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ORIGINAL PAPER

Understanding the mechanism of action of protease inhibitors in controlling the growth of the *Candida* Genus: potential candidates for development of new antifungal molecules

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Abstract

There is a growing imperative for research into alternative compounds for the treatment of the fungal infections. Thus, many studies have focused on the analysis of antifungal proteins and peptides from different plant sources. Among these molecules are protease inhibitors (PIs). Previously, PIs present in the peptide-rich fractions called PEF1, PEF2 and PEF3 were identified from *Capsicum chinense* seeds, which have strong activity against phytopathogenic fungi. The aim of this study was to evaluate the mechanism of action and antimicrobial activity of PIs from PEF2 and PEF3 on the growth of yeasts of the genus *Candida*. In this work, analyses of their antimicrobial activity and cell viability were carried out. Subsequently, the mechanism of action by which the PIs cause the death of the yeasts was evaluated. Cytotoxicity was assessed in vitro by erythrocytes lysis and in vivo in *Galleria mellonella* larvae. PEF2 and PEF3 caused 100% of the growth inhibition of *C. tropicalis* and *C. buinensis*. For *C. albicans* inhibition was approximately 60% for both fractions. The PEF2 and PEF3 caused a reduction in mitochondrial functionality of 54% and 46% for *C. albicans*, 26% and 30% for *C. tropicalis*, and 71% and 68% for *C. buinensis*, respectively. These fractions induced morphological alterations, led to membrane permeabilization, elevated ROS levels, and resulted in necrotic cell death in *C. tropicalis*, whilst demonstrating low toxicity toward host cells. From the results obtained here, we intend to contribute to the understanding of the action of PIs in the control of fungal diseases of medical importance.

Keywords Anti-*Candida* activity · Membrane permeabilization · Plant antimicrobial peptide · Low toxicity

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Introduction

Antifungal resistance to the main therapeutic agents is one of the biggest challenges of the 21st century, and the excessive use of those drugs is singled out as the main contributor to the crisis in the first place. Fungal pathogens threaten global public health, and for the first time, the genus *Candida* was cited in a priority list of pathogens in the WHO report (WHO 2022). Invasive infections caused by *Candida* species are often associated with higher mortality rates and prolonged hospital stays. These hospitalizations, prolonged exposure to drugs, and prophylaxis in high-risk patients have contributed to the emergence of multidrug-resistant strains to the main therapeutic agents available (classes of polyenes, azoles and echinocandins) (Ben-Ami et al. 2021; Partha et al. 2022). In Brazil, approximately 14.5% of infections in patients at intensive care units are caused by fungi, a percentage that will only increase (CFM 2015). The estimates indicate that over 6.55 million people are affected each year by fungal disease, currently account for approximately 3.8 million annual deaths worldwide (Denning 2024).

The genus *Candida* is responsible for serious infections mainly in immunocompromised patients. Species belonging to this genus can cause superficial and systemic diseases such as candidiasis, meningitis and sepsis caused by the species *Candida albicans* (Rodrigues et al. 2023). *C. albicans* is responsible for invasive candidiasis that causes infections in the blood, heart, central nervous system, eyes, bones, and internal organs with a high mortality rate. Among *Candida* species, this is the most prevalent, although nonalbicans species are increasing (Macias-Paz et al. 2023). Approximately 20 species of *Candida* are responsible for causing human disease; however, the five pathogens that cause more than 90% of invasive infections are the species *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* (Ksiezopolska et al. 2018; Lamoth et al. 2020). In particular, another pathogen that has become of great clinical relevance and a threat to global health is *C. auris*. This multidrug-resistant species is spreading rapidly around the world, with several outbreaks described (Sabino et al. 2020). With the emergence of these strains, there is a great interest in identifying natural compounds that act on multiresistant microorganisms.

The use of antimicrobial peptides (AMPs) provides a solution to combat this serious problem of resistance. Thus, AMPs have some advantageous characteristics that classify them as potential therapeutic agents: fast action, broad spectrum of activity against several microorganisms, activity at low concentrations, and low toxicity to mammalian cells (Campos et al. 2018). It is believed that the development of resistance to AMPs is very difficult to occur due to the speed with which microorganisms are killed because they have multiple mechanisms of action, and mainly because the plasma membrane is the target of these peptides (Matsuzaki et al. 2009).

Among the different families of AMPs are protease inhibitors (PIs). PIs reduce and/or inhibit the activity of target proteases in the most diverse organisms and have aroused the interest of researchers since they are important candidates for the development of new promising molecules for the sustainable biological control of pathogens in plants and various infections in humans (Zida et al. 2017; Koparde et al. 2019). In recent studies conducted by our research group, we isolated and characterized serine protease inhibitors from three distinct peptide-enriched fractions (PEFs), known as PEF1, PEF2, and PEF3, extracted from *Capsicum chinense* seeds (Dias et al. 2013; Silva et al. 2023). In the present investigation, we extended our analysis to assess the antimicrobial activity and mechanism of action of PEF2 and PEF3, derived from *C. chinense* seeds, against *Candida* yeasts.

Materials and methods

Microorganisms

The yeasts *Candida albicans* (CE022), *Candida tropicalis* (CE017), and *Candida parapsilosis* (CE002) were provided from Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. The yeast *Candida buinensis* (3982) was provided from Micoteca URM of Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil. Yeasts were preserved on Sabouraud agar (1% peptone, 2% glucose and 1.7% agar) (Merck) in the LFBM of the Centro de Biociências e Biotecnologia (CBB) of UENF, Campos dos Goytacazes, Rio de Janeiro, Brazil.

Extraction and purification of the protease inhibitors

Protease inhibitors were extracted and purified as described by Silva et al. (2023). Firstly, the *C. chinense* seeds were macerated with the help of liquid nitrogen. This flour was delipidated in petroleum ether in a ratio of 1:4 (flour/ether) for 1 h at 4 °C, and then centrifuged at 15,000 x g for 10 min at 4 °C. Subsequently, to the precipitate was added the extraction buffer [1% trifluoroacetic acid (TFA), 1 M HCl, 5% formic acid, 1% NaCl, 1 µg mL^{-1} pepstatin A], for 1 h at 4 °C and subjected to centrifugation at 14,000 x g for 10 min at 4 °C.

The peptide-rich extract was subjected to reversed-phase chromatography in an HPLC system (high performance liquid chromatography), using a μRPC C18 column (Shim-pack CLC-ODS, Shimadzu, Japan), coupled to a C18 guard cartridge (Pelliguard; Sigma–Aldrich). Chromatography was performed using 100% solvent A (0.1% TFA (Sigma–Aldrich) for up to 8 min, followed by an increasing up to 50% solvent B (100% 2–propanol (Sigma–Aldrich) in 0.1% TFA) up to 47 min. At a flow rate of 0.5 mL min^{-1}, monitored by absorbance at 280 nm.

Analysis of the antimicrobial activity of protease inhibitors on yeasts of the genus *Candida*

To analyze antimicrobial activity, cells from yeast species of the genus *Candida* (1×10^4 cells mL^{-1}) were incubated in Sabouraud broth (Merck) with different concentrations of PIs (200 to 1.56 μg mL^{-1}), in the final volume of 100 μL. The test was carried out in a 96-well microplate (Nunc), incubated at 30 °C for 24 h. Cell growth was determined by optical density, monitored at the end of the assay, in a microplate reader at a wavelength of 620 nm. The entire procedure was performed under aseptic conditions in a laminar flow hood, according to the method described by Silva et al. (2017). After 24 h of incubation, the bottom of the well was photographed. The microplate was positioned on a transilluminator (Hoefer - MacroVue), and the images were taken in black and white.

Cell viability analysis of cells treated with protease inhibitors

To evaluate the effect of PIs present in the PEF2 and PEF3 on the cell viability of *Candida* yeasts, the same procedure described in the subsection "Analysis of the antimicrobial activity of protease inhibitors on yeasts of the genus *Candida*" was performed with the following modification: cells were incubated only with the highest tested concentration of each fraction. Control cells (untreated) were washed and diluted 1000-fold. Posteriorly, 200 cells mL^{-1} were spread with the aid of a Drigalski loop on the surface of a Petri dish containing Sabouraud agar (Merck) and maintained at 30 °C for 36 h. At the end of this period, colony-forming units (CFU) were indicated for each species, and images of the Petri dishes assays were taken (Soares et al. 2017). Results are presented assuming that the control matches 100% viability.

Effects of protease inhibitors on membrane permeabilization in yeast cells

The permeabilization of yeast membranes treated in the absence and in the presence of PIs was evaluated using the fluorescent dye SYTOX Green (Thermo Fisher Scientific), according to the methodology described by Thevissen et al. (1999), with some modifications. SYTOX Green is a dye that has a high affinity for nucleic acids (DNA) that easily penetrates cells when their membrane is compromised; by binding to nucleic acids, it becomes fluorescent. At 24 h of the yeast growth inhibition assay, aliquots of each sample were incubated under constant agitation for 30 min with SYTOX Green at a concentration of 0.2 μM according to instructions provided by the manufacturer. Then, the samples were evaluated in a microscope (Axio Zeiss, Imager A,2) equipped with fluorescent filters for fluorescein detection (with an excitation wavelength between 450 and 490 nm and an emission wavelength of 500 nm).

Late apoptosis cell death analysis

Late apoptosis in *Candida* yeasts was analyzed by fluorescence optical microscopy with the aid of a propidium iodide fluorescent probe (Sigma Aldrich). This probe is not permeable to intact plasma membranes or cells in early apoptosis but is permeable to cells undergoing apoptosis and binds to nucleic acids in necrotic cells. The test was performed as described in the subsection "Effects of protease inhibitors on membrane permeabilization in yeast cells." Control and treated cells were incubated with the fluorescent probe for 15 min and observed under an optical microscope (excitation wavelength 506 nm, emission wavelength 530 nm), according to the methodology described by Deere et al. (1998).

ROS induction assay in yeast cells

The fluorescent probe H_2DCFDA (2′,7′-dichlorofluorescein diacetate) was used to measure intracellular reactive oxygen species (ROS), and to assess whether the mechanism of action of PIs involves the induction of oxidative stress, according to the methodology described by Mello et al. (2011). Initially, cells from different yeast species were incubated in the presence and absence of PIs. At 24 h of the growth inhibition assay, the cells were incubated with the ROS-specific probe (20 μM H_2DCFDA). After 2 h of incubation at room temperature with constant agitation, the cells were analyzed under a microscope (Axio Imager.A2, Zeiss) equipped with fluorescence filters (450–490 nm for excitation and 500 nm for emission).

Analysis of the importance of oxidative stress for *Candida* yeast death induced by protease inhibitors

In this experiment, 10.000 cells mL^{-1} were transferred to a 96-well cell plate with 100 μL of Sabouraud liquid medium incubated with 200 μg mL^{-1} PIs and 70 mM antioxidant ascorbic acid (AsA) (Sigma–Aldrich), diluted from a 1 M stock solution in water previously filter sterilized (Millex-GV 0.22 µm, Merck Millipore). AsA is a nonenzymatic antioxidant that protects cells against the oxidizing action of free radicals. At 24 h of incubation at 30 °C, the entire well volume was resuspended and spread over Petri dishes containing Sabouraud solid medium. The plates were incubated for periods of 36 to 48 h at 30 °C, and after this period, the plates were photographed. Cells grown in the presence of 70 mM AsA in solid medium or only in solid medium were used as controls (Soares et al. 2017).

Analysis of mitochondrial functionality of yeast cells

This assay was performed with the tetrazolic salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitro-phenyl)-2 H-5-tetrazolio]-1,3-benzene sulfonate) (Roche Applied Science) to quantify the effects of PIs on mitochondrial functionality. Through a complex cellular mechanism that occurs especially at the cell surface, WST-1 is cleaved to soluble formazan. The NADH formed by the mitochondrial Krebs cycle is responsible for this bioreduction, which allows colorimetric quantification of mitochondrial metabolic activity (Berridge et al. 2005). The test was performed as described in the subsection “Analysis of the antimicrobial activity of protease inhibitors on yeasts of the genus *Candida*” with the following modifications: At 24 h of incubation with PIs, WST-1 solution (10 µL) and 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone, 2 µL) (final concentration 0.24 mM in dimethyl sulfoxide) as an electron couple reagent were added and incubated for 2.5 h at 37 °C. Yeast cells detergent-treated with 1% Triton X-100 were used as a positive control. For formazan detection, optical densities were measured at 450 nm. The percentage of mitochondrial functionality was calculated as follows: % mitochondrial functionality = [(ABS_{450nm} of test samples × 100)/(ABS_{450nm} of control)- ABS_{450nm} of liquid medium)] (Taveira et al. 2022).

Regulated cell death analysis

The CaspACE FITC-VAD-FMK (Promega) marker was used to detect caspase activity. The test was performed as described in the subsection “Late apoptosis cell death analysis” according to Taveira et al. (2018). At 24 h of incubation with the PIs, the yeast cells were resuspended in Sabouraud medium, washed once in 500 µL of phosphate-buffered saline (PBS) (10 mM NaH_2PO_4, 0.15 M NaCl) pH 7.4 and resuspended in 50 µL of staining solution containing 50 µM FITC-VAD-FMK. After 20 min of incubation at 30 °C with constant shaking at 500 rpm, the cells were washed once again in 500 µL of PBS and resuspended in 20 µL of PBS. The negative control cells (incubated in the absence of PEF2 and PEF3) had the same treatment as the cells treated with the peptides. Cells were analyzed by DIC using a microscope equipped with a fluorescence filter to detect fluorescein (excitation wavelength: 450–490 nm, emission wavelength: 500 nm).

Transmission electron microscopy (TEM)

For analysis of the ultrastructure of *C. albicans* yeast cells, cells treated and not treated with PEF2 and PEF3 (100 µg mL^{-1}) were fixed for 1 h in sodium cacodylate buffer 0.1 mol L^{-1}, pH 7.4 containing 2.5% glutaraldehyde and 4% formaldehyde. Then, the cells were washed (2×) in PBS for 10 min and postfixed for 1 h in a solution containing 1% osmium tetroxide (OsO4) and 1.6% ferrocyanide in 0.1 M sodium cacodylate buffer. After these processes, the cells were washed in the same buffer, dehydrated in different concentrations of acetone and embedded in Epon. Finally, ultrathin cuts were made and stained with uranyl acetate and lead citrate, and then viewed by transmission electron microscopy (Zeiss 900) (Moreira et al. 2021).

Zymography

The yeasts were incubated in Sabouraud broth supplemented with 10% bovine serum albumin (BSA) for 24 h at 30 °C. After this period, the cell suspension obtained was centrifuged at 7,000 × g for 5 min at 4 °C. The sodium phosphate buffer 100 mM (pH 5.6) was used to ressuspend the cell pellet before ultrasonic treatment in a probe sonicator (Sonifier 150, Branson). The ultrasonic treatment cycle was 30 min, in which five 1 min pulses were performed and five 5 min intervals between pulses, in ice bath (Gebara et al. 2023). After using the sonicator, the samples were subjected to a new centrifugation at 10,000 × g for 15 min at 4 °C. The resulting crude extract was subjected to electrophoresis and zymography, again in ice bath.

The extracts were diluted in sample buffer and analyzed on SDS–PAGE plus 0.5% gelatin according to Laemmli (1970). Then, the gel was washed twice with Tris-HCl buffer (0.05 M pH 8.0) containing 2.5% Triton X-100 for 40 min. Subsequently, the gel was incubated with Tris-HCl (0.05 M pH 8.0) containing 0.01 M CaCl2 at 30 °C for 120 min. Finally, the gel was fixed for 30 min and stained with Coomassie Blue R-250 (Ito et al. 2010).

Hemolytic activity

Defibrinated sheep red blood cells (sRBCs) were used to establish the hemolytic activity of PIs. sRBCs were washed with saline solution (0.15 M NaCl), centrifuged at 2400 × g for 10 min, and then resuspended again in saline solution.

PIs were prepared in Microcentrifuge tubes containing saline solution. Posteriorly, 50 µL PIs at concentrations of 200, 100, 50, and 25 µg mL^{-1} were incubated for 1 h at 37 °C with 50 µL of sRBCs (1% v/v). At the end of incubation, all samples were centrifuged for 10 min at 2400 × g, and the supernatants were transferred to 96-well microplate (Nunc). Free hemoglobin was measured at a wavelength of 405 nm, as described by Oren and Shai (1997). As a positive hemolysis control (C^+) was used a solution containing 1% Triton X-100 and as a negative control (C^-) was used erythrocytes in saline solution. The hemolysis percentage was calculated admitting that the control (C^+) represents 100% of hemolytic activity (group treated with Triton X-100).

Effect of PEF3 in vivo toxicity on *Galleria mellonella*

Ten last-instar *G. mellonella* larvae of similar weight and size (between 250 and 350 mg) were used in each of the treatment and control groups. A Hamilton syringe was used to inject 10 µL of the PEF3 fraction at a 200 µg concentration into the hemocoel of each larva through the last left pro-leg. Two groups were included as controls for the general viability of the larvae: one group was inoculated with PBS, and the other mock inoculated to mimicry the injury of the injection needle. After the inoculations, the larvae were incubated in Petri dishes at 37 °C, and the number of dead larvae was counted every 24 h for a period of 7 days (Mylonakis et al. 2005). Larvae were considered dead when they showed no movement in response to touch. Percent survival curves were plotted, and estimates of differences in survival (log rank Mantel–Cox and Breslow-Wilcoxon tests) were analyzed by the Kaplan–Meier method using GraphPad Software. The experiment was performed in duplicate.

Statistical analysis

The data from fungal growth inhibition assays, mitochondrial functionality assays and hemolytic activity were evaluated by one-way analysis of variance (ANOVA), and mean differences were considered significant for the values of *** $p < 0.001$. All experiments were performed in triplicate unless otherwise stated. The GraphPad Prism software and Tukey's test was used to evaluate the statistical analyzes.

Results

Anti-*Candida* activity

A growth inhibition assay was performed to determine the effect of PIs on the growth of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. buinensis*. The yeasts were incubated with PIs present in the PEF2 and PEF3 at concentrations ranging from 200 to 1.56 µg mL^{-1} (Fig. 1a, b). PEF2 and PEF3 showed significant inhibitory activity against *C. albicans*, *C. tropicalis*, and *C. buinensis* yeasts.

The concentration of 200 µg mL^{-1} PEF2 and PEF3 inhibited 100% of the growth of *C. tropicalis* and *C. buinensis*. For *C. albicans* cells, the most significant inhibition was approximately 60% for both fractions at a concentration of 200 µg mL^{-1}. For *C. parapsilosis*, no growth inhibition was observed for the two fractions at any of the tested concentrations.

To verify the viability of the yeast cells, we used the highest tested concentrations of PEF2 and PEF3. After the growth inhibition assay, the cells were plated in a new culture medium, and after 36 h, the colony forming units (CFU) were counted (Fig. 2). We observed that PEF2 and PEF3 caused 100% cell death for the yeasts *C. tropicalis* and *C. buinensis*, indicating that the inhibitory effect of PEF2 and PEF3 is fungicidal. For *C. albicans* cells, PEF2 and PEF3 at 200 µg mL^{-1} caused 83.4 and 86.4%, of cell death that corresponded to lethal doses (LD) of 83.4 and 86.4%, respectively. For *C. parapsilosis*, PEF2 and PEF3 (200 µg mL^{-1}) caused 11.6 and 22.7% of cell death that corresponded to LD of 11.6 and 22.7%, respectively.

Plasma membrane permeabilization and necrosis and late apoptosis assay

The assays with *C. albicans* were performed with PEF2 and PEF3 at 200 µg mL^{-1}. For the yeast *C. tropicalis*, the assay was performed with PEF2 at 100 µg mL^{-1} and PEF3 at 50 µg mL^{-1}. For *C. parapsilosis*, the assay was performed with PEF2 and PEF3 at 200 µg mL^{-1}. The yeast *C. buinensis* was treated with the respective concentrations of PEF2 at 25 µg mL^{-1} and PEF3 at 12.5 µg mL^{-1}.

In view of the results obtained in the growth inhibition assay with PEF2 and PEF3, aliquots of the tests and controls were incubated with SYTOX Green dye for analysis of membrane permeability and investigation of the possible mechanism of action (Fig. 3). No fluorescent labeling was observed for the yeast *C. parapsilosis* with the two fractions, indicating that there was no permeabilization of its membranes. However, PEF2 and PEF3 were able to permeabilize the membranes of *C. albicans* and *C. tropicalis* cells, indicating that these fractions compromised their membrane structure, allowing the entry of the dye. By DIC microscopy, it was observed that the cells of this yeast presented structural alterations different from those observed in the control, such as cell agglomeration and formation of pseudohyphae. For the yeast *C. buinensis*, only the PEF2 fraction was able to permeabilize its membrane.

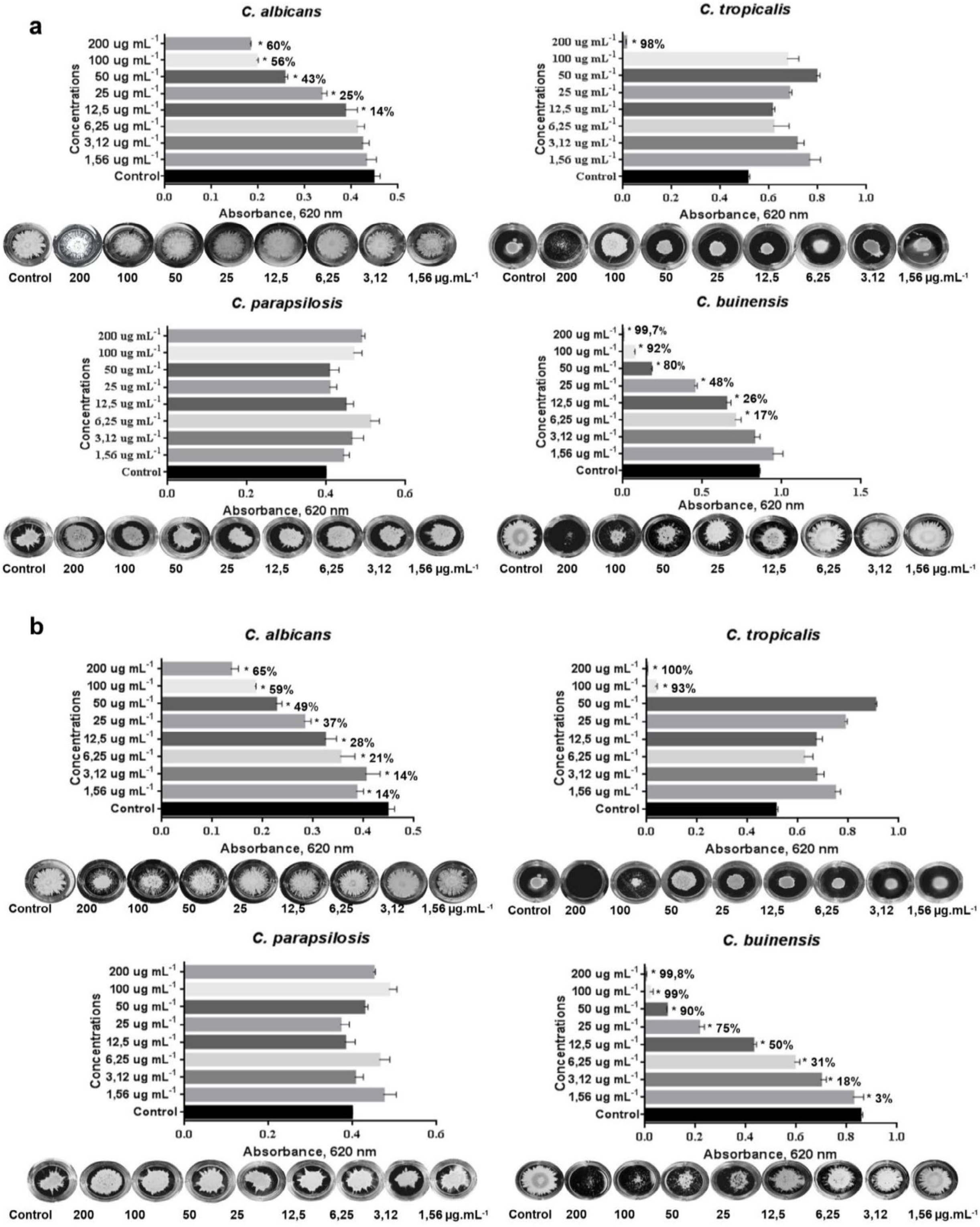


Fig. 1 Graphs and photographs of the effect of PEF2 and PEF3 on yeast growth **a** Yeast suspensions (1×10^4 cell mL^{-1}) were treated or untreated with different concentrations of PEF2; **b** Yeast suspensions with different concentrations of PEF3. *C. albicans*, *C. buinensis*, *C. parapsilosis* and *C. tropicalis* were treated or untreated with different concentrations (200, 100, 50, 25, 12.5, 6.26, 3.12–1.56 µg mL^{-1}) for 24 h. Yeast growth in the resulting cultures was quantified by optical density (620 nm). The values are the means (±SD) of triplicates. The asterisks represent significant ($P < 0.05$) differences between treatments and controls pointed out by Tukey's test. After incubation with the PIs, photographs were taken of all concentrations tested in the assay

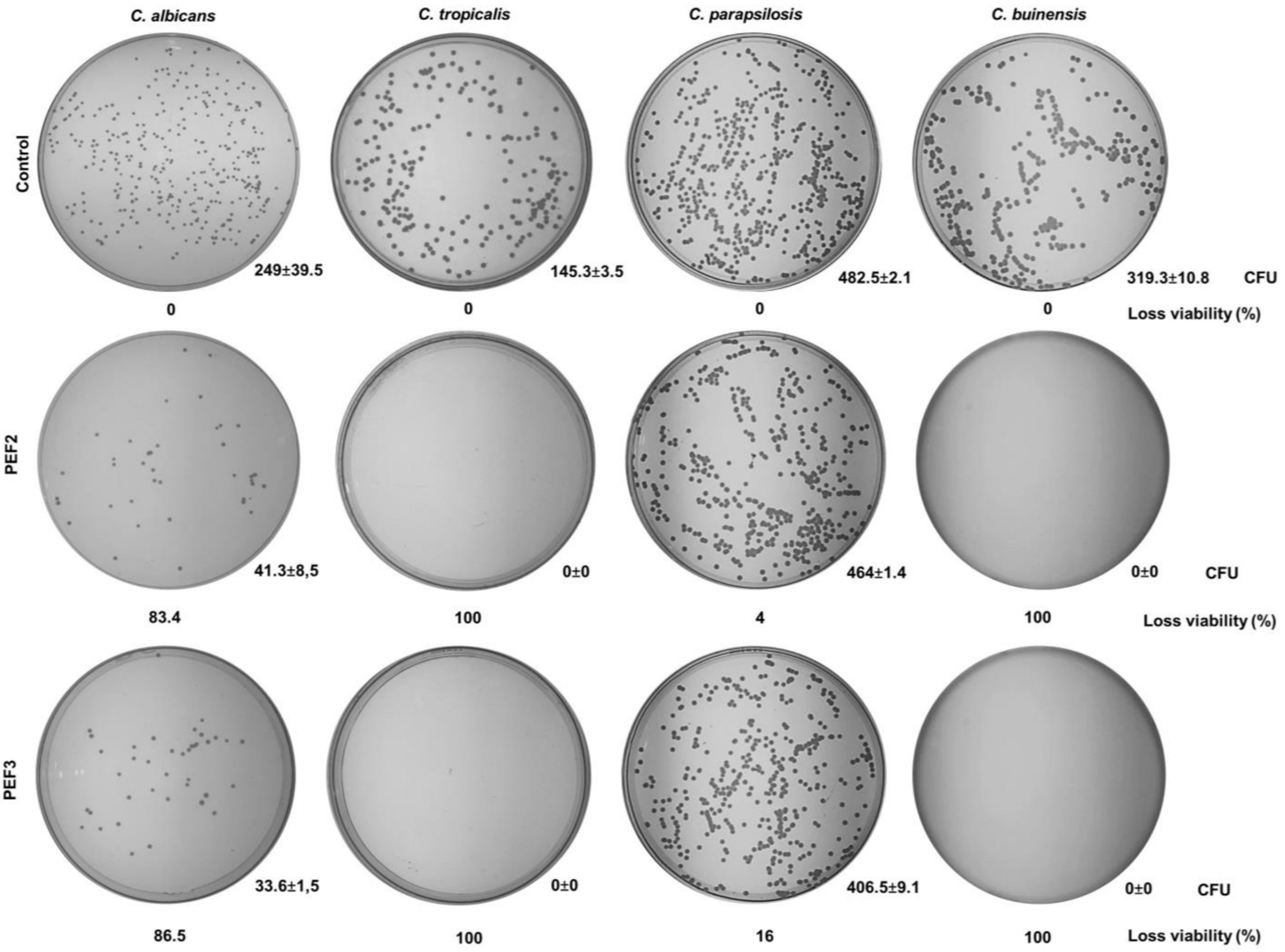


Fig. 2 Effect of treatment with PEF2 and PEF3 on the viability of yeast cells. *C. albicans*, *C. tropicalis, C. parapsilosis* and *C. buinensis* were treated with PEF2 and PEF3 (200 µg mL^{-1}), and after 24 h, cell viability was measured by direct counting of colony forming units (CFU). The cell death percentage was calculated in relation to the control, untreated cell (cell viability – 100%). The data presented are mean values obtained over three experiments, each performed in triplicate

A fluorescence microscopy assay with the necrosis and late apoptosis marker propidium iodide was also performed. By this assay, we found that three of the four *Candida* species tested showed positive labeling for propidium iodide, indicating that these cells present late apoptosis, and the presence of necrotic cells. The *C. albicans* and *C. tropicalis* cells showed markings for both fractions and *C. buinensis* only for PEF2, where it is possible to notice a greater intensity of the fluorescence signal compared to the control.

ROS induction assay

The endogenous ROS production in the different yeast species was analyzed at 24 h of the growth inhibition assay by using the H_2DCFDA probe (Fig. 4). For the *C. parapsilosis* and *C. buinensis* yeasts, ROS overproduction was not detected. However, for *C. albicans* and *C. tropicalis*, there was intense staining of the dye in the cells cultured in the presence of both fractions, which indicated an increase in the endogenous ROS production.

Involvement of oxidative stress in yeast cells death

In this assay, we utilized AsA, a ROS scavenger, to investigate whether treating yeast cells with this compound could shield them from the toxic effects of PEF2 and PEF3. AsA was administered at a final concentration of 70 mM concurrently with PEF2 and PEF3 in the culture medium and evaluated over a 24 h period (Table 1). The results revealed that the presence of the antioxidant provided protection to *C. albicans* cells, resulting in protection of 22.2% for PEF2 and 38.5% for PEF3. For *C. tropicalis*, the antioxidant protected 32.8% of cells for PEF2 and almost entirely preserved growth in the presence of PEF3, with 98.6% cell protection. Unfortunately, *C. parapsilosis* cells showed no protection when exposed to the antioxidant in any of the fractions. For

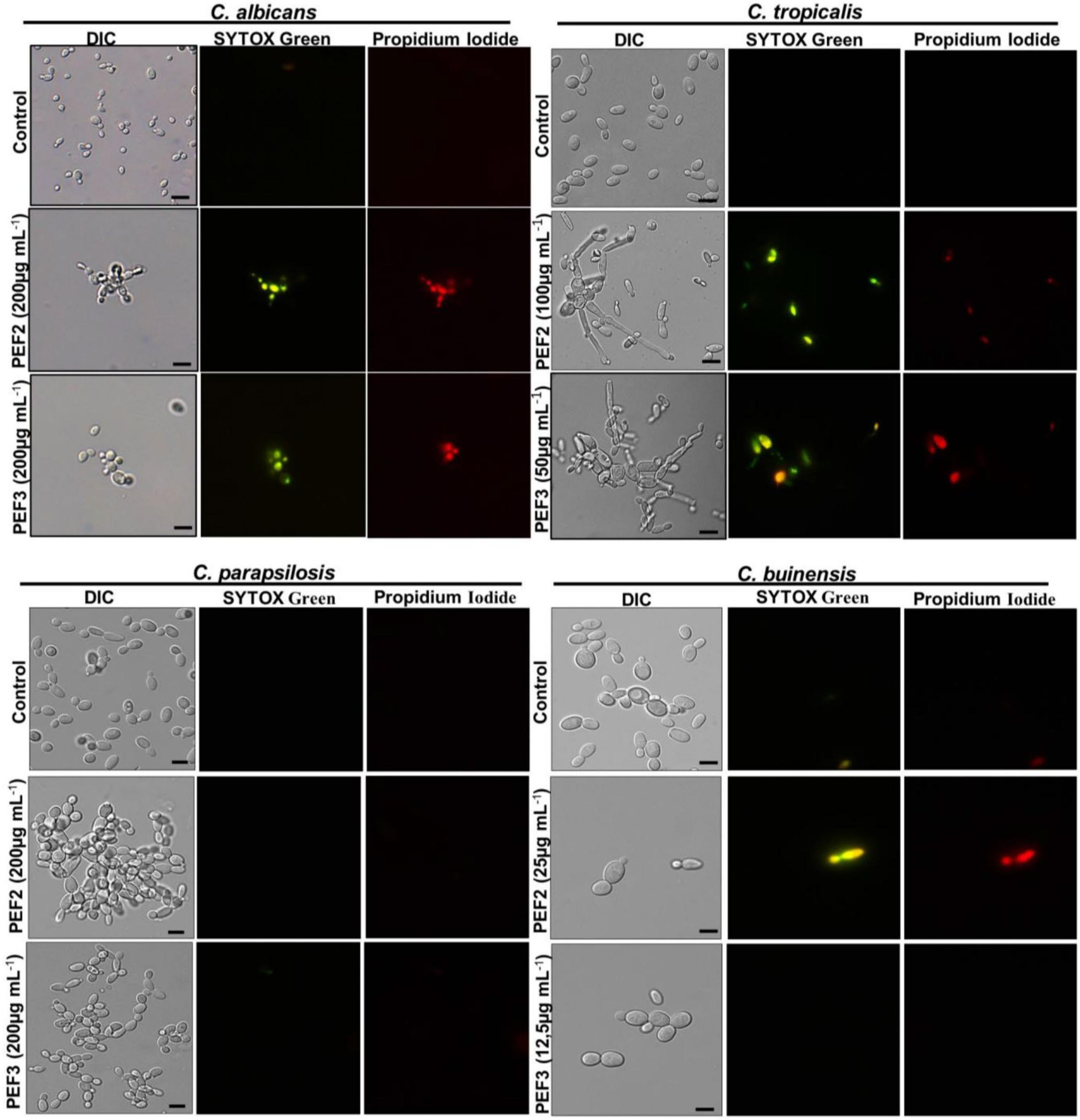


Fig. 3 Membrane permeability assay and verification of necrosis and late apoptosis. Images of *C. albicans*, *C. tropicalis, C. parapsilosis* and *C. buinensis* cells after treatment with different concentrations of PEF2 and PEF3 for 24 h, visualized by fluorescence microscopy using the fluorescent probe SYTOX Green and propidium iodide fluorescent probe. Control cells were treated only with SYTOX Green and propidium iodide probe. Bars = 50 µm

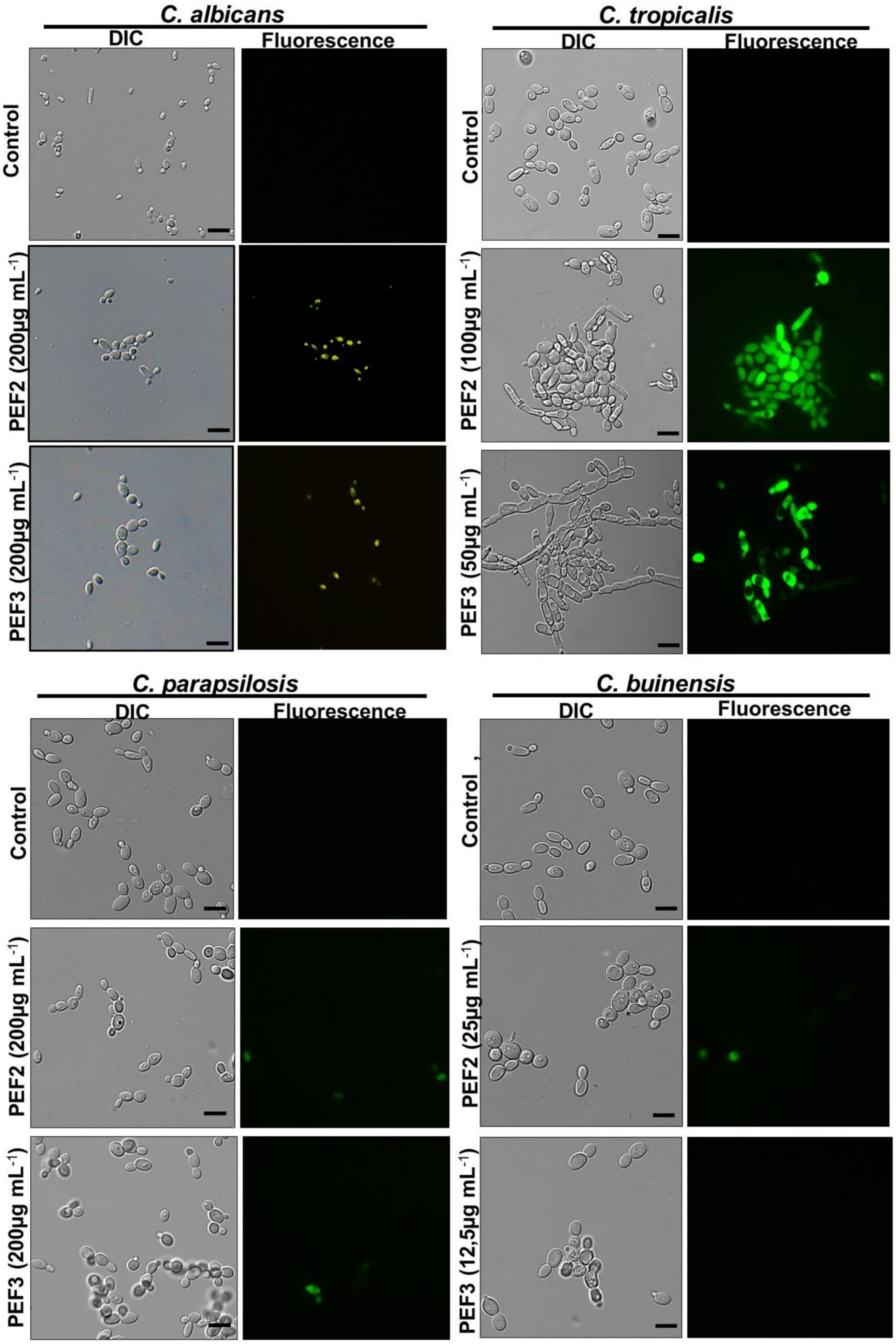


Fig. 4 Oxidative stress assay by ROS detection. Images visualized after reactive oxygen species assay detection in yeast incubated with different concentrations of PEF2 and PEF3 for 24 h. Yeast treated with the fluorescent probe 2’,7′-dichlorofluorescein diacetate (H_2DCFDA). Control cells grown in the absence of PEF2 and PEF3. Bars: 50 µm

C. buinensis, the antioxidant protected growth by 20% for PEF2 and 13.4% for PEF3.

Mitochondrial functionality assay

The mitochondrial functionality of different yeasts tested in this study was quantified by the cleavage of WST-1 to formazan, a process that is influenced by the overall activity of cellular mitochondrial dehydrogenases. We observed that the PEF2 and PEF3 fractions cause a reduction in mitochondrial functionality of 54% and 46%, respectively, for *C. albicans* cells, 26% and 30%, respectively, for *C. tropicalis*, 71% and 68%, respectively, for *C. buinensis*, and for *C. parapsilosis* cells, only PEF2 caused a significantly reduction of 21% of mitochondrial function (Fig. 5).

Table 1 Relationship of oxidative stress to PEF2- and PEF3-induced yeast death

Species of yeasts		Control	Control with ascorbic acid	PEF2	PEF2 with ascorbic acid	PEF3	PEF3 with ascorbic acid
C. albicans	CFU	284.6 ± 10.6	176 ± 22.1	61.3 ± 9.2	63 ± 4.2	76.3 ± 0.5	109.6 ± 9.2
	Cell viability %	100%	62%	21,6%	22,2%	26,8%	38,5%
C. tropicalis	CFU	282.5 ± 4.9	260 ± 7.2	0 ± 0	92.6 ± 6.6	0 ± 0	278.5 ± 20.5
	Cell viability %	100%	92%	0%	32,8%	0%	98,6%
C. parapsilosis	CFU	172 ± 2.8	206.5 ± 19.0	144.5 ± 6.3	194.5 ± 0.7	176.5 ± 2.1	216.3 ± 5.5
	Cell viability %	100%	100%	84%	100%	100%	100%
C. buinensis	CFU	165 ± 22.0	80 ± 12.2	0 ± 0	33.5 ± 10.6	0 ± 0	22 ± 1.8
	Cell viability %	100%	48,5%	100%	20%	100%	13,4%

Note Yeast were treated with PEF2 and PEF3 (200 µg mL^{-1}) and 70 mM ascorbic acid for 24 h. Cell viability was measured by direct counting of colony forming units (CFU). The cell viability percentage was calculated in relation to the control, untreated cell (cell viability – 100%). The presence of ascorbic acid recovers the cell viability loss induced by PIs in some yeasts

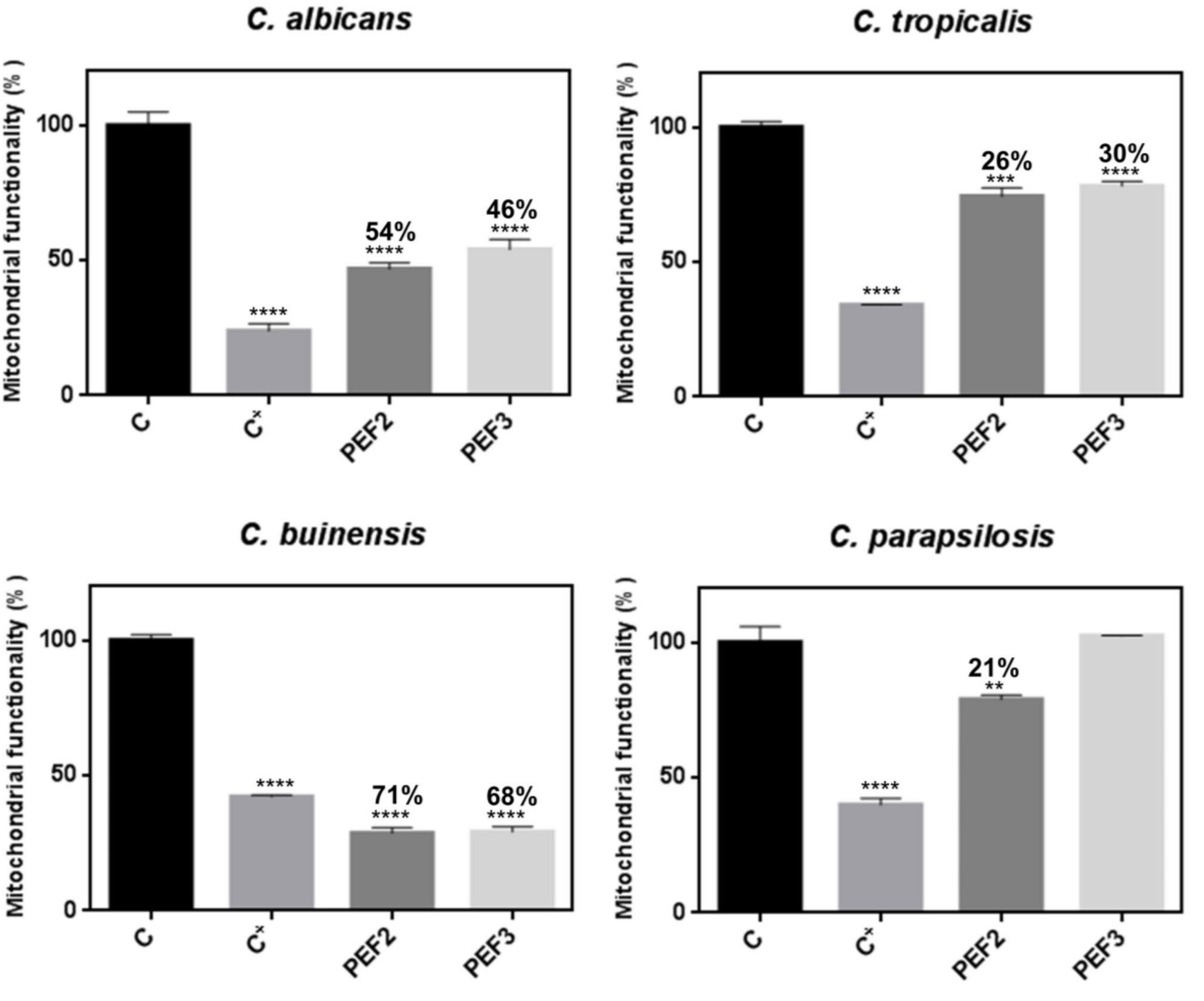


Fig. 5 Mitochondrial functionality. Effect of PIs on the reduction of tetrazolium salt (WST-1) to formazan by yeast cells. *C. albicans*, *C. tropicalis*, *C. buinensis* and *C. parapsilosis* were treated with 12.5, 50, 100 and 200 µg mL^{-1}, respectively, for 24 h. Formazans were measured at 450 nm. **$P < 0{,}01$; ***$P < 0{,}001$; ****$P < 0{,}0001$ compared to the control determined by Tukey's test

Metacaspase activity detection assay

To verify whether apoptotic events contribute to the cell death process in *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. buinensis* cells induced by the presence of PEF2 and PEF3 in the medium, we conducted an assessment of caspase activity. Our findings reveal that exposure to PIs did not lead to caspase activation in different *Candida* yeast cells (data not shown).

Transmission electron microscopy (TEM)

An analysis of *C. albicans* cells treated with PEF2 and PEF3 at 24 h was conducted using transmission electron microscopy. The analysis revealed notable alterations in cells cultivated in the presence of PIs, including a thicker cell wall, increased difficulty in bud release from the mother cell, and the presence of larger vacuoles. Additionally, the presence of PEF3 induced the disruption of the cell wall, potentially leading to the release of cytoplasmic material, as depicted in Fig. 6. These observed changes in cell development can be attributed to the presence of PIs, as none of these characteristics were evident in the control group.

Zymography from yeast protein extracts

To detect proteases in yeast species belonging to the *Candida* genus, we utilized BSA as a substrate for proteolytic digestion. In the SDS–PAGE analysis (Fig. 7a), distinct bands ranging from 20 to 66 kDa were discerned in the yeasts *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. buinensis*. In the zymography assay, a single band with a molecular mass ranging from 36 to 45 kDa was visualized within the yeast extract of *C. tropicalis* (Fig. 7b). To ascertain whether the inhibitors contained in PEF2 and PEF3 effectively suppressed these proteases, we introduced the inhibitors into the yeast extract and subsequently examined them using zymography. Notably, PEF2, and to a lesser extent PEF3, resulted in a reduction in band size. However, complete inhibition was not achieved.

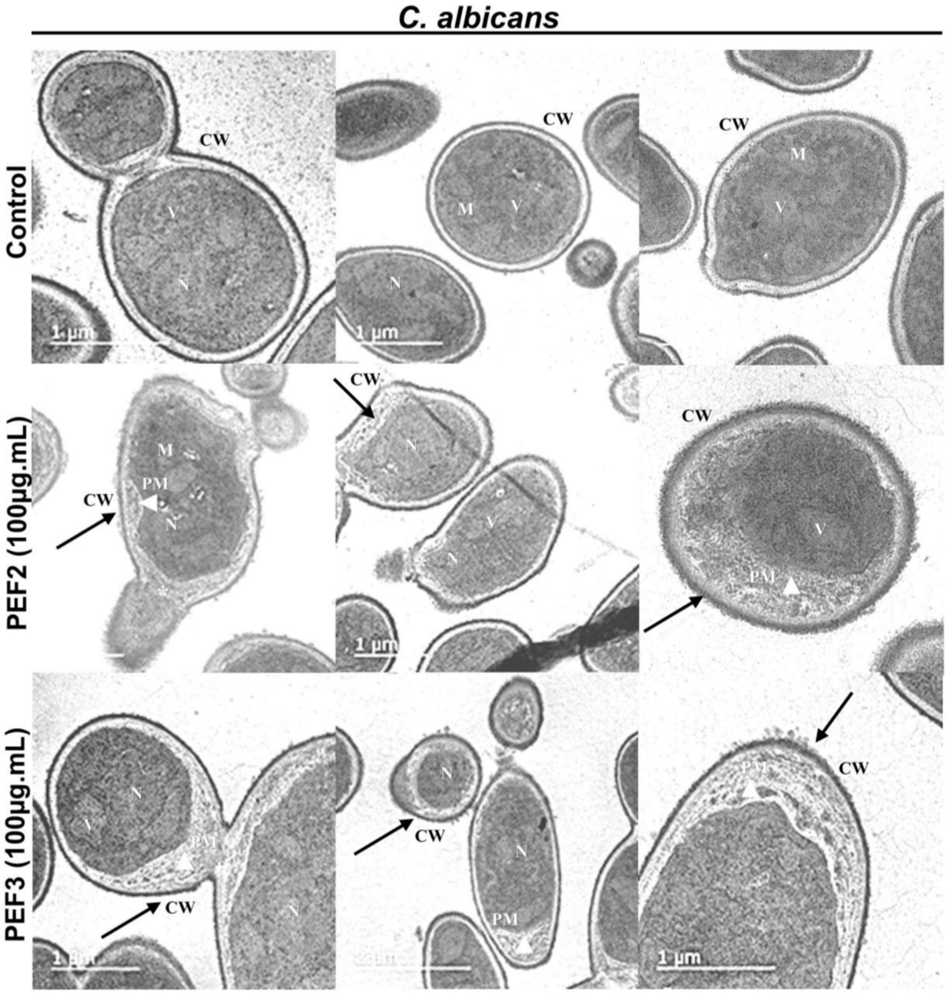


Fig. 6 Transmission electron microscopy of *C. albicans* cells treated or not treated with PEF2 and PEF3. Control cells have a normal ultrastructure of organelles, such as the plasmatic membrane and vacuole. In cells treated with PEF2 and PEF3 (black arrows), alterations in the cell wall, (arrowheads) cell wall thickening, (white arrow) cell wall disruption and * = bud scar are indicated. M = mitochondria, N = nucleus, CW = cell wall, V = vacuole and PM = plasma membrane

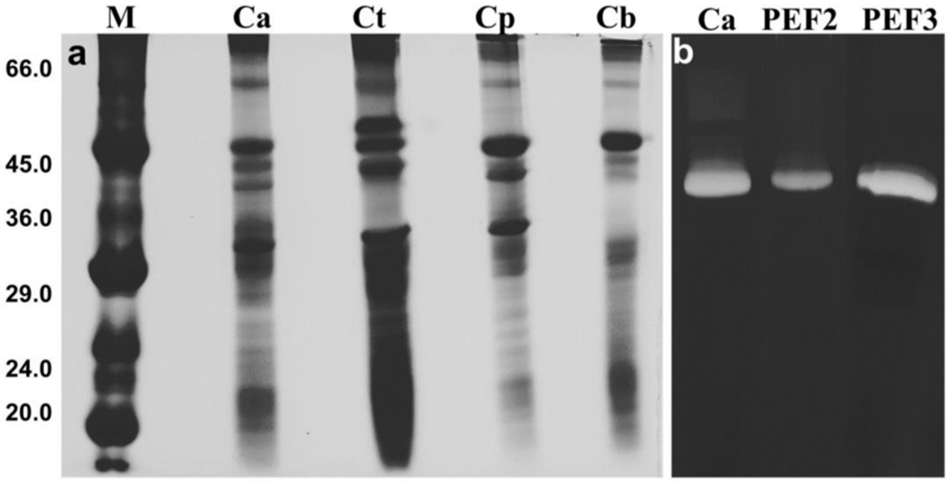


Fig. 7 Detection of proteolytic activities from cell extracts of *Candida* species by zymography **a** SDS–PAGE of *C. albicans, C. tropicalis, C. parapsilosis* and *C. buinensis*. M: molecular mass markers (kDa); **b** Zymography gel electrophoresis from *C. tropicalis* and PEF2 and PEF3 (50 µg mL^{-1})

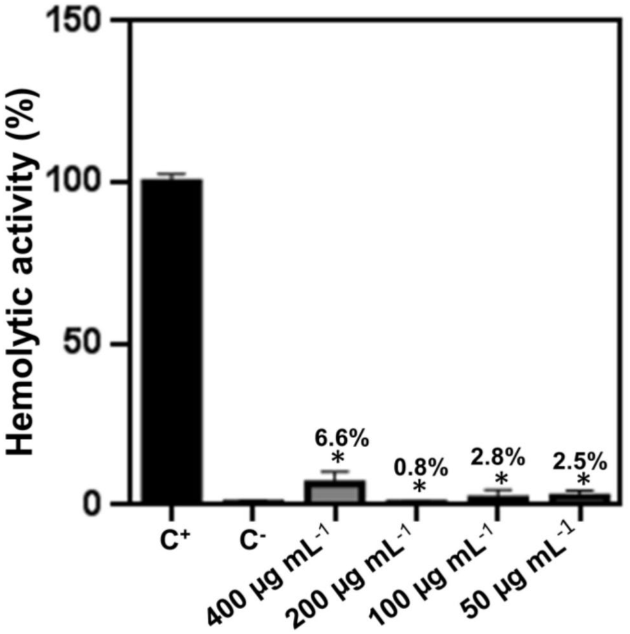


Fig. 8 Effect of the PEF3 fraction on sheep red blood cells. Data represent the percentage of hemolytic activity of cells treated with the PEF3 fraction (400, 200, 100 and 50 µg mL^{-1}), treated with 1% (v/v) Triton X-100 detergent at 37 °C for 1 h as a positive control (C^+) and untreated (0.15 M NaCl) as a negative control (C^-). The asterisks represent significant ($P < 0.05$) differences between treatments and controls pointed out by Tukey's test

Hemolytic potential on sheep red blood cells

The cytotoxicity of PEF3 was verified by measuring the lysis of fresh defibrinated sheep red blood cells, where we observed the low cytotoxic potential of the PIs present in this fraction. The potential hemolytic activity verified at concentrations of 400, 200, 100 and 50 µg mL^{-1} was only 6.6, 0.8, 2.8 and 2.5%, respectively (Fig. 8). Due to the low yield of PEF2, this test was carried out with PEF3 only. Hemolytic activity is an excellent indicator of cell viability, which confirms the low toxicity to the erythrocyte membrane.

Toxicity effect on *G. mellonella* larvae

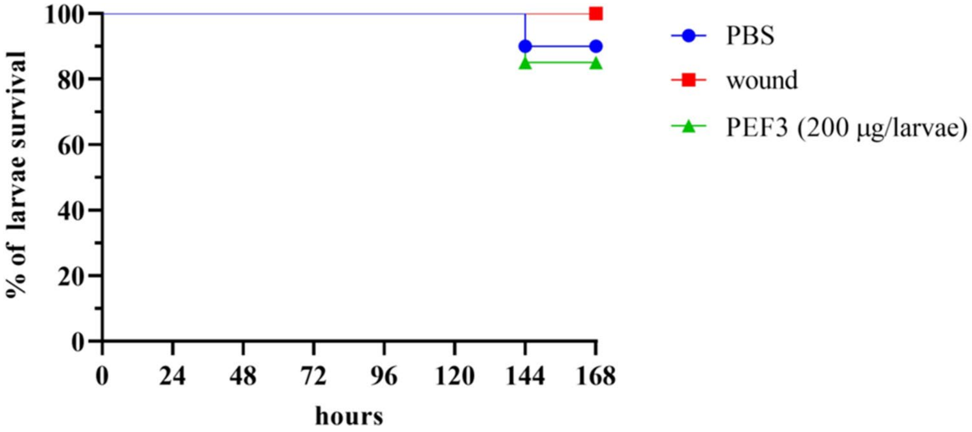
To evaluate PEF3 toxicity in vivo, we used *G. mellonella* larvae. No significant toxic effect was observed for larvae inoculated with 200 µg mL^{-1} PEF3 when compared to controls consisting of groups of larvae subjected only to needle injury and a group of larvae injected with PBS (Fig. 9).

Discussion

Due to their natural origin and characteristics, AMPs are considered as new potential class of therapeutic agents. They are promising molecules for antifungal development because of their selectivity and broad spectrum of antimicrobial activity (Tang et al. 2018). In turn, the genus *Capsicum* has been of great interest to scientific research, since their species present AMPs (Santos et al. 2017; Pereira et al. 2018; Aguieiras et al. 2021; Ribeiro et al. 2022; Cherene et al. 2023).

Plants produce a variety of AMPs that are represented by several families, such as defensins, cyclotides, thionins, heveins, knotins, lipid transfer proteins (LTPs), snakins and PIs (Jing et al. 2020). PIs are plant defense proteins, as they are known to reduce or inhibit the activity of target proteases in diverse organisms. They also act in defense against insects and inhibit digestive enzymes, in addition to inhibiting proteolytic degradation (Clemente et al. 2019; Hellinger et al. 2019). PEF2 fraction contained protein bands approximately 5.0 kDa to 8.0 kDa, PEF3 fraction showed protein bands approximately 5.0 kDa to 14 kDa. These PIs show inhibitory activity on trypsin enzymes, *T. molitor* larval and human salivar α-amylases. In addition to having inhibitory activity on the phytopathogenic fungi *Colletotrichum*

Fig. 9 Toxicity of PEF3 toward *G. mellonella* larvae. Larvae were inoculated with 200 μg mL^{-1} of PEF3. The red line represents controls consisting of groups of larvae subjected only to needle sticks. The blue line represents a group of larvae injected with PBS



gloeosporioides, *C. lindemuthianum*, *Fusarium oxysporum* and *F. solani* (Silva et al. 2021, 2023). In this work, we tested the antifungal activity and studied the mechanism of action of PIs from *C. chinense* seeds on yeasts of the genus *Candida*.

PIs significantly inhibited the growth of almost all yeasts, with the exception of only *C. parapsilosis*. This case may be linked to the absorbance of the medium. Since the measurement of absorbance is an indirect measure of cell growth, this increase may be related to other events, such as morphological changes in cells (cell aggregation and formation of pseudohyphae), extravasation of cytoplasmic content and release of extracellular vesicles (Dias et al. 2013). An important result was observed for *C. tropicalis* and *C. buinensis*, where 100% inhibition was found, and these results indicate that the inhibitory effect of PIs on these yeasts is fungicidal (Fig. 2), which represents a desired mode of action (Pfaller et al. 2004). According to Pfaller et al. (2004), an antifungal has fungicidal activity if there is a decrease in the number of colonies by 99% in CFU/mL and fungistatic activity if there is a decrease in the number of colonies by <99% in CFU mL in relation to the initial inoculum.

Although the mechanism of action of AMPs has been studied for decades, it is still not fully understood. Basically, the most accepted approach says that these molecules bind to the components of the membrane such as phospholipids and sphingolipids and lead to the formation of pores, which can lead to cell death (Nordström et al. 2018). Our results indicate that for the yeasts *C. albicans* and *C. tropicalis*, the permeabilization of the plasma membrane may be the mechanism of action used by these PIs to inhibit the growth of these yeasts (Fig. 3). As a consequence of membrane permeabilization, leakage of intracellular content may occur, leading to an imbalance in cell homeostasis and, consequently, cell death. Using DIC microscopy, we observed cellular dimorphism for *C. albicans* and *C. tropicalis*, such as the formation of hyphae and pseudohyphae. This phenotype may have been triggered by pH variations in the growth medium caused by the PIs action on the plasma membranes of these yeasts. Another observation was with the use of propidium iodide dye, which has the purpose of visualizing cell necrosis and late apoptosis, and in fact, marking was observed for these cells, indicating that the membranes of these cells were ruptured (Fig. 3). Other studies also show treatment with different PIs that cause permeability of the cytoplasmic membrane in various fungi (Oliveira et al. 2018; Dib et al. 2019; Silva et al. 2021).

In fact, positive dye labeling was observed for these cells, indicating that the membranes of these cells were ruptured (Fig. 3). Other studies also show treatment with different PIs that cause permeability of the cytoplasmic membrane in various fungi (Oliveira et al. 2018; Dib et al. 2019; Silva et al. 2021).

Next, we evaluated the increase in intracellular ROS production, which can be generated as a consequence of membrane permeabilization. Once again, ROS was observed for the yeasts *C. albicans* and *C. tropicalis* when incubated with the PIs, corroborating their growth inhibition (Fig. 4). ROS are normally generated in eukaryotic cells as a consequence of mitochondrial energy metabolism in the electron transfer chain during aerobic respiration (Saibabu et al. 2017). When excessive ROS generation occurs, the balance is lost and results in the oxidation of proteins, carbohydrates and lipids, DNA damage and activation of apoptotic pathways, leading to cell death (Soares et al. 2017). Other studies also observed an increase in ROS. Mello et al. (2019) found that *C. buinensis* cells incubated with 25 μM $_{\gamma 33-41}Pv\mathrm{D1}^{++}$ peptide showed an increase in endogenous ROS production. Likewise, in *C. parapsilosis* cells, the RcAlb-PepII peptide induced cell membrane damage and promoted endogenous ROS production (Dias et al. 2020).

Since PIs induce ROS production in *C. albicans* and *C. tropicalis* cells, a test was performed to verify whether the resulting oxidative stress is indeed relevant to cell death. Using the antioxidant AsA, the growth of *C. tropicalis* cells was almost unaffected during the 24 h of treatment with

PEF3, indicating that the toxic effect caused by this fraction in the yeast cell occurs through oxidative stress. Hayes et al. (2013) showed a defensin called NaD1 from *Nicotiana alata* plant that the antioxidant AsA decreases its antifungal activity. Soares et al. (2017) observed through assays to verify the increase in endogenous ROS production that oxidative stress is involved in the fungicidal activity of *Ap*Def1 defensin on *S. cerevisiae* cells. These reports not only reinforce our results but also indicate that the oxidative stress is involved in cell death induced by these PIs. For the other tested yeasts, the percentage of cells rescued from induced death was low, indicating that oxidative stress is not the main mechanism leading to PI-induced yeast death. In addition, in our test conditions, the AsA concentration used proved to be toxic to *C. buinensis*, out of the four tested yeasts, it is the most sensitive, and the control with AsA showed only 48.5% viable cells. The toxicity of high concentrations of AsA to *C. albicans* was reported by Ojha et al. (2009), and with increasing AsA concentration, the number of viable cell decreases.

The increase in the level of ROS in cells generates oxidative stress that can trigger the process of regulated cell death in yeast. To test this hypothesis, we analyzed the mitochondrial functionality and activation of metacaspases. All yeast species treated with PIs showed dissipation of mitochondrial membrane potential (Fig. 5), suggesting possible mitochondrial dysfunction. However, there was no evidence of metacaspase activation in any of the yeasts tested at 24 h (data not shown), which does not rule out the possibility that this process could occur at a time earlier than those analyzed in this study. Different peptides cause the loss of mitochondrial functionality and induce the apoptotic process (Lee et al. 2017; Lucas et al. 2017; Taveira et al. 2022).

To better understand the effects of PIs on yeasts, *C. albicans* was chosen to be observed by transmission electron microscopy due to its high clinical relevance. Different alterations were observed in cells grown in the presence of PEF2 and PEF3 (Fig. 6), and it is very likely that their presence in the medium caused stress conditions, preventing normal cell development. Other molecules also induce changes in the morphology of microorganisms. Padilla et al. (2022) confirmed through TEM that the ε-poly-L-lysine (EPL) damaged plasma membrane of *C. albicans* and *S. cerevisiae*. Treatment with the DvAMP peptide caused membrane invagination, alteration in mitochondria and the formation of a large vacuole in *Cryptococcus neoformans* (Yang et al. 2023).

Cell death can occur from independent events that occur individually or complement other mechanisms of action. Although the general mechanism of pore formation in the membrane is relatively expanded, studies have shown that this is not the only mechanism of action of AMPs. Several reports indicate that AMPs have multiple targets. The increasing amount of evidence suggests that these peptides have intracellular targets and can inhibit the cell wall, protein synthesis processes, and DNA/RNA in addition to inhibiting several other enzymatic functions (Klimpel et al. 2018; Li et al. 2018). In this case, we can suggest that PIs may act on intracellular targets, which could be related to cell death.

To verify whether these PIs inhibit yeast proteases, tests were performed to detect intracellular proteases of *Candida* species. To test this hypothesis, zymography was used coupled to SDS–PAGE. Zymography produced results only for active proteases in the *C. tropicalis* extract (Fig. 7). Generally, living cells produce a large amount of proteases to maintain growth, and in this case, some proteases may be inactivated in cell extracts. SDS in the sample buffer and the polyacrylamide gel can also cause irreversible conformational changes in these proteases, resulting in their inactivation. Other methods for detection of these proteases can be used, such as the native PAGE system and two-dimensional PAGE, in addition to casein supplementation in the medium (Ito et al. 2010). The *C. tropicalis* extract has enzymes with significant proteolytic activity, and this activity is significantly inhibited mainly by the PI present in PEF2. This may suggest that the mechanism of action of PIs for the yeast *C. tropicalis*, in addition to involving membrane permeabilization, ROS induction also involves the inhibition of proteases.

One of the main paradigms impeding clinical applications of AMPs as drugs in alternative therapies is their high toxicity for mammalian cells, diverse allergic reactions in patients and low oral viability due to enzymatic degradation (Lyu et al. 2016; Greco et al. 2020). In this work, we investigated the therapeutic potential of PI and found that PEF3 has very low hemotoxicity against mammalian cells in vitro (Fig. 8), with twice the concentrations of PI used in assays against yeast. Even using 400 μg mL^{-1} PI, the hemolytic activity did not exceed 6.6%. Contrary to the expected results for melittin, a potent AMP on *Candida* species has limited application due to its high hemolytic activity (Lee et al. 2015). Similar results were found for the peptides RR, D-RR and WR, which have low toxicity to mammalian cells (Toledo et al. 2021). Another assay was conducted using *G. mellonella* larvae to investigate the potential toxic effects of PEF3. This model has gained significant importance due to its resemblance to mammalian humoral and cellular immune responses, its ability to incubate pathogens at human physiological temperature (37 °C), the ease of inoculating therapeutic agents due to the larvae's size, associated with economic and ethical advantages (Wojda et al. 2017; Ménard et al. 2021). Notably, no significant toxic effects were observed in larvae inoculated with 200 μg mL^{-1} of

PEF3 (Fig. 9). Our findings are consistent with those of Ramalho et al. (2022), who reported that the synthetic peptide IKR18, when tested at various concentrations (400–1 µM), did not exhibit any signs of toxicity after 72 h of observation.

Conclusion

In this study, we investigated the antimicrobial properties of protease inhibitors (PIs) isolated from *C. chinense* seeds, specifically focusing on two distinct fractions, namely, PEF2 and PEF3. Our study revealed that these PIs possess the ability to inhibit the growth of *C. albicans*, *C. tropicalis*, and *C. buinensis*. Notably, we observed significant inhibitory effects, amounting to fungicidal activity, particularly in the case of *C. tropicalis*. Furthermore, our findings indicate that PIs induce cytoplasmic membrane permeabilization and lead to an overproduction of reactive oxygen species (ROS) in *C. albicans* and *C. tropicalis*. In vitro experiments demonstrated that PEF3 exhibited low cytotoxicity even at high concentrations when tested on mammalian cells. Moreover, in vivo toxicity assessments of PEF3 were conducted using *G. mellonella* larvae, confirming the absence of toxicity at the tested concentrations.

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Author contributions All the authors contributed extensively to the work presented in this manuscript. The study was conceived by MSS and VMG. Experimental procedures were carried out by MSS, GBT, RSG, LAS, MBC, TAMS, FFM and PSR. Data analyses were performed by MSS, GBT, OVM, SHS, RR, and VMG. The paper was written by MSS, GBT, AOC and VMG.

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Data availability All data generated or analysed during this study are included in this published article and its supplementary information.

Declarations

Conflict of interest The authors declare no conflict of interests.

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