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# Antimicrobial Activity of ILTI, a Kunitz-Type Trypsin Inhibitor from *Inga laurina* (SW.) Willd

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**Abstract** Over the last few years, a growing number of proteinase inhibitors have been isolated from plants and particularly from seeds and have shown antimicrobial activity. A 20,000 Da serine peptidase inhibitor, named *ILTI*, was isolated from *Inga laurina* seeds and showed potent inhibitory enzymatic activity against trypsin. The aim of this study was to determine the effects of *ILTI* on the growth of pathogenic and non-pathogenic microorganisms. We observed that *ILTI* strongly inhibited in particular the growth of *Candida tropicalis* and *Candida buinensis,* inducing cellular agglomeration. However, it was ineffective against human pathogenic bacteria. We also investigated the potential of *ILTI* to permeabilize the plasma membrane of yeast cells. *C. tropicalis* and *C. buinensis* were incubated for 24 h with the *ILTI* at different concentrations, which showed that this inhibitor induced changes in the membranes of yeast cells, leading to their permeabilization. Interestingly, *ILTI* induced the production of reactive oxygen species (ROS) in *C. tropicalis* and *C. buinensis* cells. Finally, *ILTI* was coupled with fluorescein isothiocyanate, and subsequent treatment of *C. tropicalis* and *C. buinensis* with DAPI revealed the presence of the labeled protein in the intracellular spaces. In conclusion, our results indicated the ability of peptidase inhibitors to induce microbial inhibition; therefore, they might offer templates for the design of new antifungal agents.

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## Introduction

Serine proteinase inhibitor molecules (PIs) are found throughout the plant kingdom and have been described in many plant species [9, 16, 23, 28]. In plants, serine PIs can be found as constituent components present in the reserve tissues (in the tubers and seeds) or expressed in response to insects and pathogens by inhibiting the action of the digestive peptidases present in mammals and especially insects, as well as the enzymes present in bacteria and fungi [5, 7, 9, 10], and its expression levels can also increase in response to abiotic stresses [3]. Plant Kunitz inhibitors are mainly concentrated in the leguminous seeds of subfamilies Mimosoideae, Papilionoideae, and Caesalpinioideae. The majority of these inhibitors are molecules between 3 and 25 kDa and can inhibit either trypsin or chymotrypsin, with some capable of inhibiting both enzymes [20, 26].

More recently, especially among plant isolates, PIs concomitantly active toward specific proteinase with antimicrobial activity have attracted the attention of many, and various researchers have shown these functions. A protein called PSC-AFP, with molecular mass of 18 kDa, was isolated from *Psoralea corylifolia,* and by partial sequencing of the N-terminal region, this protein was found to be homologous with plant trypsin inhibitors. The antimicrobial activity of this protein was also tested against different fungi, including *Alternaria niger, Aspergillus brassicae*, *Fusarium oxysporum*, and *Rhizoctonia cerealis,* and it was observed that, in the presence of 10 μM of PSC-

AFP, all of the fungi tested were inhibited [30]. Studies related to the antimicrobial roles of serine proteinase inhibitors were also performed by Lopes et al. [15]. Isoform inhibitors of Kunitz-type serine proteinase, with a molecular mass of 20 kDa each, were isolated from *Acacia* seeds, purified, and named ApTIA, ApTIB, and ApTIC. After characterization tests were performed of their structural properties, as well as tests for biological activity in the presence of different phytopathogenic fungi, it was observed that all of the inhibitors were able to inhibit the growth of *Aspergillus niger*, *Thielaviopsis paradoxa*, and *Colletotrichum* sp. More recently Ribeiro et al. [25] showed that a 6000 Da peptide, called *CaTI*, isolated from *Capsicum annuum* L. seeds showed potent inhibitory enzymatic activity against trypsin and chymotrypsin. It was also observed that *CaTI* inhibited the growth of *Saccharomyces cerevisiae, Kluyveromyces marxiannus*, and *Candida albicans*. Chilosi et al. [4] showed that STI, a family proteinase inhibitor of Bowman–Birk, showed strong antimicrobial activity in vitro against the growth of filamentous fungi. This inhibitor was able to inhibit 50 % growth of *Botrytis cinerea*, *Colletotrichum acutatum*, *Didymella bryoniae*, *Fusarium culmorum*, *Fusarium graminearum*, and *Septoria tritici*.

In 2011, antibacterial activity from corms of 15 species of the Xanthosoma genus was also shown. A Kunitz peptidase inhibitor from *X. blandum* called A Xb-KTI, with a molecular mass of approximately 24 kDa, inhibited the growth of the pathogenic bacteria *Staphylococcus aureus*, *Salmonella typhimurium*, and *Escherichia coli* at a concentration of 0.1 mg $mL^{-1}$ [13]. However, small Kunitz inhibitors are the most described, e.g., PT-1 and potide-G were isolated from different cultivated varieties of potato tubers (*Solanum tuberosum*), showing molecular masses of 5600.0 and 5578.9 Da, respectively [11, 12]. PT-1 was active against *Rhizoctonia solani* and *Clavibacter michiganensis* and inactive against *S. aureus* [11], while potide-G inhibited *S. aureus*, *Listeria monocytogenes*, *C. michiganensis*, and *E. coli* [12].

*Inga laurina* (SW.) Willd. is a tree belonging to the subfamily Mimosoideae of the Leguminoseae. It is a tropical plant with a widespread distribution in Central and South America. A protein inhibitor of trypsin (*ILTI*) was isolated from its seeds, yielding a single band with an MM of approximately 20 kDa [16]. *ILTI* previously showed insecticidal activity against the lepidoptera *Diatraea saccharalis* and *Heliothis virescens* [22] and the coleoptera *Homalinotus coriaceus* [17]. In this work, we aimed to analyze the antimicrobial role of *ILTI* against pathogenic and non-pathogenic bacteria and fungi. Here, we demonstrated the strong potential of *ILTI* as a new fungi growth inhibitor, especially against *Candida* yeasts.

## Materials and Methods

### Biological Materials

The yeasts *Candida parapsilosis* (CE002), *Candida tropicalis* (CE017), *Candida albicans* (CE022), and *Candida buinensis* (URM4674) were maintained in 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).

The bacteria *E. coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 13883, and *S. aureus* ATCC 25923, used as the bacterial model strains, were maintained in the Laboratório de Purificação de Proteínas e suas Funções Biológicas, Universidade Federal de Mato Grosso do Sul. A single colony of each was isolated by the streak method in Mueller–Hinton (MH) solid medium and was propagated in MH broth at 37 °C, and the cell suspension was stored in sterile 10 % (v/v) glycerol at −80 °C. All of the subsequent experiments were performed from this original stock.

### Extraction and Purification of ILTI

*ILTI* was purified from seeds of *I. laurina* (SW.) Willd., as previously described by Macedo et al. [16].

### Preparation of Yeast Cells and Determination of the Effect of ILTI on Yeast Growth

The yeast growth assay was performed following the protocol developed by Broekaert et al. [2] with some modifications described below. To assay the effects of *ILTI* fraction on yeast growth, cells of *C. albicans*, *C. tropicalis*, *C. parapsilosis,* and *C. buinensis* ($10^3$ cells/mL of Sabouraud broth) were incubated at 30 °C in 100 µL in 96-well microplates in the presence of different concentrations of *ILTI* fraction (15, 30, 62.5, 125, and 250 µg $mL^{-1}$) for *C. tropicalis* and *C. buinensis* and in the presence of 250 µg $mL^{-1}$ for *C. albicans* and *C. parapsilosis*. The growth of each strain 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 *ILTI* fraction was also determined.

### SYTOX *Green* Uptake Assay

Plasma membrane permeabilization was measured by SYTOX *Green* (Invitrogen, Grand Island, NY, USA) uptake, as described by Thevissen et al. [29]. Cells of *C. tropicalis* and *C. buinensis* were grown in the presence of *ILTI* (30 µg $mL^{-1}$). Aliquots (100 µL) 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 using 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 controls without *ILTI* were also run to evaluate membrane permeabilization.

### Visualization of Reactive Oxygen Species (ROS) Using Fluorescence

To determine whether the *ILTI* mechanism involves the induction of oxidative stress, it was used a fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate ($H_2DCFDA$) that indicates the presence of reactive oxygen species. Induction of endogenous production of ROS in yeast cells grown in the absence or presence of 30 μg mL$^{-1}$ *ILTI* was evaluated after growth inhibition assay, using the fluorescent probe, according to the methods described by Mello et al. [18] with some modifications. Aliquots (50 μL) of the yeast cell suspension were incubated with 20 μM of the fluorescent probe for 2 h at room temperature with periodic agitation. After this period, the cells were transferred to slides, covered with cover slips and analyzed by fluorescence microscopy (Axiophoto Zeiss), with a fluorescence filter set for fluorescein detection (excitation wavelengths 450–490 nm and emission wavelength 500 nm). The results are representative of one triplicate experiment.

### Localization of ILTI Conjugated with FITC for Optical Microscopy

*ILTI* (100 μg) was coupled to fluorescein isothiocyanate (FITC, 50 μg mL$^{-1}$ solubilized in DMSO) following the manufacturer's instructions (Sigma). After coupling, 50 μg mL$^{-1}$ *ILTI*-FITC was incubated with cells (10$^{3}$ cells mL$^{-1}$ of Sabouraud broth) of *C. tropicalis* and *C. buinensis* for 24 h in 96-well microplates. Subsequently, an aliquot of each cell suspension was removed and incubated with 50 μg mL$^{-1}$ 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min for nuclear staining. Cells were analyzed with a DIC optical microscope (Axio Imager.A2, Zeiss) equipped with a fluorescent filter set for detection of the fluorescein (excitation wavelength, 450–490 nm, emission wavelength 500 nm).

### Antibacterial Assay

The growth rate of bacterial cells from the original culture was established by measuring optical density (OD) at 595 nm and monitored at 30-min intervals in MH broth at a constant temperature (37 °C) and with shaking (240 rpm). The direct relationship of OD to colony forming units (CFUs) was established based on the drop surface plate method [21], and a standard growth curve was constructed and calibrated. The evaluation of the antibacterial activity of *ILTI* was performed at a final concentration of 200 μg mL$^{-1}$ following the microdilution assay, according to the CLSI document M07-A8 [6]. Briefly, the bacterial culture was adjusted in MH broth supplemented with *ILTI* to 5 × 10$^{5}$ UFC mL$^{-1}$ and incubated (37 °C) for 12 h, and the suspension turbidity was monitored at 595 nm every 30 min. Chloramphenicol (40 μg mL$^{-1}$) was used as the positive control for bacterial growth inhibition, and ultrapure water was the bacteria growth control.

## Results

### Antimicrobial Activity of ILTI Fraction

In this work, we tested the effects of *ILTI* as an inhibitor of the growth of pathogenic and non-pathogenic yeast strains and of human pathogenic bacteria. We noted complete growth inhibition of *C. tropicalis* in the presence of 250 and 125 μg mL$^{-1}$ *ILTI* and partial inhibition of 83, 31, and 6 % to 62.5, 30, and 15 μg mL$^{-1}$ *ILTI*, respectively (Fig. 1). Regarding *C. buinensis,* complete growth inhibition was observed only at 250 μg mL$^{-1}$ *ILTI* concentration, while for 125, 62.5, 30, and 15 μg mL$^{-1}$ *ILTI*, inhibition of 89, 45, 13, and 0 % was noted, respectively (Fig. 1). The growth inhibition curve for *C. albicans* and *C. parapsilosis,* determined only at the concentration of 250 μg mL$^{-1}$, *ILTI* inhibited 9 and 12 %, respectively (data not shown). Nevertheless, the antibacterial assay determined the inability of *ILTI* (200 μg mL$^{-1}$) to inhibit the growth of the human pathogenic bacteria *E. coli*, *K. pneumoniae*, and *S. aureus* (data not shown).

### SYTOX *Green* Uptake Assay

The ability of *ILTI* to permeabilize the plasma membrane of *C. tropicalis* and *C. buinensis* cells was examined in this study. Membrane permeabilization was assessed after 24 h of growth in the presence of *ILTI* 30 μg mL$^{-1}$ and 30 min after the addition of SYTOX *Green*. When observed with a fluorescence microscope, the cells of both yeasts showed SYTOX *Green* fluorescence in the presence of *ILTI*, compared to controls in which cells were grown in the absence of *ILTI* (Fig. 2).

### Visualization of Reactive Oxygen Species (ROS) Using Fluorescence

Using a fluorescence microscope, we demonstrated ROS induction in *C. tropicalis* and *C. buinensis* cells after preincubation with 30 μg mL$^{-1}$ *ILTI* for 24 h, compared to

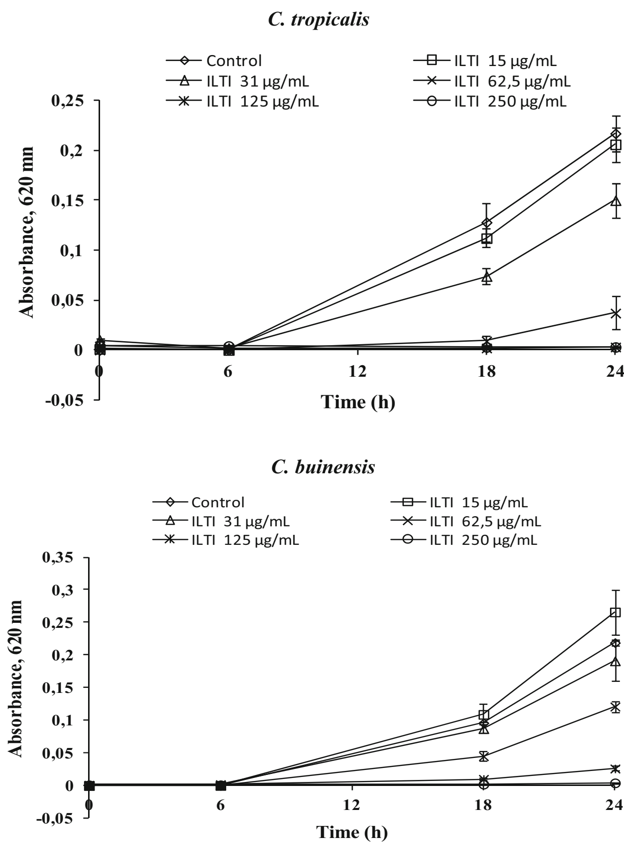


**Fig. 1** Antimicrobial activity of *ILTI* against *Candida tropicalis* and *Candida buinensis*

control cells (Fig. 3). *ILTI* could induce higher levels of ROS, which might be involved in different metabolic mechanisms of yeast growth arrest or death.

### Localization of ILTI Conjugated with FITC for Optical Microscopy

To determine the localization of *ILTI* in *C. tropicalis and C. buinensis, ILTI* was coupled to FITC and used for antifungal activity assay. Subsequently, after 24 h of growth, the cells were subjected to DAPI labeling. In this assay, we could observe the internalization of *ILTI* (Fig. 4). An overlay of the images labeled with FITC and DAPI (Fig. 4) suggests that *ILTI* could target the intercellular organelles of this organism, such as the nuclei.

## Discussion

The effects of *ILTI* on the growth of different species of yeasts were observed (Fig. 1). These effects are interesting because this activity might be associated with the presence of important proteinases responsible for the synthesis of fungal components. Other authors have shown the antimicrobial effects in vitro of some proteinase inhibitors of plants and have shown that some proteinases secreted by fungi could be important factor in determining pathogenicity and that its inhibition could significantly

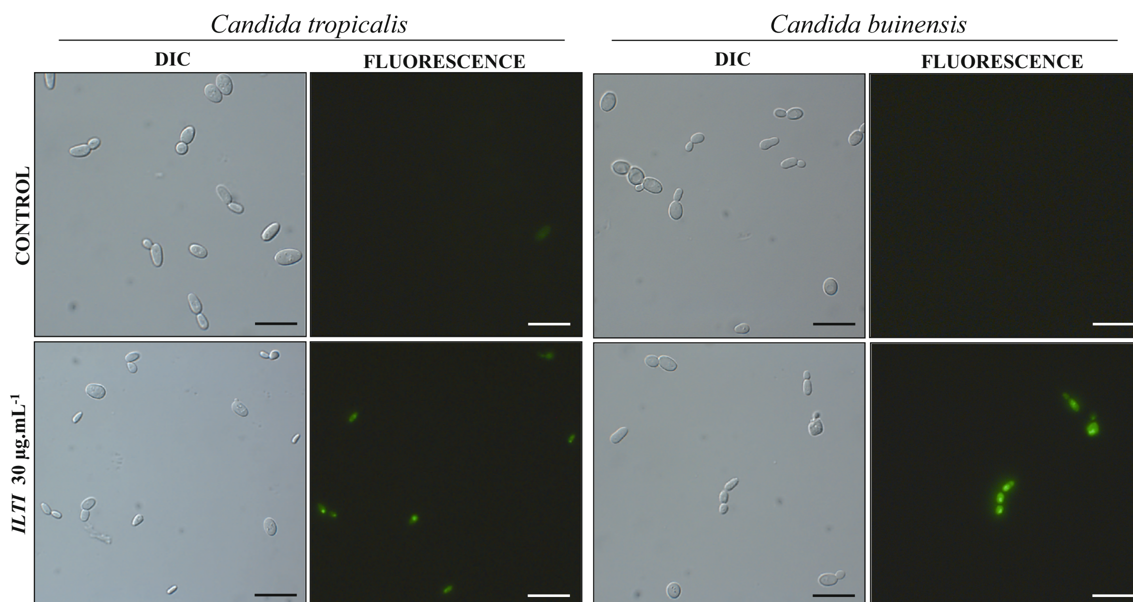


**Fig. 2** Membrane permeabilization assay performed with fluorescence microscopy of *Candida tropicalis* and *Candida buinensis* yeast cells treated with SYTOX *Green* for a period of 30 min after being treated with *ILTI* (30 µg $mL^{-1}$) for 24 h. *Bars* 20 µm (Color figure online)

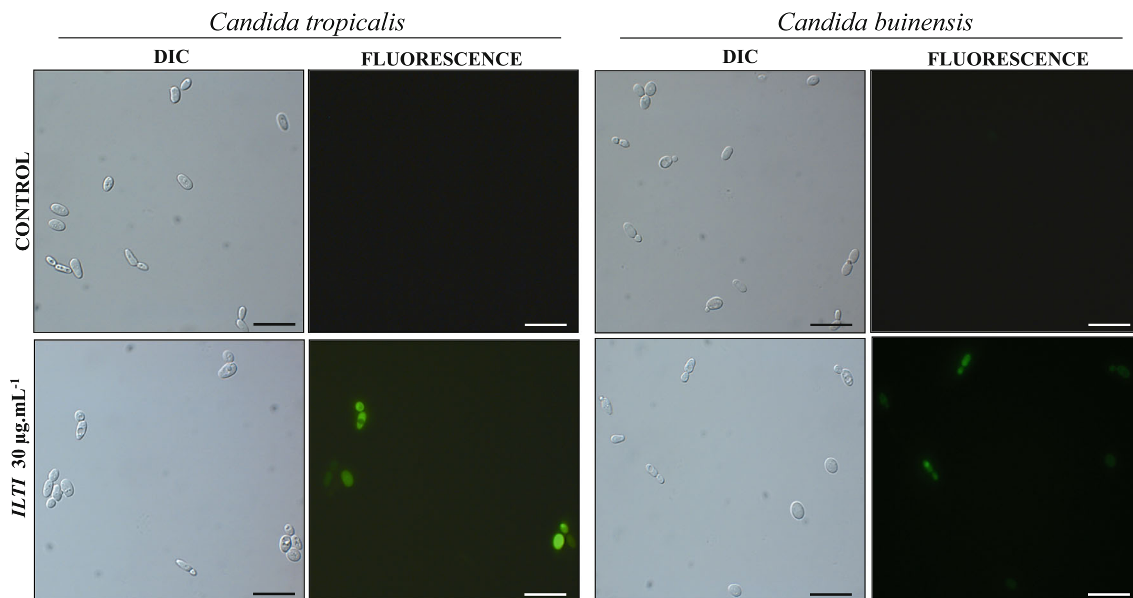


**Fig. 3** Oxidative stress assay performed with fluorescence microscopy of *Candida tropicalis* and *Candida buinensis* yeast cells for ROS detection. Yeasts cells were incubated with *ILTI* (30 µg $mL^{-1}$) for 24 h and after this period with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate ($H_2DCFDA$) for 2 h. *Bars* 20 µm

reduce the infections caused by these fungi [12, 27]. Despite its antifungal potential, *ILTI* was ineffective against both of the bacterial strains evaluated. However, *ILTI* could be selective against other bacteria not tested. However, further experiments should be performed with an extensive list of bacterial strains before disregarding any antibacterial proprieties of *ILTI*. Selectivity is recurrently observed in peptidase inhibitors [11, 13] and was also seen in *ILTI* antifungal activity. Many classes of endopeptidases and exopeptidases secreted by pathogenic fungal and bacteria are referred as virulence factors [8, 19]. Furthermore, the variety of secreted peptidases on the surface of fungal and bacterial, as well those in extracellular environment, also varies among fungal [19] and bacterial species [8], what may explain the *ILTI* selectivity.

In this study, we observed the capacity of *ILTI* to damage the plasma membranes of the yeast *C. tropicalis* and *C. buinensis* (Fig. 2), allowing for the permeation of same through the use of SYTOX *Green* dye. This ability to induce cellular damage has been viewed in different families of proteins/peptides and plants, and it is capable of causing changes to the cell membrane and regulating its flow of ions, making these molecules ideal models for understanding the functioning of vital proteins that act as ion channels.

Fluorescence assays demonstrated that *ILTI* increased ROS levels in yeast cells (Fig. 3). However, most ROS can be produced in basal levels in cells when grown under normal conditions free of stress, which might explain the low or absent fluorescence in the control cells. Other authors have reported that the plant defensin Rs-AFP2, isolated from *Raphanus sativus*, is involved in the induction of oxidative stress in cells of the yeast *Candida albicans* [1]. From the results obtained in this study, it is reasonable to suggest that *ILTI* could have some mitochondrial target, causing an increase in the production of ROS. Ribeiro et al. [24] also showed that *CaTI*, a proteinase inhibitor, induced the generation of nitric oxide and interfered in a dose-dependent manner with glucose-stimulated acidification of the medium, mediated by $H^+$-ATPase from *S. cerevisiae* cells.

The next experiments were designed to analyze whether *ILTI* was able to enter *C. tropicalis* and *C. buinensis* actively. To that end, FITC-tagged *ILTI* was monitored by fluorescence microscopy. Because *ILTI* entered yeasts cells, we suggest that a possible intracellular target for this proteinase inhibitor might be part of a complex mechanism responsible for the death of *Candida* species. FITC-tagged *ILTI* overlapped with DAPI staining, suggesting that one of its targets is nuclear (Fig. 4). Important results were shown by Lobo et al. [14], who found that the defensin isolated from *Pisum sativum* (*Ps*D1) could in fact have a nuclear target. This study and other related studies have suggested that the antifungal activities of plant defensins are not restricted to the plasma membranes of fungi because the defensins can enter cells and target different intracellular compartments. More recently, Zottich et al. [31] purified a new protein from coffee, called Cc-GRP, which is involved in the plant defense system against pathogens by acting

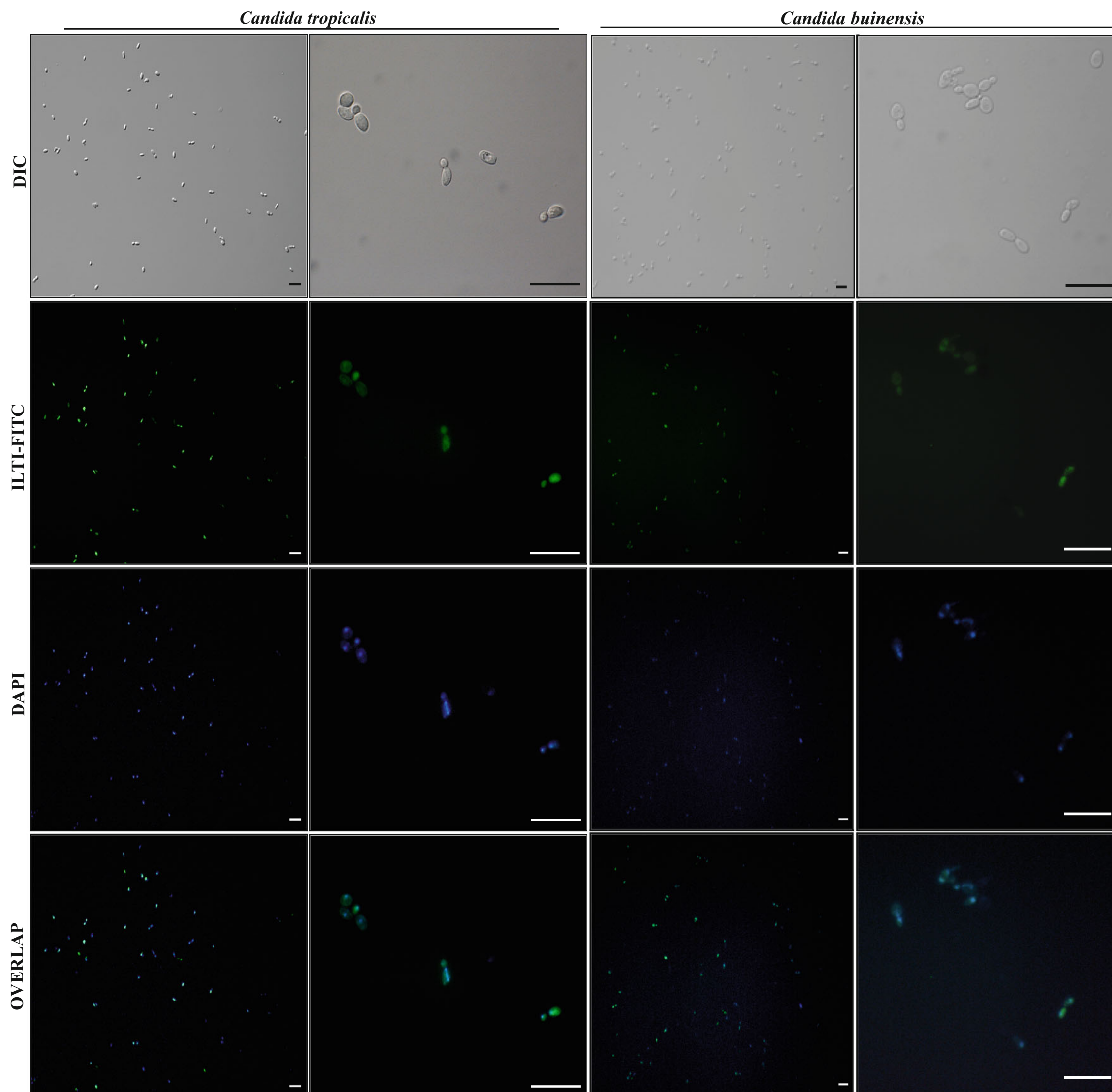


**Fig. 4** Fluorescence microscopy analysis of *Candida tropicalis* and *Candida buinensis* cells, incubated for 24 h with 50 µg $mL^{-1}$ FITC-tagged *ILTI* (*green fluorescence*). After the incubation period, the nuclei were stained with DAPI (*blue fluorescence*). *Bars* 20 µm (Color figure online)

through a membrane permeabilization mechanism, as well as being localized in the nuclei of fungal cells. Our work opens new perspectives regarding the antimicrobial mechanisms of plant-derived proteinase inhibitors suggesting that the toxicity of these proteins might have different mechanisms of action.

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**Compliance with Ethical Standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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