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Characterization and Antimicrobial Activity of Protease and α-Amylase Inhibitors from Immature Fruits of *Capsicum chinense* Jacq.

Luiza Basso Bramusse[1]
https://orcid.org/0000-0002-9552-8582

Marciele Souza da Silva[1]
https://orcid.org/0000-0002-7914-4087

Layrana de Azevedo dos Santos[1]
https://orcid.org/0000-0002-8488-5656

Larissa Maximiano Resende[1]
https://orcid.org/0000-0002-8271-1169

Érica de Oliveira Mello[1]
https://orcid.org/0000-0002-1965-4560

Gabriel Bonan Taveira[1]
https://orcid.org/0000-0002-1733-3034

André de Oliveira Carvalho[1]
https://orcid.org/0000-0002-0031-1357

Rosana Rodrigues[2]
https://orcid.org/0000-0002-8180-7265

Valdirene Moreira Gomes[1*]
https://orcid.org/0000-0003-4145-428X

[1]Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Biociências e Biotecnologia, Campos dos Goytacazes, Rio de Janeiro, Brasil; [2]Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Ciências e Tecnologias Agropecuárias, Campos dos Goytacazes, Rio de Janeiro, Brasil.

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*Correspondence: valmg@uenf.br; Fax: +55-22-27486490 (V.M.G.)

HIGHLIGHTS

- Immature fruits of *C. chinense* presents AMPs with antifungal activity against yeasts.
- D1 fraction has greater antifungal activity.
- D1 fraction shows activity against *C. albicans* and *C. tropicalis*, two yeasts of medical interest.

Abstract: Antimicrobial peptides (AMPs) are small groups of proteins obtained from plants and animals. AMPs participate in the immune response, as they provide a quick line of defense against infections, while others may be related to the plant's defense against certain pests and pathogens. The objective of the present study was to evaluate an inhibitory activity of fractions obtained from immature fruits of *Capsicum chinense* Jacq. (accession UENF 1755) on trypsin, chymotrypsin and α-amylase families and on yeast growth. The peptides were obtained from the immature fruits using saline extraction. The extract was semi-purified by DEAE-Sepharose chromatography into two fractions: D1 (non-retained fraction) and D2 (retained fraction), and analyzed using SDS-tricine-gel electrophoresis. The antifungal activity of these fractions was tested on *Candida albicans*, *Candida buinensis*, *Candida parapsilosis* and *Candida tropicalis*. To elucidate the antimicrobial mechanism of these fractions, membrane permeabilization and endogenous reactive oxygen species (ROS) induction assays were performed. The fractions were also tested for inhibition of trypsin, chymotrypsin and α-amylase enzymes. The two fractions, D1 and D2, inhibited yeast

growth at a concentration of 100 µg.mL^{-1}, promoted membrane permeabilization and caused an increase in the induction of endogenous ROS in *C. albicans* and *C. tropicalis*. Both these fractions were able to inhibit trypsin and α-amylase enzyme, while only D1 inhibited chymotrypsin activity. Thus, D1 was found to possess a greater antifungal and enzymatic inhibition activity on trypsin, chymotrypsin and α-amylase.

Keywords: *Capsicum*; plant antimicrobial peptides; antifungal activity; protease inhibitors.

INTRODUCTION

The adverse effects of chemical pesticides, the frequent emergence of drug-resistant bacteria and fungi, and consequently, the discontinued use of some traditional antibiotics, has led to select increasingly resistant microorganisms, which directs us toward identifying new antimicrobial agents [1,2].

Plant antimicrobial peptides (AMPs) are molecules that exhibit hydrophobic and cationic properties rich in positively charged arginine and lysine residues, favoring their interaction with microbial cytoplasmic membranes. Rarely, there are also anionic AMPs, mainly in the plant kingdom, that adopt an amphipathic structure, carrying a high proportion of hydrophobic residues [3].

Generally, AMPs contain cysteine-rich residues and show different secondary structures, such as β sheets stabilized by two or three disulfide bonds and often exhibit a helical amphipathic structure [4]. Plant AMPs are grouped into different classes based in some features such as type of charge, cyclic structure, presence of disulfide bonds, and mechanism of action. The most common classes of AMPs reported so far include defensins, hevein-like proteins, cyclotides, knotin-like proteins, lipid transfer proteins (LTPs), thionins and protease inhibitors (PIs) [5]. Among these peptides: PIs, thionin-like peptide, defensins, vicilin-like peptide and some other AMPs have been isolated and identified from the *Solanaceae* plant family [6].

AMPs of plant origin have a variety of amino acid compositions and structures, many of which exhibit strong broad-spectrum antimicrobial activity and are capable of rapidly killing microbes [5]. Several AMPs have been isolated from different plants and plant organs, such as stem, root, seed, flower and leaf, which have exhibited antimicrobial activities against different microorganisms (fungi, viruses, bacteria, parasites and protozoa) [7].

In addition to antimicrobial activity, AMPs may have other biological properties, such as protease and carbohydrase inhibition. The antimicrobial mechanism by which these inhibitors act is attributed to their action on protein digestion, where reduction in the availability of amino acids prevents the synthesis of new proteins necessary for the normal development of the pathogen's metabolism. This property suggests the potential of these proteins as biotechnological tools [8,9].

Over the last few years, our research group has isolated and characterized antimicrobial peptides present in seeds of different plant species, which have shown inhibitory activity on proteases, especially serine proteases. In plants of the genus *Capsicum*, peptides characterized as inhibitors of trypsin and chymotrypsin-protease with antifungal activity (MIC 50-250 µg.mL^{-1}) were identified, mainly in *C. annuum* and *C. chinense* [10,11]. The antifungal activity of these AMPs was characterized by visualization of cell agglomeration and pseudohyphae formation or by hyphal morphological changes as well as by membrane permeabilization due to reactive oxygen species (ROS) induction [11,12].

MATERIAL AND METHODS

Plant material

Capsicum chinense seeds accession UENF 1755 were provided by the Laboratório de Melhoramento Genético Vegetal (LMGV), Centro de Ciências e Tecnologias Agropecuárias (CCTA), Universidade Estadual do Norte Fluminense Darcy Ribeiro, (UENF), Campos dos Goytacazes, Rio de Janeiro, Brazil. The seeds were sown in a 128-cell Styrofoam tray containing commercial substrate (4% nitrogen; 14% phosphorus; 8% potassium) (Vivatto, Brazil) and maintained in a growth chamber with a controlled temperature of 28 ºC and a photoperiod of 12 h light/dark. After seedlings were more than 10 cm high (about 20 days), they were transplanted into 5 L pots and placed in a greenhouse where they were irrigated with water once a day. The flowers were marked at anthesis, and after a period of 30 days after anthesis for immature fruits and 45 days for ripe fruits. Fruits were harvested and used for protein extraction.

Microorganisms

The yeasts *Candida albicans* (CE022)*, Candida parapsilosis* (CE002)*, Candida buinensis* (3982) and *Candida tropicalis* (CE017) were cultivated in Sabouraud agar culture medium at 30 °C and refrigerated at 4 °C in the Laboratório de Fisiologia e Bioquímica de Microrganismos (LFBM), at Centro de Biociências e Biotecnologia, UENF, Campos dos Goytacazes, Rio de Janeiro, Brazil.

Protein extraction from *C. chinense* fruits

The protein extraction from immature and ripe *C. chinense* fruits was performed according to the methodology proposed by Taveira and coauthors (2014) [13]. Briefly, the peduncles of all the fruits were removed and discarded, and 40 g of each immature and ripe fruit (without seeds) were collected and weighed. Further, they were separated and used for protein extraction. The fruits were ground in 200 mL of phosphate buffer (15 mM NaH_2PO_4; 10 mM Na_2HPO_4; 100 mM KCl; 1.5% ethylenediamine tetraacetic acid (EDTA), pH 5.4) (Sigma-Aldrich Co., St. Louis, MO, USA), for 15 min with the aid of a multiprocessor (Mix 3x1, Philips) and the homogenate was placed in a refrigerator under stirring for 2 hours. Further, the homogenate was centrifuged at 15400 $\times$ *g* for 45 min at 4 °C, where the supernatant was filtered using a paper filter, and the precipitate was discarded. Ammonium sulfate was then added at 70% saturation under agitation for 40 minutes and placed in a refrigerator overnight. The solution was centrifuged at 15400 $\times$ *g* for 45 min at 4 °C, the supernatant was discarded and the precipitate was heated in a water bath for 15 min at 80 °C. After centrifugation for 30 min at 15400 $\times$ *g*, the precipitate was discarded and the supernatant was dialyzed (benzoylated dialysis tube, Sigma-Aldrich) with distilled water and lyophilized (Lyotop K105 Lyophilizer, SP, Brazil). The final extract obtained was named peptide-rich extract (PRE).

Tricine SDS-PAGE

SDS-tricine-gel electrophoresis was performed according to the method described by Schagger and Von Jagow (1987) [14]. Ultra-low range molecular weight marker (MW 1060-26600 Da) (Sigma-Aldrich) was used.

Partial purification by anion exchange chromatography

A DEAE-Sepharose anion exchange column (Sigma-Aldrich) was used, with 50 mL of resin. The column was assembled under the action of gravity and was equilibrated with 100 mM Tris-HCl buffer pH 8.0 for separation of peptides. After the preparation of column, 50 mg of the protein extract from immature fruits was dissolved in 10 mL of the equilibrium buffer, centrifuged at 16000 $\times$ *g* for 3 min at room temperature and the supernatant was applied onto the column. Fractions of 3 mL were collected in a flow of 60 $mL.h^{-1}$, in a total of 80 tubes. The first 50 fractions were eluted with equilibration buffer (D1), and the retained proteins (D2) were eluted using equilibration buffer containing 1 M NaCl (Merck KGaA, Darmstadt, Germany). The absorbance of the fractions was measured at 280 nm [15] using a spectrophotometer (Spectrophotometer BEL LGS 53).

Inhibition of trypsin and chymotrypsin enzyme activities

The inhibitory activity of the peptides was determined by measuring the residual hydrolytic activity of trypsin and chymotrypsin using the substrates N-benzyol-D-L arginine-p-nitroanilide (BApNA) and N-Benzoyl-L-tyrosine p-nitroanilide (BTpNA) (Sigma-Aldrich), respectively, after pre-incubation with the protein fractions. Proteolytic activity was measured using a synthetic peptide derived from p-nitroanilide in 50 mM Tris-HCl buffer pH 8.0 at 37 °C, in a final volume of 200 µL. The reaction was stopped by adding 100 µL of 30% acetic acid (v/v). Next, the photometric reading of the treatments was measured based on the extent of p-nitroanilide release from the substrates at 405 nm using a spectrophotometer (Spectroquant Pharo 100, Merck KGaA) according to the methodology described by Ribeiro and coauthors (2013) [16].

Temperature stability

For the thermal stability assay of the trypsin inhibitory activity, fractions D1 and D2 (50 $\mu g.\mu L^{-1}$) were pre-incubated at various temperatures (40, 60, 80 and 100 °C) for 30 min in a water bath. After heat treatment, the aliquots were cooled on ice and the residual enzymatic activity of trypsin was tested as described in item “inhibition of trypsin and chymotrypsin enzyme activities” [16].

Reverse zymographic detection of protease inhibition

Protease inhibition was detected using the methodology of Felicioli and coauthors (1997) [17]. Fractions D1 and D2 were separated on polyacrylamide gel (12% SDS-PAGE) co-polymerized with 0.1% gelatin under semi-denaturing conditions (without SDS and β-mercaptoethanol in the sample buffer). After electrophoresis, the gel was washed twice using wash buffer (0.1 M Tris-HCl pH 8.0 containing 2.5% Triton X-100) for 60 min to remove the SDS present in the running buffer. The gel was then immersed in the incubation buffer (50 mM Tris-HCl pH 8.0, containing 20 mM $CaCl_2$ and 50 $\mu g.mL^{-1}$ trypsin) at 37 °C for 1 h. Thereafter, it was rinsed with distilled water to remove excess trypsin. Gel was first stained by a stained using solution containing 0.2% Coomassie Brilliant Blue G 250, 45% methanol, and 10% acetic acid for 30 min, followed by destaining. The presence of protease inhibitors was assessed based on inability of trypsin to digest gelatin according to the appearance of bands in the gel. We used 15 µL of a commercial trypsin inhibitor as control (Soybean Kunitz – Merck KGaA).

α-Amylase inhibition assay

The enzymatic activity assay for intestinal α-amylases from *Tenebrio molitor* was performed as described by Da Silva and coauthors (2018) [18] with some modifications. Larval intestines were macerated at 4 °C in sterile saline and subjected to centrifugation at 12000 × *g* for 10 min. The protein content in the supernatant was quantified using the bicinchoninic acid protein assay as described by Smith and coauthors (1985) [19]. Initially, starch hydrolysis was quantified by reducing sugar liberation based on the colorimetric assay with 3,5-dinitrosalicylic acid (DNS). A reaction mixture containing different concentrations of intestinal α-amylase extracted from *T. molitor* with 25 µL of 1% starch (Sigma-Aldrich) in a final volume of 200 µL in water was incubated at 37 °C for 30 minutes. Subsequently, 400 µL of DNS solution was added to the reaction and heated at 100 °C for 5 min, after that time the samples were read at 540 nm (Spectroquant Pharo 100, Merck KGaA). The unit activity (U) was defined as the quantity of the intestinal enzyme extract (in µg) that increased the absorbance at 540 nm by 0.1 absorbance unit over 30 min.

For the inhibition assay, D1 and D2 fractions (25, 50, 75 and 100 $\mu g.mL^{-1}$) were previously incubated with 10 U (4 µL) of intestinal α-amylase extract at 37 °C for 30 minutes. The residual enzyme activity was determined as described above. EDTA (5 mM, Sigma-Aldrich) was used as a positive control and 50 $\mu g.mL^{-1}$ of bovine serum albumin (Sigma-Aldrich) as a negative control. The percentage inhibition was calculated considering the control (enzyme only) as 100% enzyme activity.

Antifungal activity assay

Candida yeasts were transferred from the stock and grown in Sabouraud agar medium (Merck KGaA) for approximately 24 h at 30 °C. A portion of the culture was resuspended in 10 mL of Sabouraud broth (Merck KGaA). Cell numbers were quantified using a Neubauer chamber (LaborOptik Ltd, United Kingdom, UK) under an optical microscope. The quantitative test for fungal growth inhibition was performed using the protocol developed by Broekaert and coauthors (1990) [20], with modifications. To verify the effect of immature and ripe fruits fractions (D1 and D2) on yeast growth, 1×10^4 $cells.mL^{-1}$ were incubated in 100 µL of Sabouraud medium at 30 °C in 96-well microplate (Thermo Fisher Scientifc Inc, Waltham, MA, USA) in the presence of protein extracts at a concentration of 100 $\mu g.mL^{-1}$. The optical readings were measured at 620 nm (EZ Read 400, Biochrom Ltd, Cambridge, UK) after 24 hours. Fungal growth without the addition of the fractions was also determined. The experiments were performed in triplicate.

Fungal membrane permeabilization assay

The membrane permeabilization of yeast cells treated with the fractions obtained in anion exchange DEAE-Sepharose chromatography was evaluated using the SYTOX Green fluorescent probe (Invitrogen, Carlsbad, CA, USA), according to the methodology described by Thevissen and coauthors (1999) [21] with modifications. SYTOX Green dye penetrates the plasma membrane of structurally compromised cells, binds to nucleic acids and leads to cellular fluorescence. Immediately after 24 h incubation of the fungal cells with the protein fractions, 100 µL aliquots of cells were incubated in the dark, for 15 min with the fluorescent dye SYTOX Green, at a final concentration of 0.2 µM, according to the instructions provided by the manufacturer. Control cells were incubated only with the SYTOX Green dye under the same conditions. After incubation, 100 µL fungal cell suspension was incubated with 0.2 µM of SYTOX Green and 10 $\mu g.mL^{-1}$ of propidium iodide (PI) in microcentrifuge tubes for 10 min at 30 °C with constant agitation. The cells were then analyzed using an optical microscope, version 4.0 (Axioplan. A2, Zeiss, Germany), coupled to an Axio CAM MRc5 (Zeiss) camera and the images were analyzed using the Axiovision software, version 4.0

(Zeiss). The microscope is equipped with a set of fluorescent filters for the detection of fluorescein (excitation with wavelength between 450 and 490 nm and emission at 500 nm).

Determination of ROS induction

The production of intracellular ROS in the fungal cells of *C. albicans, C. parapsilosis, C. buinensis* and *C. tropicalis* with/without protein fractions was measured by incubating with the fluorescent probe 2´,7´-dichlorofluorescein diacetate (H_2DCDFA) (Calbiochem-EMD, San Diego, CA, USA), at a concentration of 20 µM. The samples were incubated in the dark for 30 min at 30 °C, under constant agitation. Fungal cells were analyzed under an optical microscope (Axioplan. A2, Zeiss) equipped with a set of fluorescent filters for fluorescein detection (excitation with wavelength between 450 and 490 nm and emission of 500 nm) [22].

Statistical analysis

Assays were performed in triplicate and with thrice repetition. All statistical analyses were performed using the GraphPad Prism software (version 8.0 for Windows). Statistical differences were assessed using Tukey's one-way analysis of variance (ANOVA) test. The test into account the analysis of variance between the means of the control and treatments, at the level of rigor 5% ($p < 0.05$).

RESULTS AND DISCUSSION

Extraction and characterization of peptides from immature and ripe fruits

Antimicrobial peptides extracted from different plant organs, such as seeds and fruits of the genus *Capsicum*, have already been described and characterized previously [13,15, 23-27]. Silva and coauthors (2014) [12], showed the antimicrobial potential of protein fractions obtained from seeds of *C. chinense* Jacq. accession UENF 1755 on the fungi *Colletotrichum gloeosporioides*, *C. lindemunthianum*, *Fusarium oxysporum* and *F. solani*, in addition to the inhibition of the activity of trypsin and α-amylase enzymes. The AMPs studied in this work were obtained from immature fruit of *Capsicum chinense* Jacq. accession UENF 1755.

In order to evaluate the type of proteins extracted, that would be more advantageous, two extractions were performed: acidic and saline extraction, according to Dias and coauthors (2013) [28] and Taveira and coauthors (2014) [13], respectively. Inhibition of the enzymatic activity of trypsin showed that the saline extraction was more significant in terms of obtaining AMPs and possible protease inhibitors (data not shown).

Protein saline extraction from immature and ripe fruits of *C. chinense* accession 1755, showed the presence of majority proteins bands of approximately 6.5 kDa (Figure 1a) in Tricine SDS-PAGE analysis in both immature fruits extract (IFE) and ripe fruits extract (RFE).

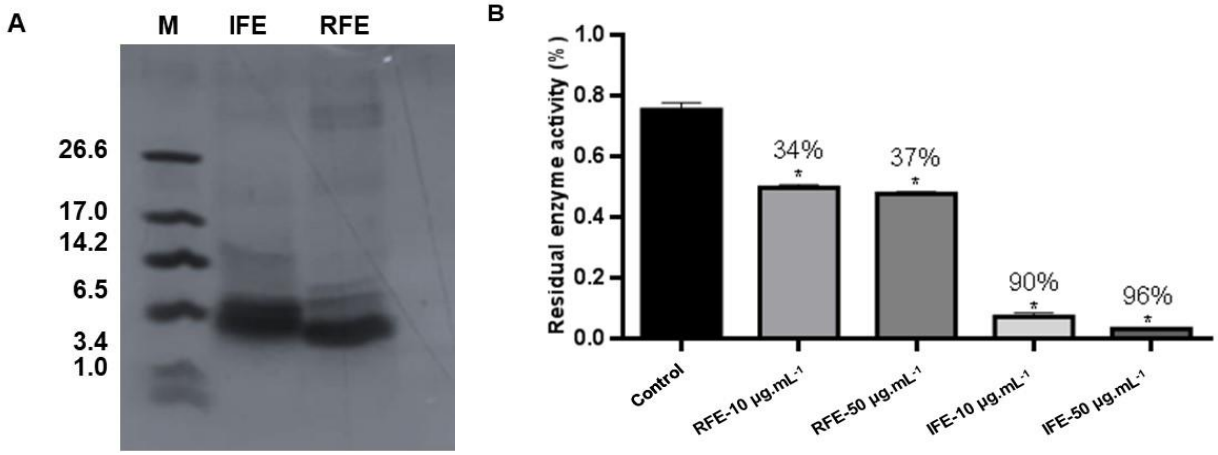

Figure 1. (a) Electrophoretic visualization by SDS-PAGE tricine of immature fruits extract (IFE) and ripe fruits extract (RFE) of *C. chinense* (accession UENF 1755). M – Low molecular mass marker (kDa); (b) Inhibitory effect of extracts from different maturation periods of *C. chinense* 1755 on trypsin enzyme activity: Ripe fruits extract - RFE 10 and 50 µg.mL^{-1}; Immature fruits extract – IFE 10 and 50 µg.mL^{-1}. Values are averages (±SD) of triplicates. Asterisks indicate a significant difference ($p < 0.05$) by Tukey's test.

Further, trypsin activity inhibition assays using immature and ripe fruits extracts was performed, to identify the highest inhibitory activity, between the two maturation times, at two different concentrations of 10 μg.mL^{-1} and 50 μg.mL^{-1}. Significant inhibition ($p < 0.05$) in trypsin activity of 34% and 37%, respectively, in the ripe fruit and 90% and 96%, respectively, of inhibition of trypsin activity in the immature fruit (Figure 1b) were observed.

Protease inhibitors are found in plants, mainly in storage tissues, acting as a defense by inhibiting digestive proteases from pests and pathogens [29,30]. Ribeiro and coauthors (2007) [31] isolated an inhibitor from *Capsicum annuum* seeds, called CaTI, with a molecular mass of 6 kDa, which showed antifungal activity against different yeasts. Silva and coauthors (2017) [11], corroborating these results, demonstrated that the CaTI inhibitor was able to inhibit the growth of the phytopathogenic fungi *C. lindemuthianum* and *C. gloeosporioides*. Dias and coauthors (2013) [28] isolated protease inhibitors from *C. chinense* seeds and demonstrated that these inhibitors were able to inhibit the growth of several yeast species, such as: *Saccharomyces cerevisiae, C. albicans, C. tropicalis, Pichia membranifaciens* and *Kluyveromyces marxiannus*.

Maracahipes and coauthors (2019) [25] demonstrated that there is a difference between the peptide fractions obtained from mature and immature *Capsicum* fruits in their expression, antifungal activity and enzymatic inhibition of trypsin. Pearce and coauthors (1988) [32] described that during the immature phase, wild tomato species showed a significant amount of protease inhibitors, acting as a means of defense in these plants against herbivores, and with the maturation of these fruits, the levels of inhibitors would decrease, making these fruits edible and promoting the dispersion of their seeds. Therefore, to confirm this result, we tested two protein extracts from the saline extraction - IFE and RFE of *C. chinense*, as well as the inhibition of the trypsin enzyme, and we observed a significant difference between the immature and ripe fruits, thus choosing to continue the experiments only with IFE (Figure 1b).

The immature fruit extracts were subjected to anion exchange chromatography in a DEAE-Sepharose column and two fractions named D1 (non-retained fraction) and D2 (retained fraction) were obtained. Figure 2a shows the chromatographic profile corresponding to these fractions. The fractions obtained by DEAE-Sepharose chromatography were revealed by SDS-tricine-gel electrophoresis. It was possible to observe in D1 fraction protein bands with molecular mass between 6.5 and 14 kDa, and in D2 fraction with a protein band of approximately 6.5 kDa (Figure 2b).

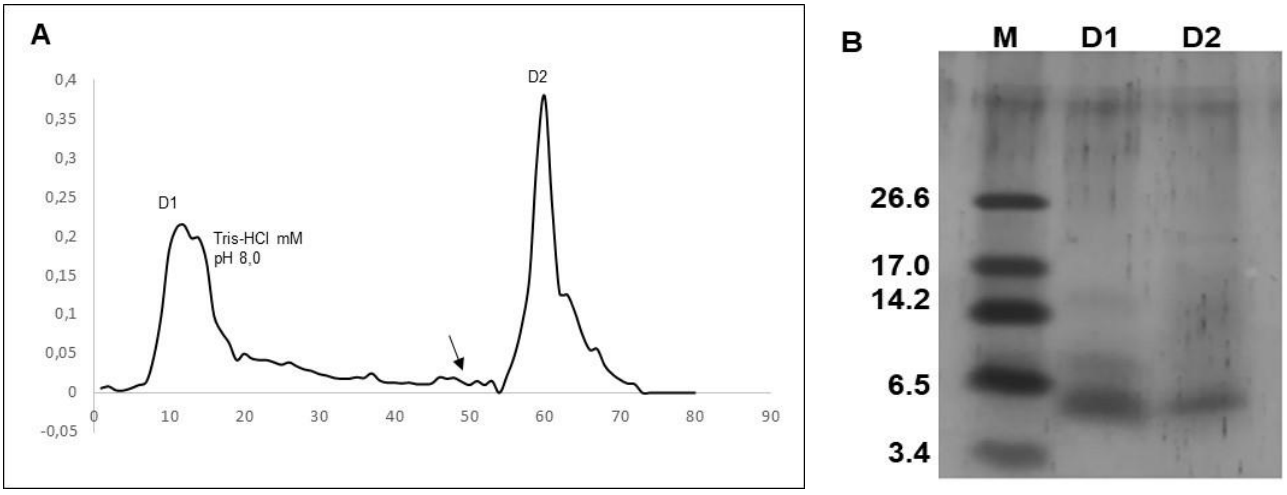

Figure 2. (a) Chromatographic profile of the protein extract from immature fruits, obtained in anion exchange chromatography on a DEAE-Sepharose column. Fraction D1 (not retained) was eluted in equilibration buffer and fraction D2 (retained) was eluted in equilibration buffer containing 1 M NaCl; (b) Electrophoretic visualization by tricine SDS-PAGE of fractions D1 and D2. M – Low molecular mass marker (kDa).

Characterization of the activities of inhibition of trypsin and chymotrypsin enzymes by fractions D1 and D2

To assess the inhibition of the serine protease activity, 10 μg.mL^{-1} and 50 μg.mL^{-1} of the fractions D1 and D2 were able to inhibit 15.5% and 94.65% in D1 and 30.75% and 85.73% in D2 for trypsin (Figure 3a), and a significant inhibition for both fractions was observed at a concentration of 50 μg.mL^{-1}.

To corroborate the *in vitro* trypsin inhibition assays, reverse zymography electrophoretic assays were performed. The assay was performed using 100 μg.mL^{-1} of D1 and D2 fractions. However, the protein bands appeared white in color, after coomassie staining instead of blue, both in the first assay and the

repetition. Despite the staining, it was possible to visualize protein bands, with molecular weights between 45 and 66 kDa, in both fractions, showing a positive result for protease inhibition and suggesting aggregation of these AMPs, as indicated by the arrows in (Figure 3b). Fractions D1 and D2 were also submitted to inhibition assays of the enzyme chymotrypsin at a concentration of 50 μg.mL^{-1}, where significant inhibition ($p < 0.05$) of the enzyme activity (93 and 30%, respectively) was observed (Figure 3c). In evaluating the stability of fractions D1 and D2 at different temperature. It is shown that D1 inhibited an average of 97% of trypsin activity after heating at 40, 60, 80 and 100 °C. Fraction D2 was also resistant to temperature variations, with an average of 95% enzyme inhibition (Figure 3d).

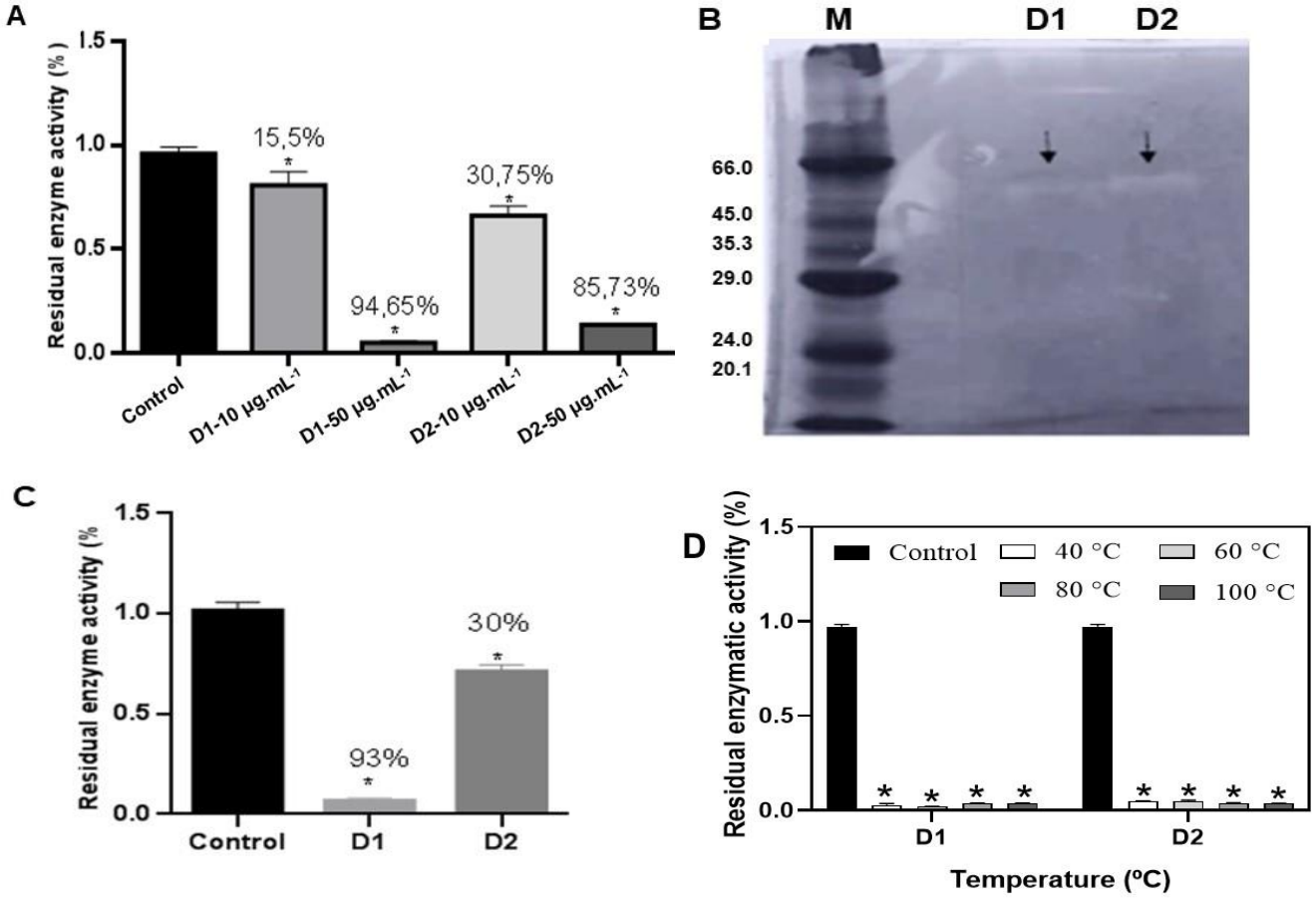

Figure 3. (a) Inhibitory effect D1 and D2 fractions of immature fruits of *C. chinense* 1755, on the activity of the trypsin enzyme: D1 (10 and 50 μg.mL^{-1}), D2 (10 and 50 μg.mL^{-1}); (b) Reverse zymogram to identify trypsin inhibitors in fractions D1 and D2 (100 μg.mL^{-1}). M - molecular mass markers (kDa). Arrows indicate trypsin inhibitor bands; (c) Inhibitory effect of fractions (D1 and D2) on chymotrypsin enzyme activity: D1 and D2 (50 μg.mL^{-1}): (d) The effect of temperature on D1 and D2 fractions analyzed for trypsin inhibitory activity at different temperatures. Values are means (±SD) of triplicates. Asterisks indicate significance by ANOVA test, and differences in mean values were considered significant ($p < 0.05$).

The main antimicrobial mechanism of the inhibitors on these microorganisms is the inhibition of protein digestion, reducing the availability of amino acids for the synthesis of new proteins, which are necessary for the metabolic development of the pathogen [9]. Some inhibitors also have the properties of inhibiting serine proteases and α-amylases together, called α-amylase/trypsin inhibitors [33-35]. As already seen in the literature that these inhibitors are thermostable at different temperatures, as shown by our results. Tamhane and coauthors (2007) [36] demonstrated a bifunctional inhibitor called CanPI-7 from *C. annuum* that was able to inhibit the activity of trypsin, chymotrypsin and intestinal proteases of the moth *Helicoverpa armigera*.

Inhibition of α-amylase enzyme activity by fractions D1 and D2

For the present study, we asssessed whether fractions D1 and D2, from the DEAE-Sepharose anion exchange chromatography (Figures 2a and 2b), would also have the inhibitory capacity not only of trypsin

and chymotrypsin, but also intestinal α-amylase from *Tenebrio molitor*. An inhibitory curve was obtained at the following concentrations of each fraction: 25 μg.mL^{-1}, 50 μg.mL^{-1}, 75 μg.mL^{-1}, and 100 μg.mL^{-1}, where differences between the inhibitions caused by D1 and D2 can be observed. The D1 fraction remains in the inhibitory range of: 71% for 25 μg.mL^{-1}, 73% for 50 μg.mL^{-1}, 76% for 75 μg.mL^{-1} and 79% for 100 μg.mL^{-1}. We observe that the D1 fraction does not need high concentrations to inhibit the action of the α-amylase. However, the D2 fraction presented a different curve, where inhibition increased considerably as the fraction concentration increased. We observed 11% for 25 μg.mL^{-1}, 28% for 50 μg.mL^{-1}, 48% for 75 μg.mL^{-1} and 72% for 100 μg.mL^{-1}, which indicates that the D2 fraction needs to be in higher concentrations to act as an α-amylase inhibitor. Asterisks indicate significance by ANOVA test, and differences in mean values were considered significant ($p < 0.05$). 5 mM EDTA was used in these assays as an inhibitor in the positive control (Figure 4). It has been shown that the D1 fraction has the ability to inhibit proteolytic enzymes (trypsin, chymotrypsin) and glycosidases such as α-amylase, while the D2 fraction has inhibitory activity for trypsin and α-amylase, requiring a higher concentration of α-amylase fraction for significant inhibition to occur, unlike D1. Thus, we can suggest that in the fractions, both in D1 and D2, there may be a bifunctional inhibitory potential which required further assessment.

Many inhibitors extracted from plants are already known, among which many are protein. A well-known inhibitor is amaranth amylase inhibitor, isolated from the plant *Amaranthus hypochondriacus,* is a Knottin-type inhibitor [37]. Within the same "Knottin" family, we found other inhibitors also extracted from plants, and with similarity in their sequence, such as: Mj-AMP1, Mj-AMP2: antimicrobial peptides from the seed of *Mirabilis jalapa* L. [38], PAFP -S: antifungal peptide from *Phytolacca americana* seed [39] WR-AI1: inhibitor of cystine node α-amylase in *Wrightia religiosa* [40], AC-AI1: α inhibitor-cystine node amylase in *Allamanda cathartica* [41]. It was recently described by Aguieiras and coauthors (2021) [15] an AMP from the defensin family, extracted from the fruits of the *C. chinense* pepper, capable of significantly inhibiting the activity of the α-amylase enzyme called *Cc*Def3. Pereira and coauthors (2018) [35] showed that the protein extract from the leaves of *C. annuum* was able to inhibit the activity of the human salivary α-amylase enzyme at all concentrations tested. In addition, these extracts showed the ability to inhibit trypsin enzyme activity. Diz and coauthors (2011) [24] found a lipid transfer protein (LTP) called Ca-LTP1, isolated from *C. annuum* seeds, was able to inhibit human salivary α-amylase activity.

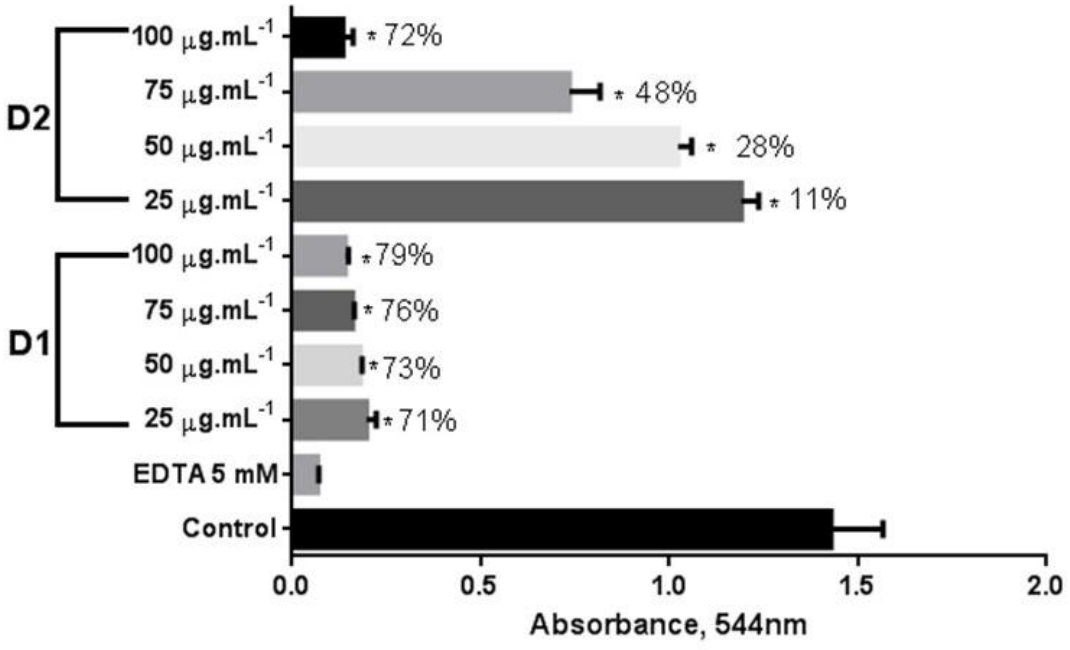

Figure 4. Inhibition curve of α-amylase activity of *T. molitor* in the presence and absence of 25 μg.mL^{-1}, 50 μg.mL^{-1}, 75 μg.mL^{-1} and 100 μg.mL^{-1} of fractions D1 and D2. EDTA was used as a positive control. Values are averages (±SD) of triplicates. Asterisks indicate significance by ANOVA test, and differences in mean values were considered significant ($p < 0.05$).

Yeast growth inhibition by fractions D1 and D2

The D1 and D2 fractions were used to verify their inhibition potential on the growth of the yeasts *in vitro*. Optical density readings of growth were taken within 24 h after incubation of cells with 100 μg.mL^{-1} of

fractions. Here, 56% inhibition for *C. buinensis* was observed in the presence of the D1 and 53% in the presence of the D2 fraction. Further, the D1 and D2 fractions showed an inhibition of 40% and 28%, respectively, for *C. tropicalis*. For yeast *C. albicans*, the D1 fraction showed a significant inhibition of 62% while D2 fraction revealed 59% inhibition. The D1 and D2 fractions displayed an inhibition of 44% and 64%, respectively, for *C. parapsilosis*. Absorbance values are represented as mean (± standard deviation) of triplicates. Asterisks indicate significance by ANOVA, and differences in mean values were considered significant at $p < 0.05$) (Figure 5).

Although the mode of action of AMPs in cells is still not well completely understood, it is already known that they have the ability to cause membrane permeabilization and, consequently, cell death [42,43].

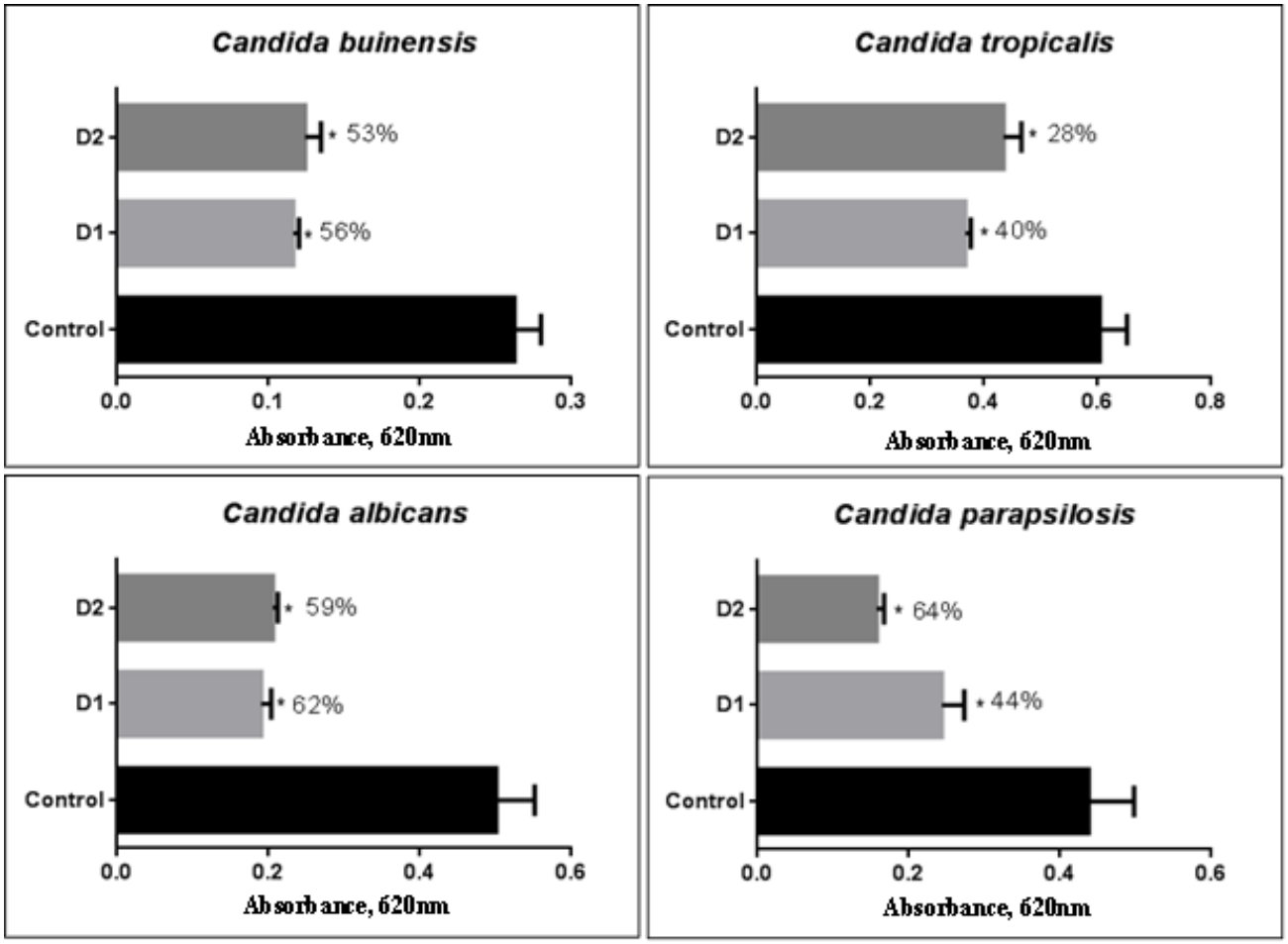

Figure 5. Inhibition of yeast growth after 24 hours of incubation with D1 and D2 at a concentration of 100 µg.mL^{-1}. (%) Percentage of inhibition caused by fractions. Asterisks indicate differences ($p < 0.05$) between the experimental and control treatments by Tukey's test.

Effect of fractions D1 and D2 on membrane permeabilization

In order to understand the possible mechanism responsible for the inhibition of yeast growth, aliquots of cells treated with fractions D1 and D2 were incubated with SYTOX Green and propidium iodide (Figure 6). A considerable decrease in the number of cells was observed after treatment when compared to that of the control *C. buinensis* cells. In the presence of 100 µg.mL^{-1} of fractions D1 and D2, the cultured cells only showed SYTOX Green and propidium iodide staining for the D2 fraction treatment, however, due to the reduced number of cells the result was not significant.

For *C. tropicalis* and *C. albicans*, a considerable decrease in the number of cells was evident when compared to that in the control. There was no significant labeling of cells in the control. When the cells were grown in the presence of D1 and D2 fractions (100 µg.mL^{-1}), SYTOX Green and by propidium iodide staining was observed, indicating that these fractions were able to cause permeabilization in the cell membrane, and these cells were no longer viable, especially cells treated with D1. For *C. parapsilosis*, we visualized a considerable decrease in the number of cells when compared to the control and morphological alterations, such as the formation of pseudohyphae were observed. When cells were grown in the presence

of 100 µg.mL^{-1} fractions D1 and D2, they did not show a significant SYTOX Green and propidium iodide staining.

Some researchers suggest the ability to damage the membrane as an efficient mechanism, reducing the chances of the pathogen developing resistance, having already been elucidated that AMPs have amphipathic characteristics, attributing to them the ability to interact with biological membranes [44].

Protease inhibitors are able to permeabilize the membrane of different pathogens, such as the trypsin inhibitor CaTI. *S. cerevisiae* and *C. albicans* cells showed SYTOX Green staining, indicating the permeabilization of their membranes [26]. Dib and coauthors (2019) [45] demonstrated a protease inhibitor (IETI), isolated from *Inga edulis* seeds, which inhibited the yeasts *C. buinensis* and *C. tropicalis*, caused membrane permeabilization and consequently affected cell viability. It is important to emphasize that membrane permeabilization and inhibition of microorganism growth are not necessarily related phenomena. It is possible to find peptides that inhibit the growth of microorganisms and do not permeabilize membranes and others that permeabilize membranes and do not inhibit growth [46].

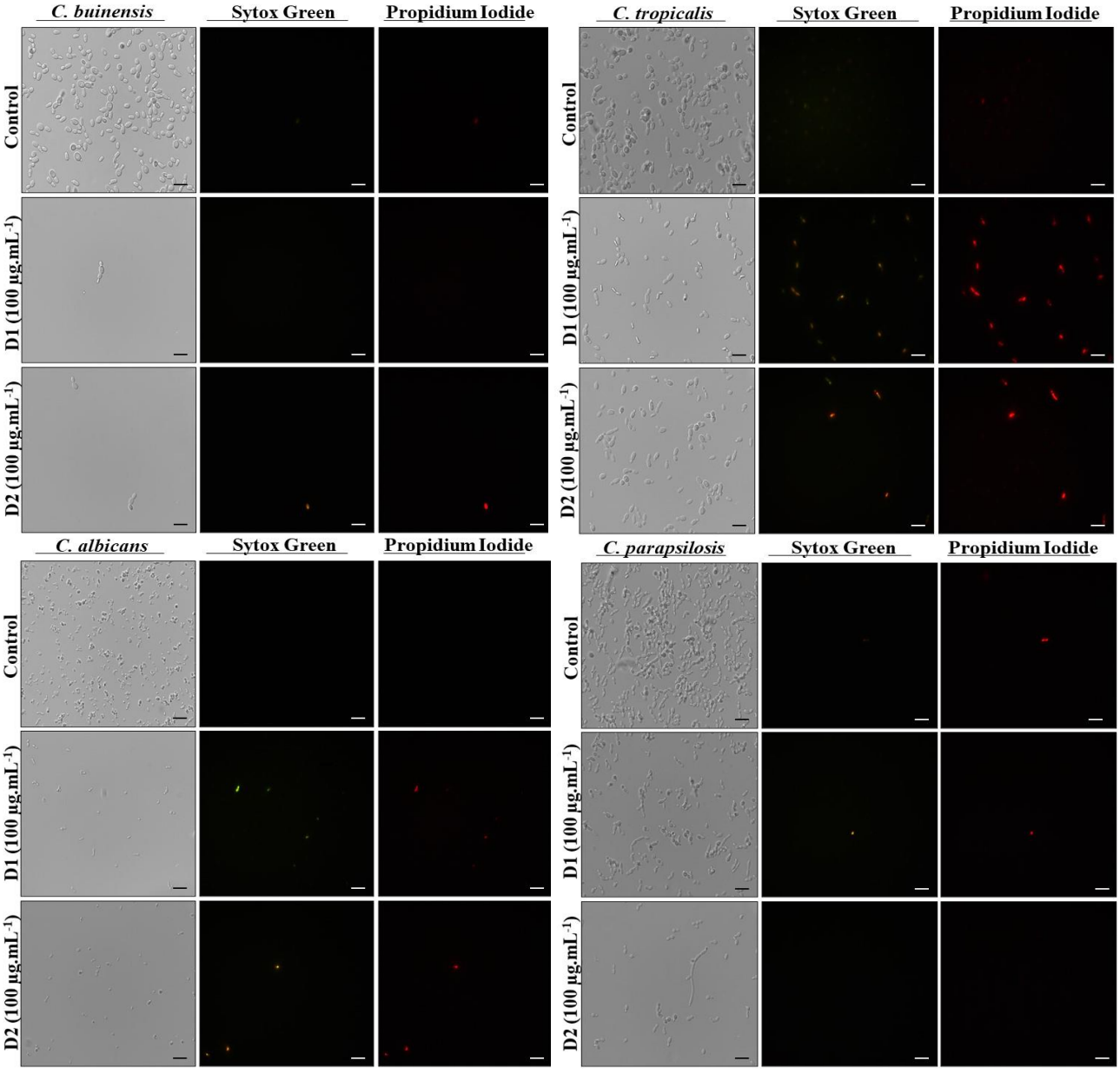

Figure 6. Images of membrane permeabilization assay in yeasts *C. buinensis*, *C. tropicalis*, *C. albicans* and *C. parapsilosis* cells after treatment with fractions D1 and D2 at a concentration of 100 µg.mL^{-1} for 24 hours. Cells were treated with fluorescente probes SYTOX Green and propidium iodide. Bars = 20 µm.

Effect of fractions D1 and D2 on the induction of intracellular ROS

Another approach to elucidate the mechanism of action of AMPs is through the induction of intracellular accumulation of ROS, which can generate DNA damage, oxidation of proteins, carbohydrates and lipids, including activation of apoptotic pathways [43]. Reactive oxygen species are generated as a natural by-product of normal oxygen metabolism and play important roles in cell signaling. However, under stress conditions, ROS levels can increase dramatically, causing significant cell damage [47].

To assess whether 100 $\mu g.mL^{-1}$ of D1 and D2 fractions would cause an increase in endogenous ROS production, the H_2DCFDA probe was used in different yeast species (Figure 7). For *C. buinensis*, fluorescence observed in the treatment with D1, but not in D2. Here, it is worth mentioning that the number of *C. buinensis* cells decreases considerably compared to the control. Moreover, for *C. albicans*, staining with the dye was not observed for both fractions. For the yeast *C. tropicalis,* we observed fluorescent staining in the treatment with D1 and D2, however there was a difference when compared to that in control, since the control cells also showed endogenous fluorescence. This yeast proved to be more sensitive to the D1 fraction, and even with an absorbance not very high for the microscopy assays, we can observe a decrease in microbial growth and several morphological changes, coupled with the fluorescent staining with propidium iodide indicating that those cells were no longer viable.

Conversely, no significant labeling was found in *C. parapsilosis* following D1 treatment, while for the D2 fraction, there was probe labeling, which leads us to believe that the D2 fraction increased the level of ROS when compared to the control.

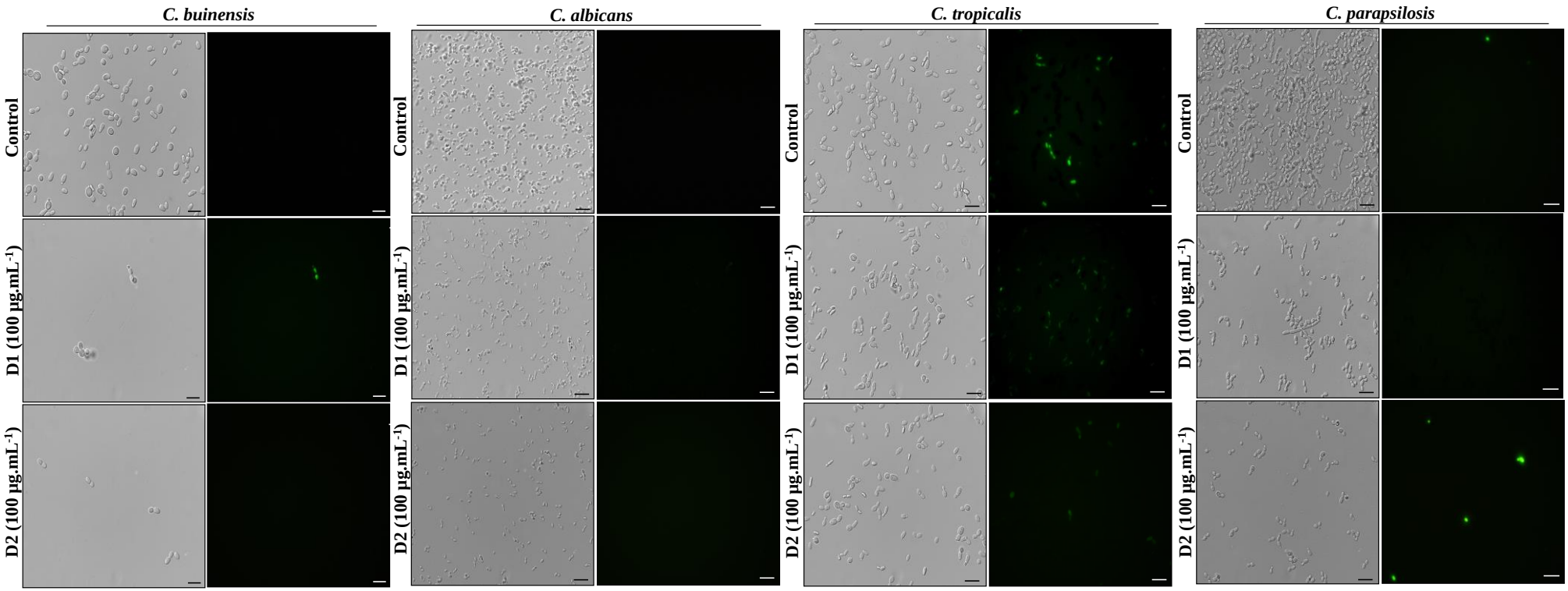

Figure 7. Images oxidative stress assay by ROS detection in yeasts *C. buinensis*, *C. tropicalis*, *C. albicans* and *C. parapsilosis* cells after treatment with fractions D1 and D2 at a concentration of 100 µg.mL^{-1} for 24 hours. Cells were treated with the 2',7'-dichlorofluorescein diacetate (H_2DCFDA) fluorescent probe. Bars = 20 µm.

CONCLUSION

The results described in this work show, for the first time, that immature fruits of *C. chinense* accession UENF 1755, presents fractions rich in AMPs with antifungal activity against yeasts. It is possible to affirm that new analyzes are necessary of these fractions obtained from immature fruits of *C. chinense* (UENF accession 1755). A purification and more specific studies for the D1 fraction are of great interest, due to the results obtained in this work, where the D1 fraction proved to be a promising protein fraction because it has the highest antifungal activity, being able to inhibit the four yeasts tested and cause morphological changes, and it is also possible to observe SYTOX Green and ROS labeling in *C. albicans* and *C. tropicalis*, two yeasts of great medical interest due to the high rates of serious hospital infections caused by these two species [48], in addition to significant inhibition of the activity of trypsin, chymotrypsin and α-amylase enzymes. It would be interesting to elucidate the peptides present in this fraction and their mechanisms of action, opening the possibility of obtaining purified peptides with possible potential for both medical and agronomic interest. The D1 fraction is the most promising fraction in view of the results obtained in this study, requiring further analysis of the peptides present in this protein fraction and assessment of their mechanisms of action in future studies.

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