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PARTIAL CHARACTERIZATION OF A IMMUNE-RELATED LIPID TRANSFER PROTEIN (LTP) AND ACTIVITY AGAINST YEASTS OF MEDICAL INTEREST FROM PROTEINS OF *CANAVALIA ENSIFORMES* (JACK-BEAN) SEEDS

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ABSTRACT

The aim of this work was to characterize antimicrobial peptides from *Canavalia ensiformis* seeds and evaluate their antimicrobial activity against fungi. The crude extract was subjected to anion-exchange chromatography in a DEAE-Sepharose column, resulting in two fractions called D1 and D2. The D1 basic fraction was subjected to reverse-phase chromatography on a C2/C18 column, where it was separated into six new fractions named (RP1, RP2, RP3, RP4, RP5 and RP6). The entire purification process was monitored using tricine gel electrophoresis. In D1 fraction a lipid transfer protein (LTP) immune related protein were also detected by ELISA. The D1 fraction inhibited the growth of the yeasts *Candida albicans*, *Candida tropicalis* and *Pichia membranifaciens* and promoted changes in cellular agglomeration in some of the yeasts tested, including the formation of pseudohyphae in *C. albicans*. In the "spot assay", different degrees of sensitivity were observed, indicating that the yeast *C. albicans* has a fungicidal effect and that the other tested strains have a fungistatic effect. Membrane permeabilization was assessed, and when observed with a fluorescence microscope, all yeast

species showed fluorescence in the presence of the D1 fraction. It was also shown that this fraction inhibits the acidification of the medium of *Saccharomyces cerevisiae* and *C. albicans*.

KEYWORDS: Antimicrobial peptides, yeast, LTP, fungi.

INTRODUCTION

Plants generally present defense mechanisms against pathogen attack and are responsible for producing a wide array of compounds to ward off these pathogens.[1] The defense repertoire of plants includes morphological barriers, secondary metabolites and the production of antimicrobial peptides (AMPs). AMPs are produced by several species, including bacteria, insects, plants and vertebrates. They constitute a heterogeneous class of low molecular mass proteins, usually small cationic peptides, and exhibit a broad spectrum of antimicrobial activity.[2-5] Despite the abundance and biologic activity of different families and the presence of some species-specific AMPs, the main groups of plant AMPs are represented by defensins, thionins, cyclotides, snakins and lipid transfer proteins (LTPs).[6,7] The mechanism of action of AMPs has been studied for selected peptides. AMPs can interact with microbial membranes, resulting in different possible modes of action, depending on the peptide and the microbial species. The peptides can be membrane-disruptive, resulting in cell lysis, or membrane interaction can lead to the formation of transient pores and the transport of peptides inside the cell, bringing them into contact with intracellular targets.[5,8] AMPs can have multiple intracellular targets; bind DNA, RNA and proteins; and inhibit cell wall synthesis and DNA, RNA or protein synthesis.[5,9,10] Thus, the concept of antimicrobial peptides as potent pharmaceuticals is firmly established in the literature, and most research articles on this topic conclude by stating that AMPs represent promising therapeutic agents against bacterial and fungal pathogens. Specifically lipid transfer proteins (LTPs) are a structurally related antimicrobial family of proteins that were originally defined by their *in vitro* ability to transfer lipids between membranes. It is a homogeneous class of small (7-10 kDa) and contains eight cysteine residues with four conserved disulfide bridges.[4]

Canavalia ensiformis (commonly known as jack-bean) is a leguminosae of the Fabaceae family that originated from South America. It is grown in the tropics and subtropics and produces high yields, especially in regions of low altitude, high temperature and relative humidity.[11] In Brazil, it is used as animal fodder and for human nutrition.[12] *C. ensiformis* is a natural source of proteins such as urease, which has already been shown to have

antimicrobial activity.[13,14] Oliveira *et al*.[15] have also found that many proteins present in the seeds of the Jack bean are detrimental to the development of the bruchid insect *Callosobruchus maculatus.* In this study, we investigate the antifungal activity of *C. ensiformis* proteins against different pathogenic yeasts to determine whether these proteins play an important role in protection against microbial infection.

MATERIALS AND METHODS

Plant material

Seeds of *Canavalia ensiformis* were provided by Profª Drª Antonia Elenir Amâncio Oliveira, Laboratório de Química e Função de Proteínas e Peptídeos from Universidade Estadual do Norte Fluminense - Darcy Ribeiro, Campos dos Goytacazes/RJ.

Yeast

Candida albicans (CE0022), *Saccharomyces cerevisiae* (1038), *Pichia membranifaciens* (CE0015) and *Candida tropicalis* (CE0017) were maintained at the Laboratório de Fisiologia e Bioquímica de Microrganismos, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil. The yeasts were maintained on Sabouraud agar (1% peptone, 2% glucose and 1.7% agar-agar, Merck).

Purification of the *Canavalia ensiformis* proteins

The purification of proteins from *Canavalia ensiformis* seeds was performed as described by Egorov *et al*.[16] An anion-exchange DEAE-Sepharose column equilibrated with 0.02 M Tris-HCl buffer, pH 6.0, was used. Elution of the bound fraction was carried out using equilibration buffer containing NaCl 1 M. The unbound fraction (D1) was pooled, diluted in a solution containing 0.1% (v/v) trifluoracetic acid (TFA) plus 0% acetonitrile (v/v) and injected into a C2/C18 ST 4.6/100 reverse-phase (RP) column (GE HealthCare UK Ltd, Buckinghamshire, UK) for high performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan). The chromatography was developed at a flow rate of 0.5 mL.min^{-1} with 100% solvent A (0.065% TFA plus 2% acetonitrile) for 10 min, 100% solvent B (80% acetonitrile containing 0.05% TFA) for 40 min and finally 100% solvent B for 5 min. Proteins were monitored by on-line measurement of the absorbance at 220 nm.

Tricine-SDS-PAGE analysis

Tricine-SDS-PAGE analysis was performed according to the method described by Schagger and Von Jagow.[17]

Elisa assay

LTP-like proteins were detected in D1 fraction by enzyme-linked immunosorbent assay (ELISA) according Engvall and Perlmann,[18] using an anti-*Vigna unguiculata* LTP antibody produced in rabbits (primary antibody),[19] diluted 1:500 and a peroxidase-conjugated anti-rabbit IgG antibody (secondary antibody) diluted 1:500. Peroxidase activity was revealed by using ortho-phenylenediamine. Assay absorbance was measured at 492 nm.

Effects of *C. ensiformis* proteins on yeast growth

The yeast growth assay was performed following the protocol developed by Broekaert *et al*.[20] with some modifications. To determine the effect of the D1 fraction on yeast growth, *C. albicans*, *S. cerevisiae*, *P. membranifaciens* and *C. tropicalis* cells (10.000 cells per 1 mL of Sabouraud broth) were incubated at 30ºC in 200 µL in 96-well microplates in the presence of different concentrations of the D1 fraction (25, 50, 100, 200 or 400 $\mu g.mL^{-1}$). The growth was evaluated by turbidity readings at a wavelength of 670 nm every 6 h for a period of 24 h Yeast growth without the addition of D1 fraction was also determined.

Spotting assay

To evaluate the antifungal effect and to discriminate fungistatic versus fungicidal activity, yeasts (1×10^4 cells per mL) were incubated with the D1 fraction at different concentrations (200 and 400 $\mu g.mL^{-1}$) using a standard spotting assay. A series of tenfold dilutions was made, and a 5 µL aliquot of each dilution was spotted onto media with Sabouraud agar (Merck, Germany). The plates were incubated at 30 ºC for 24 h.

Optical microscopy analysis

After a 24 h growth period, the yeast cells were separated from the growth medium by centrifugation, washed in Sabouroud broth (Merck, Germany) and visualized using an optical microscope at 1.000 X magnification (Axio Imager A2 Zeiss). The yeast cells grown in the absence of D1 fraction were also visualized.

Evaluation of cell permeability

The permeabilization of the plasma membrane was measured by *SYTOX Green* (Invitrogen, Grand Island, NY, USA) uptake as described by Thevissen *et al.*[21] *C. albicans*, *S. cerevisiae*, *P. membranifaciens* and *C. tropicalis* cells were grown in the presence of the D1 fraction (25, 50, 100, 200 and 400 $\mu g.mL^{-1}$). One hundred-microliter aliquots of yeast cell suspension were incubated with 0.2 µM *SYTOX Green* in 96-well microplates for 30 min at room temperature

with periodic agitation, followed by observation under an optical microscope (Axio Imager A2 Zeiss) equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths, 450 - 490 nm; emission wavelength, 500 nm). Negative (no D1 fraction added) controls were also assayed to evaluate membrane permeabilization.

Inhibition of glucose-stimulated acidification of the medium by *S. cerevisiae* and *C. albicans*

The Sabouraud broth (100 mL) (Merck, Germany) containing the *S. cerevisiae* or *C. albicans* cultures was shaken for 16 h at 30ºC with good aeration. The cells (DO_{600} = 0.2) were pelleted by centrifugation at 3,000 x *g* (5 min, 4 ºC) and washed three times with water. The effect of the D1 fraction on the yeast metabolism was determined by incubating the yeast cells (10^{-4} cells per 1 mL) with 800 µL of 0.01 M Tris-HCl buffer, pH 6.0. D1 fraction was added to a final concentration of 50, 100, 200 or 400 $\mu g.mL^{-1}$. A 0.5-M glucose solution was added to a final concentration of 0.1 M. After the incubation period, pH measurements were taken each subsequent minute for 30 min. Controls without the addition of D1 fraction were included to evaluate the influence of D1 on H^+ extrusion by yeast cells. The extent of H^+ extrusion was calculated as the difference between the initial (T = 0) and final (T = 30 min) pH (△ pH), and the resultant values were input into the equation pH = -log $[H^+]$.[22]

RESULTS

Characterization of antimicrobial peptides

The resulting suspension after extraction was fractionated by anion-exchange chromatography in DEAE-Sepharose and reverse-phase chromatography (RP) in the C2/C18 ST 4.6/100 column. Two protein peaks, named D1 and D2 fractions, were eluted with equilibrium buffer and 1 M NaCl, respectively (Fig. 1A). The non-adsorbed fraction (D1) from the anion-exchange chromatography assay was further fractionated by reverse-phase chromatography. The D1 fraction was separated into five new fractions named RP1, RP2, RP3, RP4 and RP5 (Fig. 1B). The fractions obtained after anion-exchange and RP chromatography were analyzed by SDS-tricine gel electrophoresis. The D1 and D2 fractions showed the presence of different bands with molecular weights of 8.000 Da to 16.000 Da (Fig. 2A). When we analyzed the five fractions obtained after reverse-phase chromatography, it was possible to observe two major bands in the RP2 and RP3 fractions with apparent molecular weights of 6.000 and 8.000 Da. RP4 presents three major bands, with molecular weights of approximately 9.000, 8.000 and 6.000 Da. The RP5 fraction showed a low number

of bands, ranging of 6.000 to 16.000 Da (Fig. 2B). Interestingly LTP immunerelated proteins were detected in D1 fraction by ELISA assay using an anti-*V. unguiculata* LTP antibody (Fig. 3).

Antimicrobial activity of the protein fraction against different yeasts

After the purification process, we tested the effect of the D1 fraction as an inhibitor of the growth of different yeast strains, pathogenic or not. We noted that an inhibitory effect could be observed for *C. albicans* (Fig. 4A), *P. membranifaciens* (Fig. 4C) and *C. tropicalis* (Fig. 4D). At both 200 and 400 $\mu g.mL^{-1}$, *C. albicans* have an accentuated reduction of their growth by 50% or more. We noted that an inhibitory effect could be observed for *P. membranifaciens* (Fig. 4C) and *C. tropicalis* (Fig. 4D) at all tested concentrations (200 and 400 $\mu g.mL^{-1}$). No inhibition was noted for *S. cerevisiae* in the presence of the D1 fraction (Fig. 4B). To further analyze the involvement of the antimicrobial activity of the D1 fraction and to verify its fungitoxic properties on *C. albicans*, *S. cerevisiae*, *P. membranifaciens* and *C. tropicalis* growth, different degrees of sensitivity were shown in a spot assay, among the strains and concentrations tested (Fig. 5). After the growth assay, cells were grown on Sabouraud agar (4% glucose) with or without the D1 fraction. Only *C. albicans* was sensitive to the D1 fraction (Fig. 5), where a marked inhibition was detected at both tested concentrations (200 and 400 $\mu g.mL^{-1}$), suggesting a fungicidal effect. For the rest of strains tested, *S. cerevisiae*, *P. membranifaciens* and *C. tropicalis*, no inhibition was observed (Fig. 5), suggesting a fungistatic effect. Tests with D1 fraction concentrations of 25, 50 and 100 $\mu g.mL^{-1}$ were also performed, but no inhibitory effect on the growth of the yeasts was observed (data not shown).

To understand the D1 fraction's possible mode action, we observed the yeast cells by optical microscopy (Fig. 6) to determine whether morphological changes were occurring. Photomicrographs of yeast cells were taken after 24 h of yeast growth in the absence and presence of the D1 fraction. Normal growth and development were observed for all control cells analyzed (Fig. 6). For optical analyses, a cellular agglomeration could be observed for *C. albicans* and *P. membranifaciens* (Fig. 6). For *C. albicans*, it was also possible to observe a morphological shape alteration, consistent with the formation of pseudohyphae structures. This morphological alteration confirms the feature dimorphic that *Candida albicans* present, depending on environmental conditions, growing alternately between a yeast phase and a hyphal phase. No alterations were observed for the *S. cerevisiae* and *C. tropicalis* strain. To

further analyze the D1 fraction's possible mode of action, we tested the ability of the D1 fraction to permeabilize the plasma membrane of all yeast strains used (Fig. 7). In this study, membrane permeabilization was assessed after the growth assay in the presence or absence of the D1 fraction and 30 min after the addition of *SYTOX Green*. When observed with a fluorescence microscope, all yeast species showed fluorescence in the presence of the D1 fraction compared with controls, where cells were grown in the absence of the D1 fraction (Fig. 7).

Effect of protein fraction on the glucose-stimulated acidification of medium by yeast cells

We investigated whether the D1 fraction could interfere with the functioning of yeast H^+-ATPases. Therefore, we monitored the glucose-stimulated acidification, a phenomenon dependent on the activity of H^+-ATPases, of the incubation medium by *S. cerevisiae* and C. *albicans* cells in the presence of 50, 100, 200 and 400 $\mu g.mL^{-1}$ of the D1 fraction. As shown in Figure 8, the D1 fraction was able to inhibit the acidification of the medium by the *C. albicans* strain at all concentrations tested in a dose dependent manner and with a pre-incubation time of 30 min. The plasma membrane H^+-ATPase plays an essential role in fungal cell physiology.

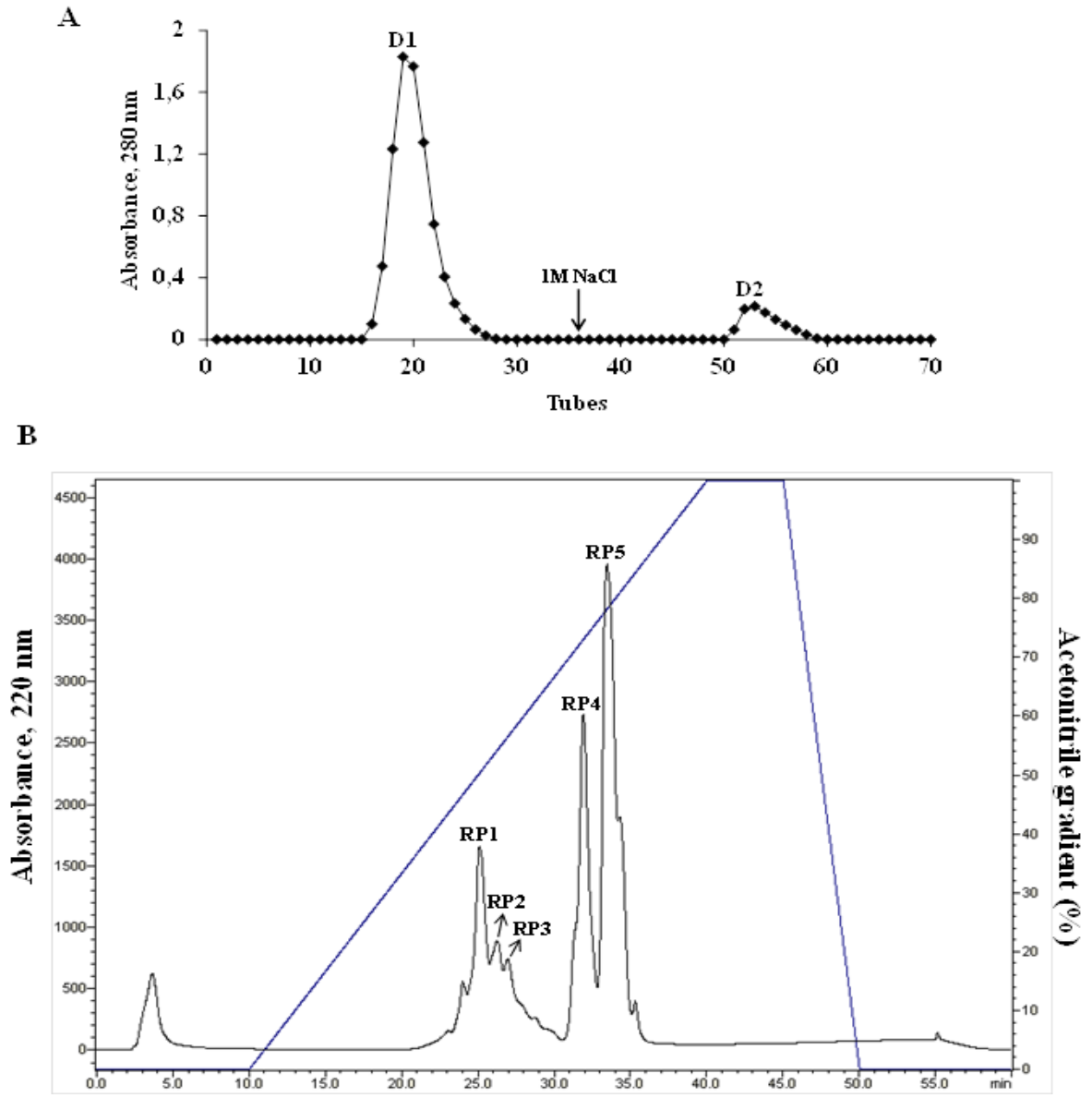

Fig. 1: Purification of the D1 fraction from *Canavalia ensiformis* seeds. (A) Anion-exchange DEAE-Sepharose chromatography. The column was previously equilibrated with 0.02 M Tris-HCl buffer, pH 6.0. Elution was carried out using NaCl 1M in the equilibration buffer, at 60 mL.h^{-1}; (B) RP-HPLC chromatography. D1 fraction was applied to a C2/C18 ST 4.6/ 100 RP column and run in a Shimadzu apparatus. The chromatography was developed at a flow rate of 0.5 mL.min^{-1} with 100% solvent A (0.065% TFA plus 2% acetonitrile) for 10 min, 100% solvent B (80% acetonitrile containing 0.05% TFA) over 40 min and finally 100% solvent B over 5min. Proteins were monitored by on-line measurement of the absorbance at 220 nm. The line represents the acetonitrile gradient and the dark line represents the protein elution profile at 220 nm.

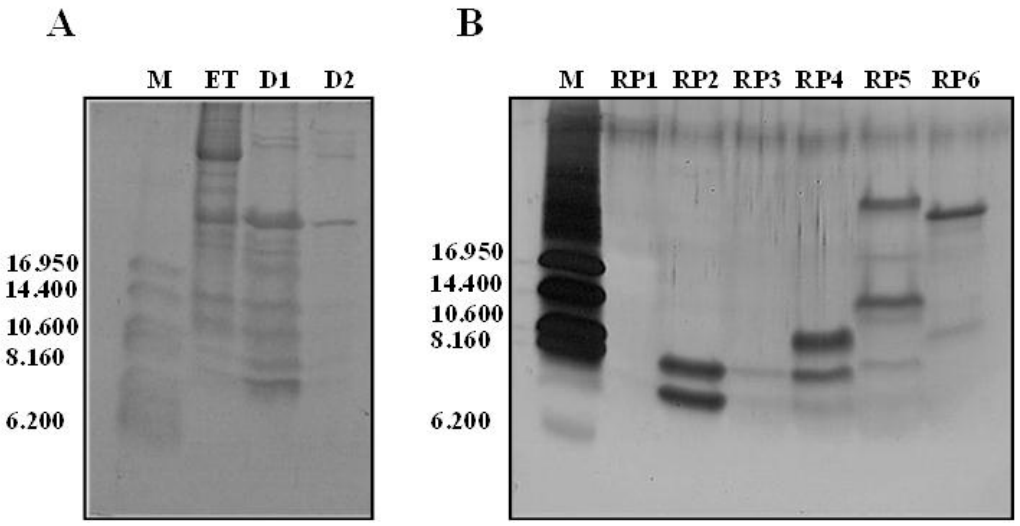

Fig. 2: SDS-Tricine-gel electrophoresis of *Canavalia ensiformes* proteins during the purification process. (A) ET, total extract; D1, non-retained fraction eluted from anion exchange column; D2, retained fraction eluted with 1M NaCl from anion exchange column. (B) Fractions eluted from the RP column: RP1, first peak; RP2, second peak; RP3, third peak; RP4, fourth peak and RP5, fifth peak. M represent the molecular mass markers (kDa).

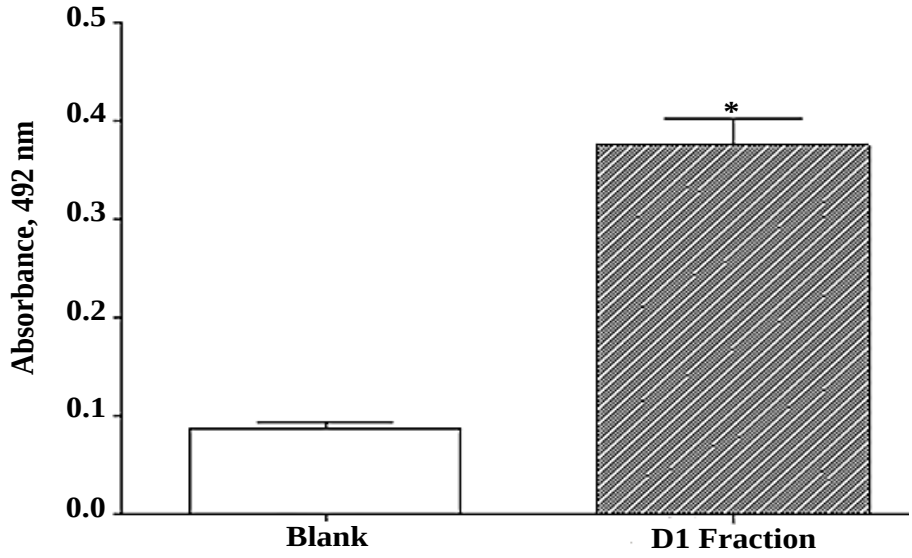

Fig. 3: Detection of LTP immunerelated protein in D1 fraction by enzyme-linked immunosorbent assay (ELISA). Primary antibody used was an anti-*Vigna unguiculata* LTP antibody produced in rabbits, diluted 1:500. As secondary antibody was used a peroxidase-conjugated anti-rabbit IgG antibody diluted 1:500. Peroxidase activity was revealed by using ortho-phenylenediamine. Absorbance was measured at 492 nm and values represent means (± SEM). * Samples with significant statistic difference from the control (p<0,0001) according Studart T test.

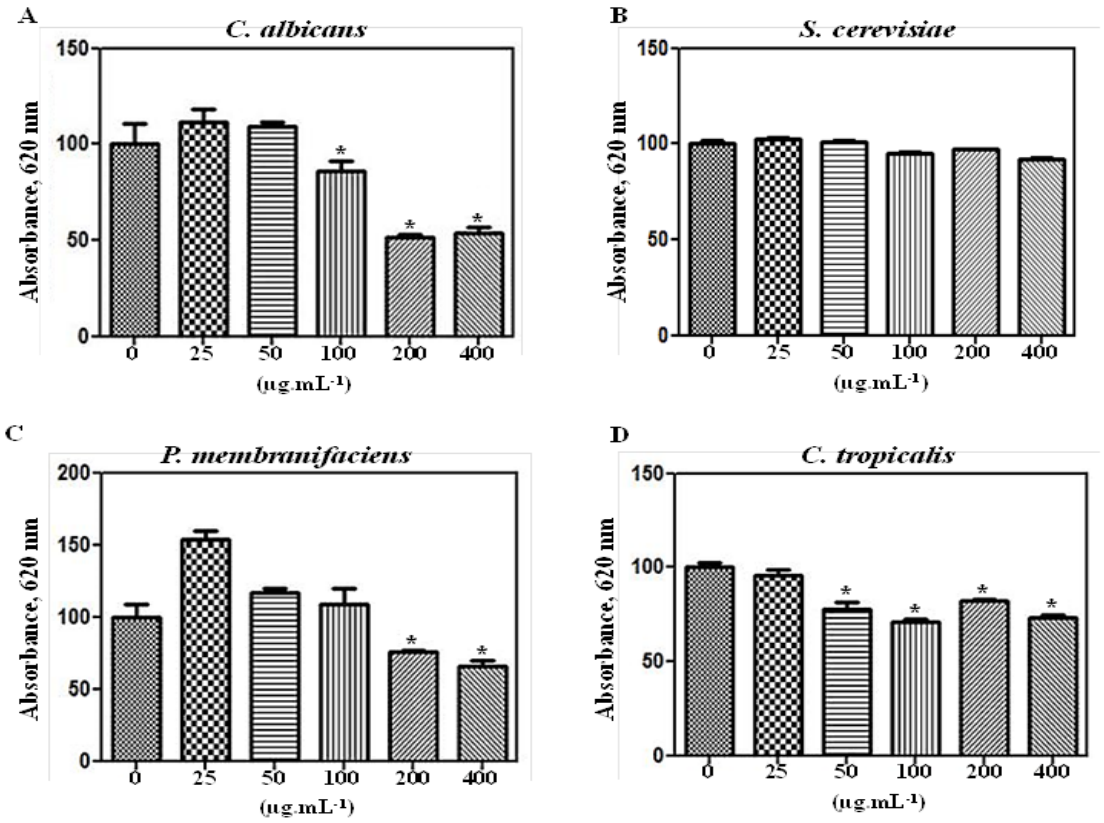

Fig. 4: Antifungal activity of D1 fraction against *C. albicans*; *S. cerevisiae*; *P. membranifaciens* and *C. tropicalis*. Cells grown in the presence of different concentrations of D1 fraction. Cells (10^4) in Sabouraud medium were incubated with the samples at 30 ºC and the absorbance of the culture was measured at 24h. (*) Indicates significance by the One-way ANOVA test ($P < 0.05$) which was calculated by the absorbance values of ex periment and its respective control.

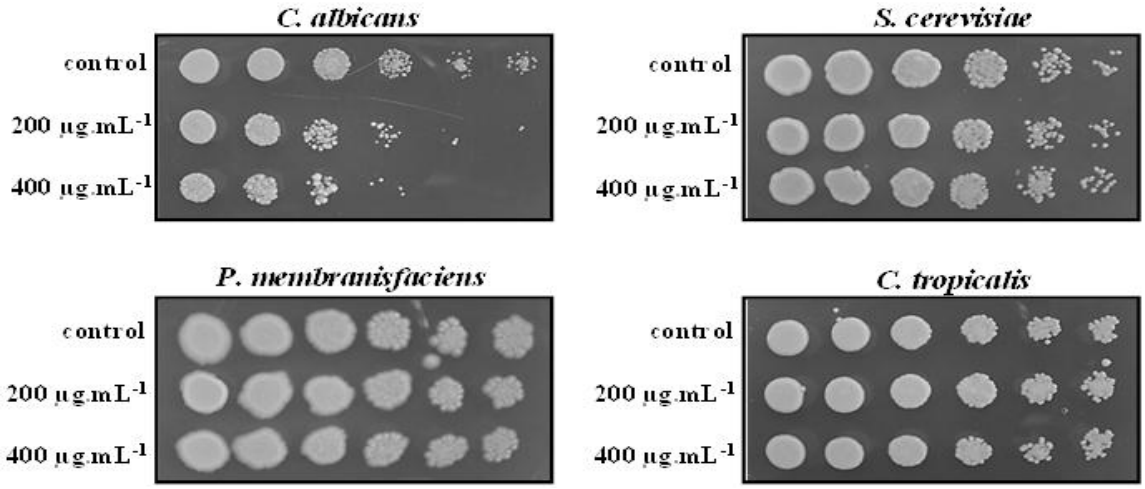

Fig. 5: Spotting assay of different yeast species after 24h exposition to D1 fraction. Treated cells were tenfold serial diluted in fresh Sabouraud medium and 5 µL of each dilution were spotted and incubated at 30 ºC.

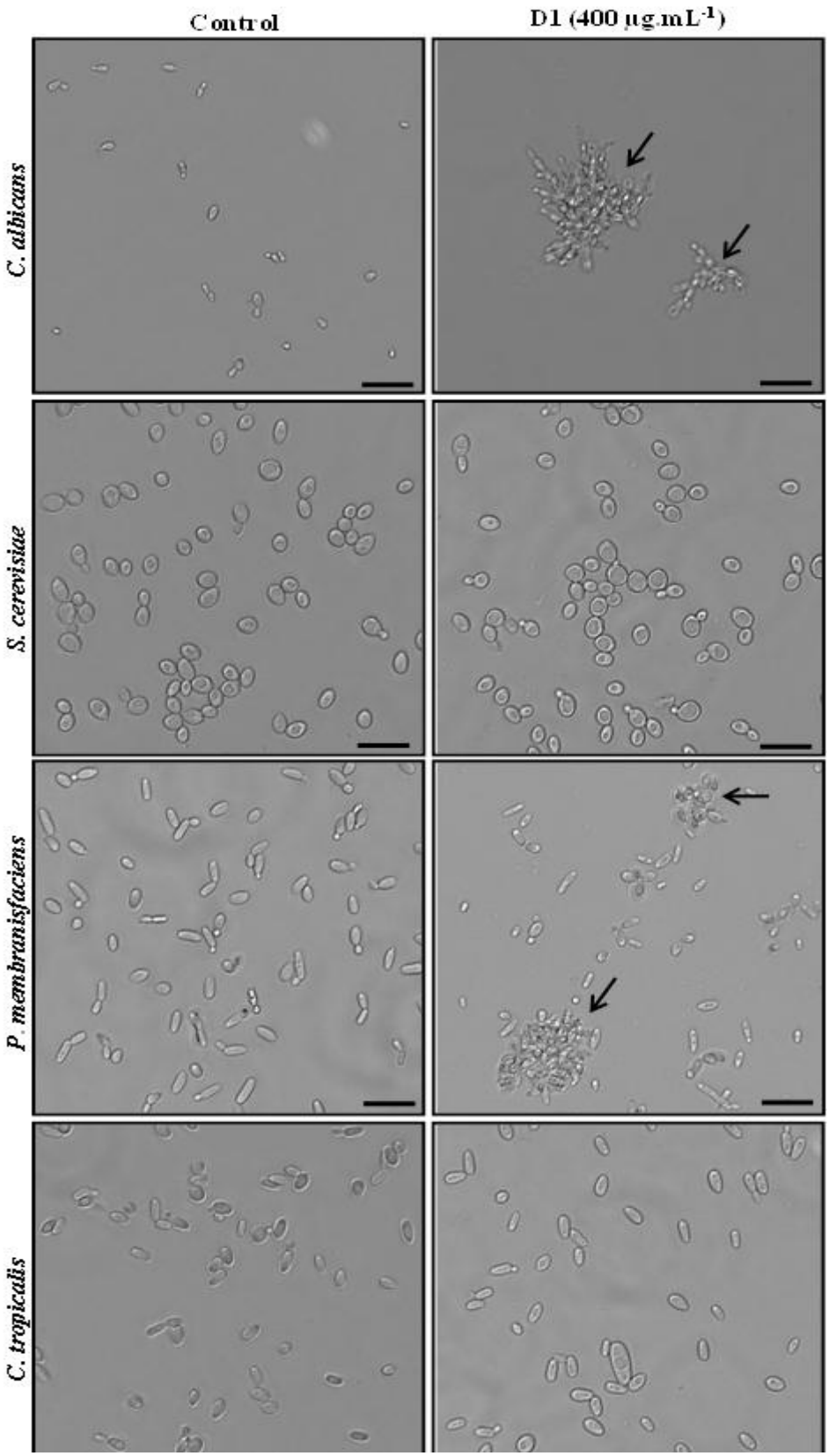

Fig. 6: Optical microscopy of yeast cells in the presence of D1 fraction. Control cells without D1 fraction and treated cells with 400 µg.mL^{-1} of D1 fraction. Arrows shows cellular agglomeration. 400x magnification.

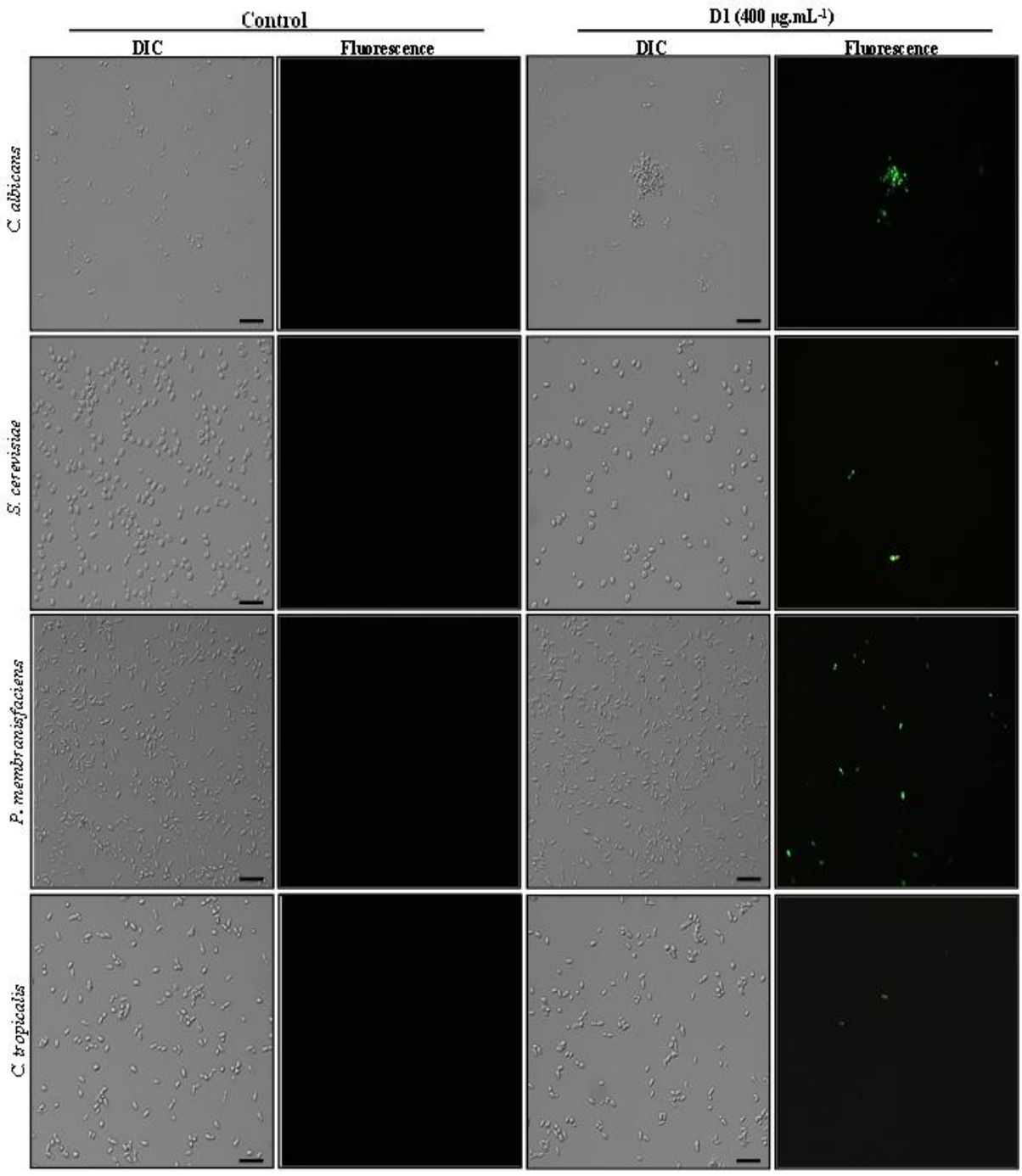

Fig. 7: Membrane permeabilization assay performed with fluorescence microscopy of different yeast cells treated with *SYTOX Green*. Control cells (without D1 fraction addition). Yeast cells treated with 100 µg.mL^{-1} of D1 fraction. 400x magnification.

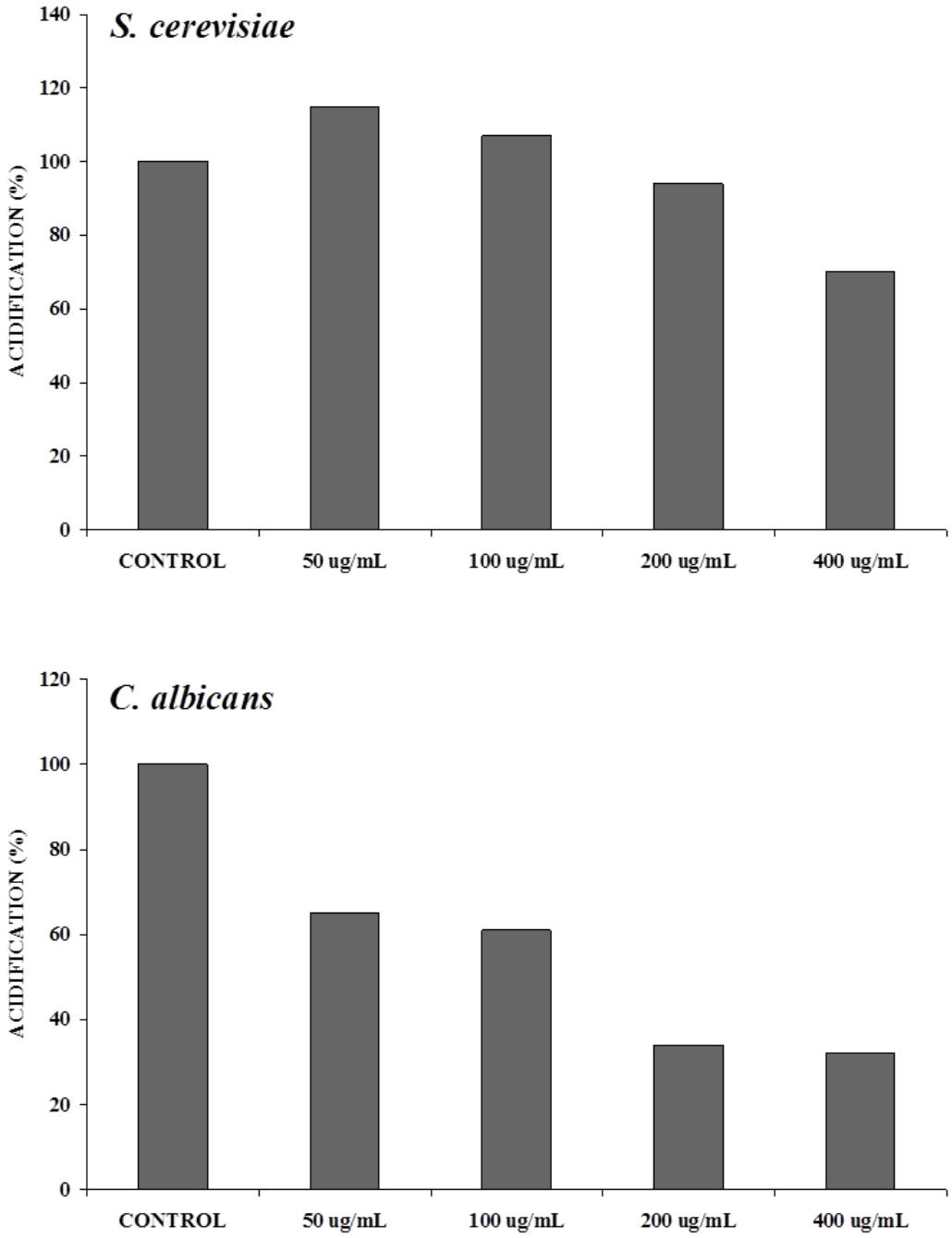

Fig. 8: Effect of D1 fraction on the glucose-dependent acidification of the medium by different yeast cells. D1 fraction was pre-incubated for 30 min at 50, 100, 200 and 400 µg.mL^{-1} concentrations before the addition of glucose to a final concentration of 100 mM.

DISCUSSION

In plants some of the antifungal peptides are classified, based on their functions and/or structures, into families including LTPs. The mechanisms of antifungal action of some antifungal proteins and antifungal peptides like defensins and lipid transfer proteins have

been elucidated.[4,5,23] LTPs, for example are able to permeabilize fungal plasma membrane to fluorescent dye, which possibly indicates that the permeabilization may be involved in the process of fungal growth arrest.[4,24-26]

Peptides with antimicrobial activities are important substances functioning in self-defense against infection by various harmful pathogens. Antimicrobial peptides are the ancient and widespread players of the defense system in all multicellular organisms.[27,28] Several families of antimicrobial peptides have been reported in plants, especially in the seeds, on the basis of sequence similarity.[29] Games *et al*.,[30] for example, purified a defensin, named *PvD1*, from *Phaseolus vulgaris* seeds and was able to inhibit a wide range of yeasts, such as *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida guilliermondii*, *Kluyveromyces marxiannus* and *Saccharomyces cerevisiae*. Different peptides, including from *C. ensiformis* have also showed antimicrobial activity. The toxic effect of urease (JBU) from *C. ensiformis* was carried and results showed that toxicity of JBU varied according to the genus and species of yeasts. Inhibition of proliferation, morphological alterations with formation of pseudohyphae was observed.[14] Zottich *et al*.,[26] showed that Cc-LTP1, a purified 9 kDa peptide that was isolated from *Coffea canephora* seeds, had homology to LTPs isolated from different plants and exhibited strong antifungal activity against the pathogenic yeast *Candida albicans*.

SYTOX Green is a dye that penetrates cells only when the plasma membrane is structurally compromised. Once inside the fungal cytoplasm, it binds to nucleic acids, resulting in a fluorescent complex.[21] Most of the antimicrobial peptides studied exhibit a broad-spectrum activity. Some act through nonspecific interaction with cytoplasmic membranes, leading to disruption of the lipid bilayer and lysis of the pathogen's cells, whereas others exploit a more sophisticated receptor-mediated mechanism. Intracellular targets for some plant AMPs have also been suggested.[31] Postal *et al*.,[14] evaluated the toxic effect of *Canavalia ensiformis* urease (JBU) on different yeast species and verified the ability of this urease to permeabilize these membranes using *SYTOX Green.* After incubation of *C. tropicalis*, *P. membranifaciens*, *K. marxiannus* and *C. parapsilosis* cells treated with JBU with the dye, the authors observed that all JBU-treated yeasts showed higher fluorescence compared the controls, indicating permeabilization of yeast cells. Other plant peptides, which have also been isolated from seeds, have been found to induce changes in yeast morphology, and these changes may be associated with the presence of these peptides in the medium. Diz *et al*.,[25,32] demonstrated

that *C. tropicalis* cells treated with Ca-LTP1 (400 µg.mL^{-1}) exhibited notable growth inhibition as well as morphological alterations, with the apparent formation of pseudohyphae, showed also permeabilization of yeast cells. The 2S albumin from *C. annuum* seeds (Ca-Alb) inhibited the growth of the yeasts *K. marxiannus*, *C. tropicalis*, *C. albicans* and *S. cerevisiae*. These yeast strains exhibited nitric oxide induction in the presence of Ca-Alb and displayed cellular agglomeration, elongated cells and the induction of pseudohyphae.[33] More recently a thionin-like peptide from *Capsicum annuum* fruits was toxic and induced changes in the membranes of all yeast strains tested, leading to their permeabilization.[34] In this work the antimicrobial activity found for D1 fraction, can be explained through the synergism between the proteins / peptides present in this fraction, among these peptides, were possible to suggest the antimicrobial activity of a LTP in this fraction.

Finally we also investigated whether the D1 fraction could interfere with the functioning of yeast H^+-ATPases. Interference on the function of H^+-ATPases in fungi by antagonists commonly leads to cell death. This ability to inhibit the glucose-stimulated acidification has been demonstrated for other plant species as well. Thevissen *et al.*,[35] demonstrated that when the fungi *Neurospora crassa* and *Fusarium culmorum* were treated with the plant defensins *Rs*-AFP2 and *Dm*-AMP1, an ion flux across the fungal plasma membrane was observed. Cruz *et al.*,[36] investigated whether the CM1 fraction could interfere with the fungal H^+-ATPase. To address this question, they monitored the glucose-stimulated acidification of the incubation medium by *S. cerevisiae* cells in the presence of various concentrations of the CM1 fraction and observed that of 8 and 16 µg.mL^{-1} of the CM1 fraction were able to inhibit this acidification by 95% and 98%, respectively, suggesting that the membrane may be a potential target for peptides of the CM1 fraction. A thionin-like peptide from *C. annuum* fruits was also capable of inhibiting acidification of the medium of glucose-induced *S. cerevisiae* cells 78% after an incubation time of 30 min[34] and Zottich *et al.*,[26] showed that Cc-LTP$_1$ isolated from *Coffea canephora* interfered in a dose-dependent manner with glucose-stimulated, H+-ATPase dependent acidification of yeast medium.

CONCLUSIONS

In conclusion, we have showed biologically active D1 fraction that has strong inhibitory effect against the pathogenic yeasts. In this fraction a lipid transfer protein (LTP) immune related protein were detected. The results presented in this work, indicate the potential of

using plant peptides and or proteins, particularly LTPs, as new anti-yeasts molecules, specially acting in synergy with other substances.

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