, (ii) CDC3: initial program for DNA denaturation at 95 °C for 5 minutes, followed by 35 cycles at 95 °C for 1 minute (denaturation), 52 °C for 1 minute (annealing) and 72 °C for 1 minute (extension). The final cycle was conducted at 72 °C for 7 minutes for the final extension, (iii) ERK1, KRE6, LOC4 and ZNF1: initial program for DNA denaturation at 95 °C for 5 minutes, followed by 35 cycles at 95 °C for 1 minute (denaturation), 55 °C for 1 minute (annealing) and 72 °C for 1 minute (extension). The final cycle was conducted at 72 °C for 5 minutes for the final extension, (iv) CPH1 and MNT2: initial program for DNA denaturation at 94 °C for 5 minutes, followed by 40 cycles at 94 °C for 1 minute (denaturation), 50 °C for 1 minute (annealing) and 72 °C for 1 minute (extension). The final cycle was conducted at 72 °C for 5 minutes for the final extension [31].

### ***Minimum inhibitory concentration and determination of synergistic action***

Both plant product (CLE, HX and EtOAc from *T. paniculatum*) and FLC were tested to determine the MIC values against the two resistant *C. albicans* strains, as well as against the reference strain of *C. albicans*, using the broth checkerboard microdilution assay, following the CLSI document M27A3 (CLSI, 2008) [32]. The tests were performed on 96-well microplates (flat-bottom, Corning Inc., NY) containing 100 mL of RPMI-1640 broth (plus phenol red, 1.04% [w/v] of glutamine, 2% [w/v] of glucose, and without bicarbonate) per well, buffered with 165 mM MOPS (pH 7.0 at 25 °C). Afterwards, the CLE or the fractions (EtOAc or HX) were diluted into the wells (the concentration range used was 4000-1.95  $\mu\text{g ml}^{-1}$  for the CLE, and 500-0.244  $\mu\text{g ml}^{-1}$  for fractions), either alone or coupled with FLC (final concentrations ranged from 32000 to 15.6  $\mu\text{g ml}^{-1}$ ). Microbial suspensions (cultures during the exponential growth phase after an overnight incubation) were centrifuged (900  $\times g$  for 6 min, 5810R Centrifuge, Eppendorf, NY, USA) and the pellet diluted in sodium chloride 0.9%, and then spectrophotometrically adjusted at  $1 \times 10^4$  colony-forming units (CFUs)/mL<sup>-1</sup> (OD<sub>530</sub> = 0.1 =  $5 \times 10^6$  CFUs/mL<sup>-1</sup>, further diluted). Afterwards, 10  $\mu\text{L}$  of inoculum were added to the wells.

The plates were then incubated at 37 °C for 48 hours. After the incubation period, readings were performed visually as previously determined [18], wherein the presence of turbidity in the wells was considered indicative of microbial growth, further confirmed using the specific dye TTC to evaluate the metabolic activity of *C. albicans* cells. The MIC<sub>99.9</sub> of the CLE, fractions (EtOAc or HX) or FLC alone, and all isoeffective combinations (plant product plus FLC) were defined when the growth of the microorganism was inhibited at the lowest concentration relative to the untreated control. The growth control was composed of 100  $\mu\text{L}$  of RPMI-1640 (as above specified) and 10  $\mu\text{L}$  of inoculum. The extract control was composed of 100  $\mu\text{L}$  RPMI-1640 and 100  $\mu\text{L}$  of the CLE or fraction (EtOAc or HX) and the sterility control contained only 100  $\mu\text{L}$  of RPMI-1640.

### ***Minimum microbicidal concentration***

The minimum microbicidal concentration (MMC) was defined as the lowest concentration of antibiotic/extract/fractions that killed 99.9% of the original inoculums from each well where growth inhibition occurred in the analysis of MIC/Synergism. For each strain of *C. albicans*, MBC values were determined by removing 100  $\mu\text{L}$  of microbial suspension from each well demonstrating no microbial growth and inoculating them with three serial dilutions of 1:10 onto nutrient agar in plates (plus 2% [w/v] of glucose; Petri plates, 90  $\times$  15 mm) and then maintaining them at room temperature for complete absorption. After that, the plates were incubated at 37 °C for 48 hours.

Finally, colony counts were performed to determine which concentrations presented microbicidal (fungicidal) or microbistatic (fungistatic) action.

### **Evaluation of the amount of intracellularly-generated oxidants in *C. albicans***

The quantification of hydroxyl radicals ( $\text{HO}\cdot$ ) generated under the different treatments was performed using the probe APF (highly specific toward  $\text{HO}\cdot$ ) and HPF [33]. First, reference strain of *C. albicans* (ATCC 10231) was grown overnight on BHI. Next, microbial suspensions (in exponential-phase growth) were centrifuged ( $900 \times g$  for 6 min) and the pellet diluted in sodium chloride 0.9% and spectrophotometrically adjusted. Afterwards, *C. albicans* cells ( $1 \times 10^4$  CFUs/mL<sup>-1</sup>) were added on wells of a 96-well microplate and previously treated (for 10 min) with CLE ( $100 \mu\text{g mL}^{-1}$ ) or fractions (EtOAc [ $50 \mu\text{g mL}^{-1}$ ] or HX [ $50 \mu\text{g mL}^{-1}$ ]). After that, FLC treatments ( $10 \mu\text{g mL}^{-1}$ ) were performed. The control group was comprised of untreated microorganisms. Finally, *C. albicans* cells were loaded with the membrane-permeable fluorescent probes APF or HPF ( $25 \mu\text{M}$ , dissolved in DMSO) for 30 min in the dark at  $35^\circ\text{C}$  and then immediately readings took place every 30 minutes over the next 18 hours, using a Varian Cary Eclipse *spectrofluorometer* ( $\lambda_{\text{excitation}}=500 \text{ nm}$ ;  $\lambda_{\text{emission}}=520 \text{ nm}$ ). The results were recorded as arbitrary fluorescent units (AFU) and the fluorescence intensity of the probe is proportional to the amount of ROS ( $\text{HO}\cdot$ ).

### **Cell-free assays**

Antioxidant activity of the CLE and fractions from *T. paniculatum*.

The free radical scavenging ability of the CLE and fractions was measured using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay [34]. The results were reported in percentage (%) of DPPH $\cdot$  scavenging activity inhibition.

### **Quantitative analysis and Chemical characterization of *T. paniculatum***

#### *Quantitative analysis*

The Folin Ciocalteu reagent was used to determine the total polyphenols content of the CLE and fractions according to Fattahi *et al.* [34]. As a standard, gallic acid was used. From the calibration curve, total polyphenol content was expressed in terms of gallic acid equivalent per gram of sample (mg/g).

#### *Chemical characterization of *T. paniculatum**

High-performance liquid chromatography (HPLC) was performed for analysis of the CLE from *T. paniculatum* using a Shimadzu UFLC 20A CLC-ODS (250-4.6 mm, C18 column and  $5 \mu\text{m}$  particle sizes). Mobile phases were composed of (A) 0.5%

aqueous acetic acid and (B) 0.5% acetic acid in methanol. It was used the mobile phases (A:B) for separation with a linear gradient from 0 to 60 minutes (90:10 to 0:100), solvent flow rate of 1.0 ml/min, and an injection volume of 25  $\mu$ L at a concentration of 1 mg/ml. We used the photodiode array detection (DAD) with UV light at 268 nm and the LC solution software (Shimadzu) for data collection. Ascorbic acid, benzoic acid, chlorogenic acid, caffeic acid, and ferulic acid were used as standards.

### Data analysis

Results were from three replicates from three independent experiments and values are presented as mean  $\pm$  SD. To assess the interactions between the CLE, HX, or EtOAc and FLC, the data obtained from the checkerboard microdilution assays were analyzed by a nonparametric model based on the no-interaction theory known as the Loewe additivity model (LA). Using the LA-based model, the nonparametric approach of the model-fractional inhibitory concentration index (FICI) was calculated as follows:  $FICA = MIC_{AB}/MIC_A$  and  $FICB = MIC_{BA}/MIC_B$ , where  $MIC_A$  and  $MIC_B$  are the MICs of samples A (FLC) and B (extract or fraction) when acting alone, and  $MIC_{AB}$  and  $MIC_{BA}$  are the MICs of samples A and B when acting in combination, respectively; thus, the FICI values were expressed as follows:  $FICA + FICB$ . "Synergy" was defined as an  $FICI \leq 0.5$ , while "antagonism" was defined as an  $FICI$  value  $> 4.0$ . A FICI between 0.5 and 1.25 was considered as "Additive effect", while between 1.25 and 4 was considered to have "No interaction" [35-37]. According to the ratio of MMC/MIC, we reported the type of antifungal action displayed by the sample. If the ratio of  $MMC/MIC = 1$  or 2, the effect was considered fungicidal, but if the ratio of  $MMC/MIC = 4$  or 16, the effect was defined as fungistatic [38]. The Selectivity Index (SI) was calculated as follows:  $SI = CC_{90}/MIC_{99.9}$ , being  $CC_{90}$  adopted from our previous study [18]. The significance of difference was analyzed by one-way ANOVA and a Tukey post-test (BioEstat 5.0, Belém, Pará, Brazil, 2007). Significance was accepted at  $p < 0.05$  ( $\alpha = 5\%$ ), unless indicated otherwise. Structural elucidation of compounds from *T. paniculatum* was performed by interpreting the second mass spectra order upon the fragmentation pattern assumption. Structures depicted in this study were constructed on ACD/Labs (Advanced Chemistry Development Inc., version 6.0).

## RESULTS

### MIC and MBC values for the CLE, EtOAc, and HX from *T. paniculatum*

As observed in **table 2**, high MIC values were observed with FLC measured against the isolates, in which both isolates of *C. albicans* were resistant to this antifungal agent ( $MIC \geq 64 \mu\text{g ml}^{-1}$ ). The MIC and MMC values for the CLE, EtOAc, HX, and FLC,

measured against the reference strains of *C. albicans* and FLC-resistant *C. albicans* strains are presented in **table 2**. It was found that for FLC-resistant *C. albicans*, the MIC of both EtOAc and HX was 150  $\mu\text{g ml}^{-1}$ , whereas the MIC against the reference strain was 31.5  $\mu\text{g ml}^{-1}$  for HX and 62.5  $\mu\text{g ml}^{-1}$  for EtOAc. The CLE presented MIC value of 500  $\mu\text{g ml}^{-1}$  against all of *C. albicans* strains. The EtOAc was as effective as FLC against the reference strain of *C. albicans*, whereas this fraction was more effective ( $< \text{MIC}$ ) than FLC against the two FLC-resistant *C. albicans* (clinical isolates). HX fraction had lower MIC values than FLC against all of *C. albicans* strains. The CLE was more effective than FLC against the two antibiotic-resistant *C. albicans*, but not against the reference strain of *C. albicans*.

### **MIC and MBC values for the associations between plant product (CLE, EtOAc, and HX) and FLC, and FICI from the associations**

The interactions from associations between plant products (CLE, EtOAc, or HX from *T. Paniculatum*) and FLC against *C. albicans*, were investigated. As demonstrated in **table 2**, treatment of *C. albicans* using the CLE, EtOAc, or HX alone, resulted in outstanding MIC values (the lowest MIC values). However, when the mixture of extract/fraction and antifungal agent was assayed to determine the type of interaction between the two compounds, FLC MICs increased 2- to 16-fold (table 3), whereas some fractions MICs were shown to be decreased as an effect experienced during these associations. Taken together, data from tables 2 and 3 were interpreted as the FICI values (table 3) in which there was no synergy for any of associations between plant product and antifungal agent. The observed effects for the associations were: “additive effect” (2 associations), “no interaction” (5 associations), or “antagonism” (5 associations).

### **Antifungal effects/actions for the crude leaf extract (CLE) and fractions**

In table 2, it is also shown that the CLE displayed fungicidal action against antibiotic-resistant *C. albicans* (sample 1). There was a fungicidal action from both EtOAc and HX against antibiotic-resistant *C. albicans*, but only HX presented this effect against the two samples (1 and 2), whereas the effect of EtOAc against sample 1 of the antibiotic-resistant *C. albicans* and both fractions against the reference strain of *C. albicans* were fungistatic. Therefore, the EtOAc and HX fractions not only presented the lowest MIC values, but also presented fungicidal action.

### **Selectivity index (SI) for the CLE and fractions from *Talinum paniculatum***

The results of the SI are presented in table 4 and the best ones were 17.7 and 4.42 (HX against the reference strain of *C. albicans*), 4.85 (EtOAc against the reference strain of *C. albicans*), and 2.76 (CLE against all strains of *C. albicans*).

**Table 2.** Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) for the alone crude leaf extract (CLE) and fractions from *Talinum paniculatum* and fluconazole against *Candida albicans* and the types of antimicrobial effects.

Microorganisms	<i>C. albicans</i> (ATCC 90028)						<i>C. albicans</i> (sample 1)				<i>C. albicans</i> (sample 2)		
	MIC ( $\mu\text{gml}^{-1}$ )	MMC ( $\mu\text{gml}^{-1}$ )	MMC/MIC	Antimicrobial Effect	MIC ( $\mu\text{gml}^{-1}$ )	MMC ( $\mu\text{gml}^{-1}$ )	MMC/MIC	Antimicrobial Effect	MIC ( $\mu\text{gml}^{-1}$ )	MMC ( $\mu\text{gml}^{-1}$ )	MMC/MIC	Antimicrobial Effect	
CLE	500	N	N/A	N/A	500	1000	2	Fungicidal	500	1000	2	Fungicidal	
EtOAc	62.5	250	4	Fungistatic	125	125	1	Fungicidal	125	250	2	Fungicidal	
HX	31.25	250	8	Fungistatic	125	500	4	Fungistatic	125	125	1	Fungicidal	
Fluconazole	62.5	250	4	Fungistatic	2000	8000	4	Fungistatic	2000	16000	8	Fungistatic	

Antimicrobial Action:  Fungicidal  
 Fungistatic

For plants products: MIC up to  $100 \mu\text{gml}^{-1}$  = promising inhibitory potential, MIC between 100 and  $625 \mu\text{gml}^{-1}$  = moderate inhibitory activity [18]. Fractions from the CLE of *Talinum paniculatum*; EtOAc = Ethyl acetate fraction and HX = Hexane fraction; *C. albicans*: samples 1 and 2 are clinical isolates from children with cleft lip and palate; *C. albicans*:  $5 \times 10^4 \text{ CFU/ml}^{-1}$ ; Antimicrobial resistance (AMR) classification: Fluconazole MIC  $\leq 8 \mu\text{gml}^{-1}$  = Sensitive, MIC  $16\text{-}32 \mu\text{gml}^{-1}$  = Intermediate, MIC  $\geq 64 \mu\text{gml}^{-1}$  = Resistant [32]. *In vitro* phenotypic “tolerance” has also been defined as an MBC that is  $\geq 32$  times the MIC; Ratio MMC/MIC = 1 or 2  $\rightarrow$  Effect was considered as microbicidal; Ratio MMC/MIC = 4 or 16  $\rightarrow$  Effect was defined as microbistatic according to Konaté *et al.*, [38]. N: Inhibition of microbial growth was not observed at maximum concentration ( $4000 \mu\text{gml}^{-1}$ ); N/A: Not applicable due to absence as for the inhibition of microbial growth at maximum concentration.

**Table 3.** Results for the contribution of each compound or plant product in the final MIC value during the association between (1) plant product (Crude leaf extract [CLE], EtOAc, or HX) from *Talinum paniculatum* and (2) antibiotic (Fluconazole) against *Candida albicans*, correspondent fractional inhibitory concentration index (FICI), and interpretation of the FICIs from the associations with the resulting interaction type.

Association	<i>C. albicans</i> (ATCC 90028)				<i>C. albicans</i> (sample 1)				<i>C. albicans</i> (sample 2)			
	Concentration ( $\mu\text{g ml}^{-1}$ )*		FICI	Interpretation	Concentration ( $\mu\text{g ml}^{-1}$ )		FICI	Interpretation	Concentration ( $\mu\text{g ml}^{-1}$ )		FICI	Interpretation
	(1)	(2)			(1)	(2)			(1)	(2)		
(1) CLE + (2) Fluconazole	625	1000	>4	Antagonism	1000	16000	>4	Antagonism	1000	16000	>4	Antagonism
(1) EtOAc + (2) Fluconazole	3.9	250	>4	Antagonism	62.5	4000	2.5	No interaction	31.25	2000	1.25	Additive effect
(1) HX + (2) Fluconazole	1.9	125	2	No interaction	62.5	4000	2.5	No interaction	62.5	4000	2.5	No interaction
(1) EtOAc + (2) HX	1000	1000	1	Additive effect	125	125	2	No interaction	62.5	62.5	1	Additive effect

Interaction index interpretation:



\*Concentration of each substance in the associations between the (1) crude leaf extract (CLE)/fraction (EtOAc, or HX) from *Talinum paniculatum* and (2) antibiotic (Fluconazole) or (1) fraction and (2) fraction in which no microbial growth was observed; Fractions from the CLE of *Talinum paniculatum*; EtOAc = Ethyl acetate fraction and HX = Hexane fraction; FICI data interpretation from the interactions/associations: 'synergy' (FICI  $\leq 0.5$ ), 'additive effect' (FICI > 0.5-1.25), 'no interaction' (FICI > 1.25-4.0), and 'antagonism' (FICI > 4.0) [35-37].

### Chemical characterization, antioxidant property, and total polyphenol content of *T. paniculatum*

Here, we identified compounds likely to be related to the bioactivities. To support the increase in MIC values of FLC in the association with plant products, table 5 shows the antioxidant activity and the total polyphenols content of the CLE and fractions. EtOAc and HX fractions presented higher polyphenols contents and the best results as for antioxidant activity. Table 6 shows some compounds identified by HPLC (retention time in minutes, compared to standards) and illustrates the proposed structures. Figure 1 presents the chromatogram of the CLE, which renders the identified structures drawn on it (compounds also depicted in table 6).

**Table 4.** Selectivity Index (SI) for the crude leaf extract (CLE) and fractions from *Talinum paniculatum*.

Extract/ Fraction	CLE		EtOAc		HX	
Sample Results	MIC	SI	MIC	SI	MIC	SI
<i>C. albicans</i> (ATCC 90028)	500	2.76	62.5	4.85	31.25	17.7
<i>C. albicans</i> (sample 1)	500	2.76	125	2.42	125	4.42
<i>C. albicans</i> (sample 2)	500	2.76	125	2.42	125	4.42

SI:=  $CC_{90}/MIC_{99}$ ; MIC:  $\mu\text{gml}^{-1}$ ; Fractions from the CLE of *Talinum paniculatum*; EtOAc= Ethyl acetate fraction and HX= Hexane fraction;  $CC_{90}$  data from our previous paper [18].

**Table 5.** Polyphenols content and antioxidant activity for the crude leaf extract (CLE) and fractions from *Talinum paniculatum*.

Extract/Fraction	CLE	EtOAc	HX
<b>Polyphenols content</b>			
Gallic acid equivalent <sup>1</sup>	20.38 <sup>a</sup>	56 <sup>b</sup>	52 <sup>b</sup>
<b>Antioxidant activity</b>			
DPPH• radical scavenging activity (%)	31.93 <sup>a</sup>	77.5 <sup>c</sup>	47.98 <sup>b</sup>
Ascorbic acid (90.45)			
BHT (63.8)			

Fractions from the CLE of *Talinum paniculatum*; EtOAc= Ethyl acetate fraction and HX= Hexane fraction; <sup>1</sup>gallic acid equivalent per gram of sample (mg/g); <sup>abc</sup>Means followed by the same letter (row) do not differ statistically by the Tukey's test ( $\alpha= 5\%$ ).

**Table 6.** Chemical compounds found in the crude leaf extract (CLE) and fractions from *Talium paniculatum*.

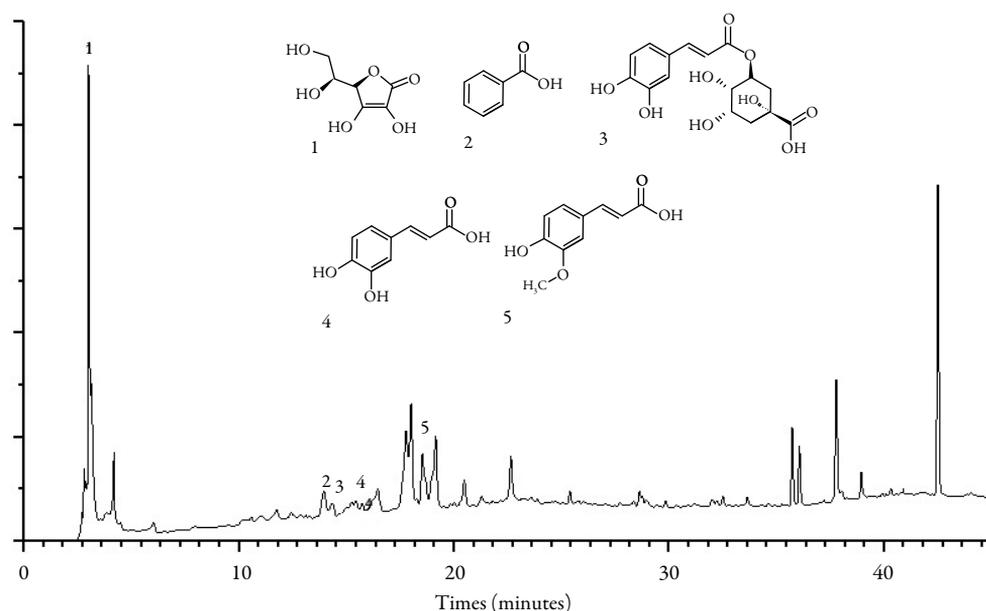
Compound	Sample	HPLC Retention time (min)	MS** <i>m/z</i> (m <sup>2</sup> / <i>z</i> )	MS** <i>m/z</i> (m <sup>2</sup> / <i>z</i> ) (second order mass spectra)	Activities/interactions (references)	Structure
Campesterol	CLE: + EtOAc: + HX: +	----	401	207, 147, and 121	Antifungal [39] Antibacterial [39] Antioxidant [40] Antibiotic interaction: ----	
Stigmasterol	CLE: + EtOAc: + HX: +	----	413	207 and 269	Antifungal [39] Antibacterial [39] Antioxidant [40] Antibiotic interaction: ----	
Sitosterol	CLE: + EtOAc: + HX: +	----	415 (M+H)	223, 267, and 355	Antifungal [39] Antibacterial [39] Antioxidant [40] Antibiotic interaction: ----	
Ascorbic acid	CLE: + EtOAc: ---- HX: ----	3.2	176	----	Antifungal no activity <sup>b</sup> , [41] Antibacterial [42,43] Antioxidant [41] Antibiotic interaction [44]	
Benzoic acid	CLE: + EtOAc: ---- HX: ----	14.5	122	----	Antifungal [45] Antibacterial [46] Antioxidant [47] Antibiotic interaction [48, 49]	

(*Continúa*)

**Table 6.** Chemical compounds found in the crude leaf extract (CLE) and fractions from *Talinum paniculatum*.

Compound	Sample	HPLC Retention time (min)	MS** <i>m/z</i> (m <sup>2</sup> /z)	MS** <i>m/z</i> (second order mass spectra)	Activities/interactions (references)	Structure
Chlorogenic acid <sup>c</sup>	CLE: + EtOAc: ---- HX: ----	15	354	----	Antifungal [50] Antibacterial [51,52] Antioxidant [53] Antibiotic interaction [54]	
Caffeic acid	CLE: + EtOAc: ---- HX: ----	16.5	180	----	Antifungal [55] Antibacterial [56-57] Antioxidant [53, 57] Antibiotic interaction [49]	
Ferulic acid	CLE: + EtOAc: ---- HX: ----	19	194	----	Antifungal [58] Antibacterial [52, 59] Antioxidant [60] Antibiotic interaction [54]	

+: presence of the compound; ----: not evaluated; \* mixture of sterols (Campesterol, Stigmasterol, and Sirosterol); <sup>b</sup> up to 250 µg ml<sup>-1</sup>; <sup>c</sup> MIC of 80 µg ml<sup>-1</sup> against *C. albicans*; \* Interaction between polyphenolic compounds and antibiotics has been reported. Fractions from the CLE of *Talinum paniculatum*; EtOAc= Ethyl acetate fraction and HX= Hexane fraction. HPLC: High performance liquid chromatography; MS: Mass spectrometry; data from our previous paper [18].



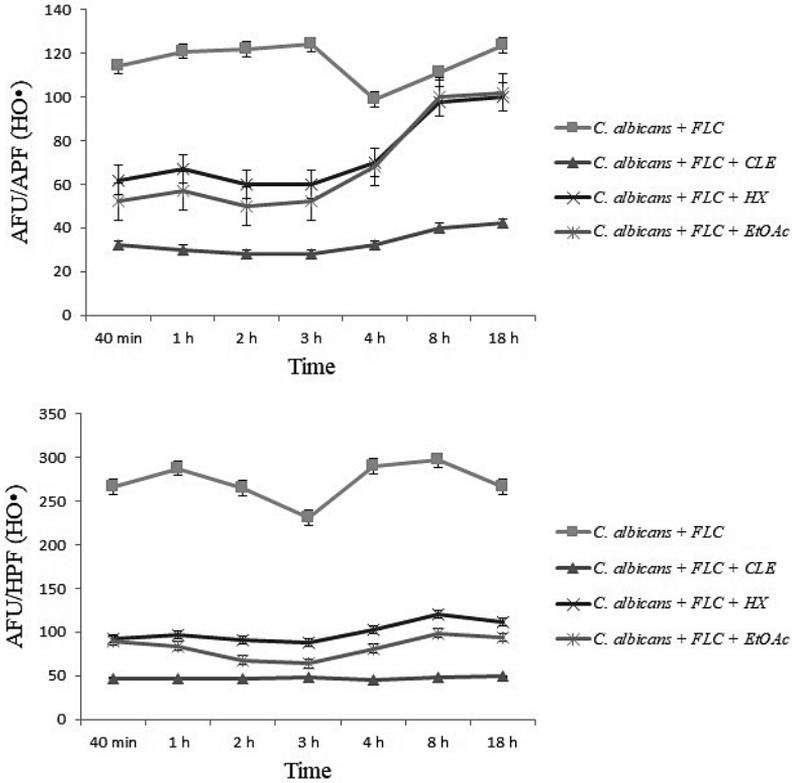
**Figure 1.** High performance liquid chromatography (HPLC) of the crude leaf extract (CLE) from *Talinum paniculatum*. Based on the peaks at each retention time (minutes) for the sample, related to the used standards, the separation permitted the identification of the following structures: 1: Ascorbic acid, Molar mass (MM)= 176.12 g.mol<sup>-1</sup>; 2: Benzoic acid, MM= 122.12 g.mol<sup>-1</sup>; 3: Chlorogenic acid, MM= 354.31 g.mol<sup>-1</sup>; 4: Caffeic acid, MM= 180.16 g.mol<sup>-1</sup>; 5: Ferulic acid, MM= 194.18 g.mol<sup>-1</sup>.

### Influence of the CLE, EtOAc, and HX from *T. paniculatum* upon oxidants in *C. albicans*

To explain the increase in MIC values of FLC, when in association with plant products, we performed tests to evaluate the influence of the CLE, EtOAc, and HX from *T. paniculatum* on the levels of oxidants (HO<sup>•</sup>) generated by *C. albicans* cells. The results are shown in figure 2. Increased ROS levels in *C. albicans* cells after FLC exposure was observed (over 18 h), as part of the antifungal action. On the other hand, decreased ROS levels were constated over the 18 h of exposure to FLC, when a previous treatment with plant product (CLE, EtOAc, or HX) was performed. CLE induced the greatest decrease in ROS levels, significant relative to *C. albicans* cells exposed to FLC, *per se*.

## DISCUSSION

In this study, we demonstrated that the CLE, EtOAc, and HX from *T. paniculatum* present outstanding antifungal activity against *C. albicans*, with “moderating” or “promising” inhibitory potential [18]. The CLE and its fractions (EtOAc and



**Figure 2.** Levels of hydroxyl radicals (HO•) in *Candida albicans* cells generated by alone fluconazole (FLC) or during the associations with plant products. CLE: crude leaf extract from *Talinum paniculatum*; EtOAc= Ethyl acetate fraction and HX= Hexane fraction. AFU: arbitrary fluorescent units.

HX) promoted growth inhibition of the reference strain of *C. albicans* (fungistatic effect). Against FLC-resistant *C. albicans*, the cell death was the principal outcome of the treatments (fungicidal effect). With focus on a selectivity of *T. paniculatum* against *C. albicans* cells, outstanding SI values were observed for the CLE, EtOAc, and HX against both clinical isolates of FLC-resistant *C. albicans* and reference strain of *C. albicans*. A previous work has shown notable SI values of *T. paniculatum* toward *C. albicans* cells (ATCC 10231) and other microorganisms [18].

The pathogenic potential (overgrowth) of *C. albicans* arises after a break in homeostasis and depends on factors of both the human host, for example, immunosuppression, and the microorganism, for example, virulence of the strain. Thus, in these cases, the invasion of *C. albicans* into vital sites of immunocompromised patients causes the so-called IFI (sometimes life-threatening infections) and accounts for the high mortality rates

from infectious diseases caused by this fungus [61, 62]. Here, the clinical isolates of *C. albicans* presented resistance to FLC, which is commonly used to treat *C. albicans* infections. The LE, HX, and EtOAc fractions from *T. paniculatum* presented significant inhibitory activity against clinical isolates of *C. albicans*. Moreover, the activity of terpenes, sterols (compounds found in *T. paniculatum*), and plant products with high terpenes/sterols contents were also demonstrated against *Candida* species [18, 63].

*In vivo*, even on antibiotic action, the killing of pathogens in humans requires a competent immune system. The immunocompetent host is usually far better equipped to eliminate *C. albicans* than an immunosuppressed host. Therefore, it is especially desirable to have a truly microbicidal drug—one that absolutely kills the microorganisms—as a treatment option for immunosuppressed patients [64]. In this context, regarding the type of antifungal effect expected for the CLE and fractions from *T. paniculatum*, as seen here, and bearing in mind the clinical considerations above mentioned, the antimicrobial agents with fungicidal action are preferred to with fungistatic action.

Interestingly, the MIC and MBC values may vary for the actions of the CLE, EtOAc, and HX, as well as for FLC, as consequences of different AMR profiles of *C. albicans*, used in this study. Furthermore, differences between chemical compositions of the fractions or CLE (i.e., in part, from the differences in solvents used to extraction) and the association with antifungal may explain the effects found in this study, since the concentration of metabolites produced may produce a combination of antimicrobial effects or inactivation of FLC [65].

The different compounds in *T. paniculatum*, here evidenced by HPLC, explain the observed bioactivities, including the effects when there were associations between plant product and antifungal. As examples, chlorogenic acid, benzoic acid, caffeic acid, and ferulic acid, and phytosterols display antifungal activity [45, 46, 50-53, 55-59]. In contrast, isolated ascorbic acid does not have antifungal activity up to 250  $\mu\text{g ml}^{-1}$ , as reported by Khalil *et al.* [41], but it displays outstanding antioxidant activity. These factors may, at least in part, contribute with the observed decrease in FLC MIC since ascorbic acid can have an outstanding antioxidant and hence it prevents FLC-induced oxidative damage. Apart from the phenolic content, other compounds present in *T. paniculatum* such as phytosterols take part in the redox control [40, 66, 67].

In this study we demonstrated for the CLE and fractions from *T. paniculatum* the presence of some compounds with outstanding antioxidant activity (some polyphenols and the most abundant plant phytosterols: campesterol, sitosterol, and stigmasterol), quantified polyphenols (flavonoids and tannins), and evidenced their antioxidant potential. It may justify—at least in part—the actions of these plant products to decrease MIC values of FLC. The process of fractioning CLE into HX or EtOAc concentrated

the polyphenols in these fractions, as seen in this study. This fact is also reflected in our results from MIC values and during the associations between plant product and FLC, since the outstanding MIC values for the alone HX fraction overcome the influence of its polyphenols content and antioxidant activity on FLC activity (“no interaction” when the associations were performed), while the greater polyphenols content and antioxidant activity of EtOAc fraction or a higher MIC values for the alone CLE influenced MIC values from the associations (“antagonism” from most associations).

We demonstrated that plant product can increase the MIC of FLC, the next question arising was whether antioxidants-containing plant product could potentially interfere with FLC susceptibility owing to a previous action upon ROS levels. The generation of ROS is an inevitable aspect of aerobic organism’s life, so that, during oxygen ( $O_2$ ) metabolism, by electron-transport chains and/or other mechanisms,  $O_2$  is converted into  $O_2^*$ , that is spontaneously or enzymatically converted into  $H_2O_2$ . Consequently, in the Fenton reaction,  $H_2O_2$  is further converted into the highly-reactive  $HO^*$  that can generate damage upon DNA. Under physiological conditions, steady-state levels of ROS are maintained in *C. albicans* cells, but the cell’s homeostatic system can be disturbed, in the so-called oxidative stress, overly burdening the cell with ROS and leading to death. Thus, external factors, including antibiotic action, as here performed, can modify this redox balance, and it could explain why FLC-induced ROS/RNS-mediated *C. albicans* death/damage or growth inhibition is hampered by antioxidants-containing plant product.

Over the last few decades, controversies about the participation of intense ROS production during antibiotic action have generated discussion [68, 69]. Reinforcing the theory that high levels of ROS are induced during antibiotic action, the presence of some exogenous redox-active compounds with pro- or antioxidant activity, such as polyphenols and other plant products, can change the antibiotic action. It has been shown that antioxidants promote a diminution in the expression of genes related to ROS detoxification systems in microorganisms, reversing expectations of an increase in gene expression—a common consequence of oxidative stress induced by antibiotics— [27], as seen in cases of antibiotic resistance because of a higher oxidative stress tolerance in *C. albicans* [70] and *P. aeruginosa* biofilms [71]. As demonstrated here, we found that these increased FLC MICs were caused by antioxidant effects attributed to the plant products accordingly decreasing  $HO^*$ ; thus, mitigating FLC-induced growth inhibition or killing of *C. albicans*. As part of its antifungal action, FLC, *per se*, increased the levels of ROS ( $HO^*$ ) in *C. albicans* cells, whereas the association between plant product (CLE, EtOAc, or HX) and this antifungal agent caused a decrease in FLC-induced ROS which in turn generated an increase in MIC values (decreased sensitivity of *C. albicans* to FLC). Similarly, Smirnova *et al.* demonstrated that plant

polyphenols and crude extracts from the leaves of various plants decreased the antibacterial effect (increased MIC value) of ciprofloxacin against *E. coli*. This effect was also assigned by the author to an antioxidant action of the compounds/extracts [27].

Of great relevance, in this study, we considered intracellularly-generated and confined oxidants in *C. albicans* cells, so that we used membrane-permeable fluorescent probes that readily diffuse through the cell membrane and then are rapidly oxidized to highly fluorescent products. The treatments with the CLE, EtOAc, and HX were performed; and these plant products present antioxidant compounds able to cross a membrane. This is further supported in previous studies, in which non-polar compounds with antioxidant action have been described for this plant [17,18].

In this study, we detected elevated HO<sup>•</sup> formation in *C. albicans* cells induced by FLC, which may, at least in part, explain the link between FLC treatment and oxidative damage in DNA (quantifying 8-oxo-7,8-dihydro-2'-deoxyguanosine [8-oxodG]), as found by Mahl *et al.* [22], since unlike the O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub>, HO<sup>•</sup> is highly reactive toward DNA and hence it could explain the FLC-dependent ROS-induced guanine pool oxidation leading to *C. albicans* death. In addition, recent evidence suggests that FLC induces oxidative DNA damage in *Candida tropicalis* after 24 hr of exposure, being this outcome of an earlier augmentation in ROS levels (oxidative stress) [23].

Here we demonstrated the generation of FLC-induced ROS in a reference strain of *C. albicans*. Silva *et al.* reported which fluconazole induced high levels of ROS only in susceptible strains of *C. tropicalis* [23]. However, different strains may differ in the sensitivity and response to oxidative stress because of a specific sensitivity to FLC. In addition, azole antifungals act in a time and concentration dependent-manner in these cases, and the associated generation of ROS after antifungal exposure to appear not to be directly related to them but by some by-products generated through their mechanisms of action [22]. Silva *et al.* [23] and Mahl *et al.* [22] have shown that resistance to FLC among *Candida* species can involve increased gene expression of products related to redox homeostasis systems, such as genes for the synthesis of antioxidant enzymes, GPx, Sod, and GST [70], provided that the antifungal activity of FLC has been reported to be dependent on an intensive ROS production, which generate DNA damage (consequence of an excessive farnesyl pyrophosphate formation) beyond the effects in inhibiting ergosterol biosynthesis and consequent changes in the fungal membrane. A recent study has also demonstrated that FLC at subinhibitory concentrations induced oxidative- and nitrosative-responsive genes *TRR1*, *GRE2*, and *YHB1*, and led to AMR profile in *C. albicans* and resistance to phagocytes [9].

In *C. albicans* cells, antioxidant-mediated protection against FLC may function in any of the two ways: chain-breaking or prevention. Thiourea, an antioxidant, was proved to protect *E. coli* from killing by norfloxacin, a fluoroquinolone antibiotic. Later, Keren *et al.* demonstrated that, at low concentrations of norfloxacin, thiourea, acting by diminishing ROS levels, protected *E. coli* cells from killing [28]. As also here observed, plant products provide protection against FLC-induced ROS/RNS-mediated *C. albicans* death.

To reinforce the plausible putative action of antioxidants as scavengers of ROS/RNS to prevent FLC activity, rather than other effects, Goswami *et al.* [44] demonstrated that wild type *E. coli* K-12 strain MG1655 has lower susceptibility toward different antibiotics (Ciprofloxacin, Ofloxacin, Streptomycin, Kanamycin, Gentamycin, Spectinomycin, Tetracycline, Chloramphenicol, Ampicillin, and Penicillin), when in the presence of antioxidants (10 mM glutathione or ascorbic acid). The results found here—protection of the *C. albicans* cells (using an experimental model of fungi [eukaryote microorganism]) by antioxidants-containing plant products against FLC—and those ones found by Goswami *et al.* (different antibiotics representing a wide diversity of mechanisms of action) against an experimental model of prokaryote microorganism (bacteria), may indicate a putative mechanism of antioxidants, as ROS scavengers, to interfere with the susceptibility of *C. albicans*, rather than, a pleiotropic effect.

The limitations of our study should be noted, which are the analysis of oxidative stress response just in exponential growth phase (i.e., yeast cells in exponential and stationary phase can respond in a different manner to oxidative stress) and a verification upon the *C. albicans* exposure to FLC more in-depth, under the influence of treatments with the CLE, HX, or EtOAc, and the correspondent *C. albicans* antioxidant response (analyses of the systems of ROS detoxification: *GPx*, *SOD*, and *GST* genes and related enzymes), total glutathione, and oxidative damage in DNA (since FLC-induced oxidative damage upon lipids and proteins appears to be no significant in this case) [22]. These tests could help to shed light whether the treatments are priming/modulating genes expressions/enzymes activities and/or whether they are creating direct antioxidant effects (most probably) or other types of interaction with FLC.

Taken together, our findings suggest that *T. paniculatum* presents potential for further studies to look at it as an antifungal. This plant presents low toxicity at concentrations of optimal antifungal activity (significant SI values). As a recommendation, additional studies are required to isolate new compounds with original antifungal actions through a bioassay-guided approach. Alternatively, a study could be conducted regarding the efficacy, safety, and pharmacokinetics for a herbal drug from *T. paniculatum* leaves to treat infectious diseases, including those caused by AMR, since the CLE from

*T. paniculatum* alone presents outstanding antifungal activity, its inability to have significant synergic effects with antibiotics notwithstanding.

Conversely, these findings also provide novel insights into redox regulation of *C. albicans* cells during the association between plant product and antifungal. We used an experimental model of antifungal action (FLC), demonstrating a plausible putative activity of antioxidants containing-plant products in preventing the effects of FLC by decreasing oxidants. Of clinical relevance, our data support the fact that some polyphenols and other antioxidant compounds, being a part of many nutrition products and medicines, may interact with antibiotics during medical treatment and modify their action. Therefore, since the dietary intake of *T. paniculatum* as a green leafy vegetable is common, a word of warning should be issued regarding the association between *T. paniculatum* and FLC, in face of the antagonistic effects here demonstrated; thus, during antibiotic therapy, the physician should take all this into account, since patients under FLC treatment parallel to an intake of this plant may cause a decrease in antifungal efficacy.

Nonetheless, these data should also prompt *in vivo* studies, focusing additional effects of exogenous products with antioxidants on the host/organism to observe resistance phenomenon and/or the failure of therapeutic regimens, since some combinations may increase the MIC of antibiotics *in vitro* and there is possibility for this also occurs *in vivo*—in addition to—be worthwhile to target these interactions between plant product and antibiotics and associated effect's underlying mechanisms as a means to enhance the killing efficacy of available antimicrobial agents.

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## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CDC. Performed the experiments: CDC MFRN JJS GOIM LFCR. Analyzed the data: CDC. Contributed reagents/materials/analysis tools: MFGB GBS MRPLB. Gave technical support and conceptual advice: GOIM MFGB MRPLB. Wrote the paper: CDC. Supervised the study: CDC MRPLB. Final approval of manuscript: CDC JJS MFRN MFGB GBS LFCR MRPLB.

## DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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