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Original Article

Antimicrobial and antibiofilm activities of *Inga cylindrica* trypsin inhibitor

Atividade antimicrobiana e antibiofilme de inibidor de tripsina de *Inga cylindrica*

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Abstract

Inga cylindrica, a tropical fruit tree of the Fabaceae family (subfamily Mimosoideae), is native to South America. The seeds from this family are an essential source of trypsin inhibitors, which display promising bioactivity for increasing host defense against pathogens. The aim of the present study was to characterize the antimicrobial and antibiofilm activities of the trypsin inhibitor extracted from *I. cylindrica* seeds, ICTI. ICTI demonstrated antifungal activity with a minimum inhibitory concentration (MIC) of 32.11 µmol.L^{-1}, and a minimum fungicidal concentration (MFC) of 32.1 µmol.L^{-1}, against *Cryptococcus gattii, Candida albicans, Candida glabrata* and *Candida guilliermondii*. Combining ICTI with Amphotericin B had a significant synergistic effect, reducing the concentration of the antibiotic by 75% for *C. albicans* and 94% for *C. gatti.* The significant increase (16 x) in activity observed with ergosterol (1.01 mol.L^{-1}) for *C. albicans* and *C. gatti,* and the lack of activity against bacterial strains, suggests that ICTI interferes with the integrity of the fungal cell membrane. Treatment with ICTI at 10 x MIC resulted in a 51% reduction in biofilm formation and a 56% decrease in mature biofilm colonies for *C. albicans.* Finally, ICTI displayed no toxicity in the *in vivo Galleria mellonella* model. Given its antifungal and antibiofilm properties, ICTI could be a promising candidate for the development of new antimicrobial drug prototypes.

Keywords: antibacterial, antifungal, *Candida albicans*, *Cryptococcus gattii*, pathogenic yeasts, protease inhibitor.

Resumo

Inga cylindrica, uma árvore frutífera tropical da família Fabaceae (subfamília Mimosoideae), é nativa da América do Sul. As sementes desta família são uma fonte essencial de inibidores de tripsina, que apresentam bioatividade promissora para aumentar a defesa do hospedeiro contra patógenos. O objetivo do presente estudo foi caracterizar as atividades antimicrobiana e antibiofilme do inibidor de tripsina extraído de sementes de *I. cylindrica*, ICTI. O ICTI demonstrou atividade antifúngica com concentração inibitória mínima (CIM) de 32,11 µmol.L^{-1} e concentração fungicida mínima (CFM) de 32,1 µmol.L^{-1}, contra *Cryptococcus gattii*, *Candida albicans*, *Candida glabrata* e *Candida guilliermondii*. A combinação de ICTI com Anfotericina B teve um efeito sinérgico significativo, reduzindo a concentração do antibiótico em 75% para *C. albicans* e 94% para *C. gatti*. O aumento significativo (16 x) na atividade observado com ergosterol (1,01 mol.L^{-1}) para *C. albicans* e *C. gatti*, e a falta de atividade contra cepas bacterianas, sugerem que o ICTI interfere na integridade da membrana celular fúngica . O tratamento com ICTI a 10 x MIC resultou numa redução de 51% na formação de biofilme e numa diminuição de 56% nas colónias maduras de biofilme para *C. albicans*. Finalmente, o ICTI não apresentou toxicidade no modelo *in vivo* de *Galleria mellonella*. Dadas as suas propriedades antifúngicas e antibiofilme, o ICTI pode ser um candidato promissor para o desenvolvimento de novos protótipos de medicamentos antimicrobianos.

Palavras-chave: antibacteriano, antifúngico, *Candida albicans*, *Cryptococcus gattii*, leveduras patogênicas, inibidor de protease.

1. Introduction

Pathogenic microorganisms, such as yeast and bacteria, are the main cause of potentially fatal infections associated with mortality and morbidity in immunocompromised patients in developing countries (Danish et al., 2020; Bhatia et al., 2021). A report presented to the United Nations, in 2019, projected that infections caused by antibiotic-resistant bacteria would cause, by 2050, 10 million deaths per year and a global economic crisis (Bhatia et al., 2021). Numerous antibiotics and antimicrobial agents are available on the market that can kill or inhibiting the growth of microorganisms, however, strains are becoming resistant to these medications (Danish et al., 2020; Salayová et al., 2021).

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The incorrect and excessive use of antibiotics (Alibi et al., 2021; Salayová et al., 2021) has led to the development of antimicrobial resistance, a threat to global health that limits the success of therapeutic management of bacterial infections (Bhatia et al., 2021).

Additionally, fungus spores abound in the world, and can be found everywhere. Pathogenic yeasts impact millions of people annually, primarily due to infections caused by *Cryptococcus spp.* and *Candida spp*., two relevant hospital genera (Mlozi et al., 2019). These yeasts are commensal in healthy humans, but can cause systemic infections, especially in immunocompromised individuals (Kumar et al., 2018; Araujo et al., 2019) and are the main cause of nosocomial infections worldwide (Pal et al., 2022). Fungal infections caused by these species are aggravated by virulence factors, including adhesion to abiotic surfaces and the formation of microbial communities (biofilms) with complex mechanisms of resistance (Pal et al., 2022).

The current treatment of systemic infections is hampered by high patient toxicity of drugs and the incidence of drug-resistant strains (Oliveira et al., 2018; Moghadam et al., 2020). Therefore, developing alternative strategies to minimize the toxic effects of current antifungals and improve their effectiveness is being strongly encouraged (Emeri et al., 2019; Mlozi et al., 2019).

Plants show a high degree of resistance in response to different pathogens. Among the families of proteins related to plant defense, protease inhibitors (PIs) are one of the most studied and characterized (Clemente et al., 2019; Amaral et al., 2022). The PIs are non-nutritional compounds, which are expressed in storage tissues and inhibit the digestive enzymes of pathogens (Cid-Gallegos et al., 2022). These molecules act as antimicrobial agents, promoting membrane and cell wall damage, and altering cell permeability. Thus, the mechanism of action of PIs is characterized by effective antimicrobial actions, causing fewer side effects to patients and lower production costs than commercial reference antimicrobials (Araújo et al., 2019; Cotabarren et al., 2020). The activity of PIs against pathogenic microorganisms has already been well clarified (Oliveira et al., 2018; Dib et al., 2018), and shows the pharmaceutical potential of these molecules.

I. cylindrica, a representative of the Fabaceae family (Mimosoideae subfamily), is a legume that is widely distributed in Brazil and has sweet fruits and seeds that are rich in macromolecules. Calderon et al. (2010) characterized a trypsin inhibitor from *I. cylindrica* seeds, called ICTI (*Inga cylindrica* trypsin inhibitor). ICTI is a protein with a molecular mass of 19,465 Da, and a single polypeptide chain. ICTI inhibited trypsin at a 1:1 molar ratio, but showed no significant inhibition against α-chymotrypsin, characteristic properties of the type Kunitz inhibitors, commonly found in this family (Macedo et al., 2016; Bezerra et al., 2016; Dib et al., 2018).

The dissociation constant (K_i) value of ICTI is 4.3 nM, clearly indicating that this molecule is a potent and competitive inhibitor of bovine trypsin. Fluorescence studies at increasing temperatures demonstrated the thermal stability of the ICTI structure, with thermal stability at pH 7.0 as indicated by a T_m of 70 °C and ΔG^{25} of 48.5 kJ.mol^{-1}. Cell dichroism (CD) studies showed that ICTI is composed of 65% β-sheets, 33% unordered structure and 6.7% α-helix secondary structures (Calderon et al., 2010). Although Calderon et al. (2010) presented the biochemical profile of ICTI, no studies were found in the literature that investigated the biological activity of the inhibitor against microorganisms.

In this context, this study aimed to investigate the antimicrobial and antibiofilm effects of ICTI against strains with pathogenic priority, in addition to evaluating the acute toxicity of this inhibitor in an *in vivo* model of *Galleria mellonella.*

2. Material and Methods

2.1. Plant material

I. cylindrica seeds were obtained from the company, ArboCenter Seed Trade. Seeds were stored (- 20 °C) in the Laboratory of Protein Purification and its Biological Functions (LPPFB) seed bank, Federal University of Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil. The access to genetic heritage was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under ID number A315278.

2.2. ICTI purification

I. cylindrica seeds were ground and delipidated with hexane. The protein extraction was performed with 0.1 M sodium phosphate buffer pH 7.6 (1:10, w/v) for 4 h at room temperature. After centrifugation (6000 g for 30 min at 4 °C), the supernatant was dialyzed and lyophilized, and Crude Extract (CE) was obtained. The purification process was carried out according to the description of Calderon et al. (2010). For inhibitor purification, 300 mg of CE was loaded on a DEAE-Sepharose column (3 × 12 cm) equilibrated with 0.05M Tris-HCl buffer, pH 8.0. An elution was performed in a NaCl gradient (0 to 1 M) at a 60 mL/h. The chromatography was monitored by at 280 nm. The fraction with inhibitory activity was pooled, dialyzed, lyophilized and named ICTI. ICTI was resuspended in 0.9% NaCl solution, in concentrations ranging from 514 to 4 µmol.L^{-1}, and finally filtered through 0.45 µm syringe filters to ensure sterility.

2.3. Polyacrylamide gel electrophoresis under denaturing conditions

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (12.5% SDS-PAGE) was performed as described by Laemmli (1970). Amersham ECL Low-Range Rainbow Molecular Weight Markers (Mr 3500 to 38000) were used to determine the relative molecular weight of the sample.

2.4. Protein quantification

Protein concentrations were determined by Coomassie blue staining using the Bradford (1976) method, adapted to a microplate protocol, with bovine serum albumin (BSA)

serial dilution, from 1 to 0.06 mg.mL^{-1}, as the standard concentrations.

2.5. Trypsin activity assay

For trypsin inhibition activity (Erlanger et al., 1961), different concentrations of the fraction eluted from the DEAE Sepharose column (0 to 1.4 µg.mL^{-1}) were incubated in sodium phosphate buffer (0.1 M, pH 7.6 and 0.15 M NaCl) and bovine trypsin (0.25 mg.mL^{-1}) for 10 min at 30 °C in a total volume of 70 µL. Subsequently, 200 µL of Benzoyl-Arginine-p-nitroanilide colorimetric substrate (1 mM BAPNA) were added to each of the wells. The assay was performed for 30 min at 30°C, and the hydrolyzed substrate was monitored at 410 nm for 30 min in a Varioskan Lux microplate reader (Thermo Scientific). Assays were performed in triplicate and the results are presented in nmol/µg/min.

2.6. Microorganisms

The strains were obtained from the Laboratory of Physiology and Biochemistry of Microorganisms at UENF (North Fluminense State University) and were stored at –80 ºC in the LPPFB. The strains were grown in medium enriched with brain heart infusion (BHI agar; Merck, Germany). The inoculum suspensions were prepared according to the Clinical and Laboratory Standard Institute (CLSI), M27-A3 (CLSI, 2008), M27-S3 (CLSI, 2002) and M07-A9 (CLSI, 2012) protocols. Four American Type Culture Collection (ATCC) strains of bacteria were used to assess antibacterial activity: *Staphylococcus aureus* ATCC 80958, *Staphylococcus epidermidis* ATCC 35984, *Staphylococcus saprophyticus* ATCC 49453, *Escherichia coli* ATCC 35218 and *Salmonella enterica* ATCC 51741. Eight ATCC strains of yeasts were used to assess antifungal activity: *Candida albicans* (90028), *Candida albicans* (MYA5314), *Candida parapsilosis* (2209), *Candida glabrata* (9030), *Candida guilliermondii* (6260), *Candida krusei* (6258), *Candida tropicalis* (750) and *Cryptococcus gattii* AFLP4.

2.7. Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

The antibacterial activity of ICTI was evaluated by the broth microdilution assay, protocol M07-A9 (CLSI, 2012), performed in 96-well microplates. The bacterial inoculum was prepared using the direct growth method. Muller Hinton Agar (MHA) isolated colonies were placed in sterile 0.9% NaCl solution until turbidity reached 0.5 on the McFarland scale (1.5 × 10^8 CFU.mL^{-1}). The bacterial suspension was diluted 1:20 (v/v), reaching a concentration of 1.5 × 10^6 CFU.mL^{-1}. The diluted suspension was added to the microplate wells containing the Muller Hinton (MH) medium, ICTI, and antibiotic, with a final concentration of 1.5 × 10^5 CFU.mL^{-1}. ICTI was prepared in sterile 0.9% NaCl solution at concentrations ranging from 0.156 mM to 10 mM. The microplate was incubated at 37 ºC under shaking and monitored for 18 h with intervals of 30 min in a Multiskan GO microplate reader (Thermo Fisher Scientific, Brazil) at 595 nm. To determine the minimum bactericidal concentration (MFC), 10 µL aliquots were cultured on MHA plates, and incubated for 24 h at 37 °C. The MBC was determined as the lowest concentration of the ICTI at which there was no visible growth in the solid medium.

2.8. Determination of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC)

The MIC and MFC promoted by ICTI were determined by the broth microdilution method, as outlined by the Clinical and Laboratory Standard Institute (CLSI), M27-S3 and M27-A3 protocols. Yeast cells (1.5 × 10^3 cells. mL^{-1}) were incubated in Sabouraud broth containing serial concentrations of ICTI (from 514 to 4 µM). The assay was performed in 96-well microplates at 37 ºC for 24 h – 48 h, depending on the yeast tested. Amphotericin B was used as a positive control. A visual reading of the results was performed, and the MIC was determined from the wells in which there was no fungal growth (no turbidity). To determine the minimum fungicidal concentration (MFC), 10 µL aliquots were cultured on Sabouraud dextrose agar (SDA) plates, and incubated for 24 h – 48 h at 37 °C. The MFC was determined as the lowest concentration of ICTI for which there was no visible growth in the solid medium. The MFC/MIC ratio was calculated to determine fungistatic (MFC/MIC ≥ 4) and fungicidal (MFC/MIC < 4) properties. Each assay was performed in triplicate using three independent experiments (Sardi et al., 2017).

2.9. Determination of combined antifungal activity

Synergism between ICTI and the standard antifungal, Amphotericin B, was evaluated using the checkerboard microdilution assay against *Candida albicans* (90028) and *Cryptococcus gattii* yeasts in RPMI 1640 culture medium. The substances (ICTI and Amphotericin B) were tested alone, and in combination, against yeasts in a progression of dilutions from 2 x MIC (as previously found). Combinatorial activity was determined by checkboard assay using 96-well microplates incubated at 37 °C for 24 h – 48 h. A visual reading of the results was performed. A mathematical calculation was used to generate the fractional inhibitory concentration index (FICI), calculated as (MIC compound 1 in combination/ MIC compound 1 alone) + (MIC compound 2 in combination/ MIC compound 2 alone). Combinations were classified as synergistic (FICI ≤ 0.5), additive (0.5 < FICI ≤ 1.0), indifferent (1.0 < FICI < 4.0), or antagonistic (FICI ≥ 4.0). All experiments were performed in independent triplicates (Sardi et al., 2016).

2.10. Exploring the antifungal mechanisms of action in the presence of sorbitol of ergosterol

Previously determined MIC values were used as starting concentrations in MIC assays that were performed in the presence of anhydrous D-sorbitol (0.8 mol.L^{-1}) or exogenous ergosterol (1.01 mol.L^{-1}) to investigate the mechanism of action against yeast (*C. gattii* AFLP4 and *C. albicans* ATCC 90028). ICTI microdilutions (from 514 to 4 µM) were tested in triplicate, and the results were read visually, analyzing the turbidity of the wells after 24 h – 48 h. Sorbitol can prevent damage to the cell wall structure, while ergosterol

protects the fungal plasma membrane. An increased value of supplemented MIC indicates that ICTI was bound to the supplemented molecule, decreasing its effect against yeast and confirming its mechanism of action (Sardi et al., 2016).

2.11. Determining the effects of ICTI on biofilm formation

A 100 µL aliquot of *C. albicans* inoculum (1 x 10^5 CFU. mL^{-1}) was added to the wells of a 96-well microplate. The microplate was incubated at 37 °C for 2 h to allow pre-adhesion of cells using BHI supplemented with 0.1% glucose (BHIg). Subsequently, wells were washed with 0.9% saline to remove planktonic cells. The adhered cells were then treated with ICTI at 1X and 10X MIC for 24 h at 37 °C. After the incubation period, the biofilm formed on the plates was scraped using a micropipette, and 50 µL of 0.9% saline were used to dissociate cells. A serial dilution was performed in tubes for each microplate well. Aliquots of each dilution were plated on Sabouraud agar with Drigalski handle, and the CFU/mL count was determined by comparing treated and untreated biofilms after 24 h at 37 °C (Sardi et al., 2017). Amphotericin B was used as a standard drug. Culture medium alone was included as a negative control.

2.12. Determining the effects of ICTI on mature biofilm

To investigate the effects of ICTI on mature biofilm, BHIg was used, and the microplate was incubated at 37 °C for 24 h to induce biofilm formation. After removing planktonic cells by washing with 0.9% saline, BHIg-containing treatments (1X ICTI or 10X MIC) were added. Amphotericin B was used as a standard drug. Culture medium alone was included as a negative control. After the incubation period, the biofilm formed on the plates was scraped using a micropipette, and 50 µL of 0.9% saline were used to dissociate cells. A serial dilution was performed in tubes for each microplate well. Aliquots of each dilution were plated on Sabouraud agar with a Drigalski handle, and the CFU/mL count was determined by comparing treated and untreated biofilms after 24 h at 37 °C (Sardi et al., 2017).

2.13. Fluorescence microscopy

Confocal microscopy is commonly used to image yeast biofilms in 3D. Biofilms formed on acrylic resin surfaces were stained with SYTO-9 (for staining of viable cells) and propidium iodide (PI, for staining of non-viable cells), following the manufacturer's instructions. The biofilm was visualized using a Leica DM 2000 LED microscope, equipped with a Leica DFC 7000 camera (SYTO9: excitation at 490 nm and emission at 520 nm; PI: excitation at 490 nm and emission at 635 nm). Image reconstruction was performed with Andor iQ3 software (Andor, Ireland). The entire image was used for quantification, and the analysis was performed with ImageJ software.

2.14. Evaluation of toxicity in the in vivo Galleria mellonella model

G. mellonella, also known as the wax moth larvae, is an *in vivo* model used in several research fields because it shares some similarities with the mammalian innate immune system. For evaluating the potential acute toxicity of ICTI, late instar *G. mellonella* larvae weighing 0.2-0.3 g were randomly divided into groups for each treatment. Larvae were maintained in sterile Petri dishes, and treatments consisted of ICTI (1X and 10X MIC in 0.9% sterile saline), Amphotericin B (1X and 10X MIC) and 0.9% sterile saline as a negative control. Treatments were administered to larvae by injecting 10 uL into the larval hemocoel (the body cavity). For each treatment and control, ten larvae were used in triplicate. Larval survival was recorded at selected intervals for 96 hours. Larvae were considered dead when immobile to the touch and showed a high level of melanization (Megaw et al., 2015).

2.15. Statistical analysis

All assays were performed in triplicate and three independent experiments. The data were submitted to the Shapiro-Wilk test to assess the normality of the distribution. Biofilm assays were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test at a significance level of 5% ($p < 0.05$ was considered statistically significant).

3. Results

3.1. ICTI purification

The protein content in the material obtained from the extraction, called crude extract (CE), was quantified as 0.29 $mg.mL^{-1}$. The chromatogram revealed that the CE separated into two fractions, the second being eluted in the NaCl gradient (Figure 1a). CE and fraction 2 from the HiPrep™ DEAE FF 16/10 column were analyzed by 12.5% SDS-PAGE to accompany the inhibitor purification process (Figure 1a). Three major bands were observed in the CE, and one in fraction 2. The first band, between the 17 and 24 kDa markers, appears in the CE and in fraction 2. The bands that appear between the 3.5 and 8 markers, 5 kDa are only present in the CE. Calderon et al. (2010) determined that ICTI was an inhibitor with a molecular weight of approximately 20 kDa, as observed for the protein present in fraction 2 (ICTI). The inhibitory activities of CE and ICTI against trypsin were determined by measuring their hydrolytic activity against BAPNA. As shown in Figure 1b, stoichiometry studies reveal a dose-dependent inhibition, reaching 100% inhibition from a concentration of 1 $µg.µL^{-1}$.

3.2. Antibacterial activity

The bactericidal activity of ICTI against pathogenic strains of Gram-positive (*S. aureus*, *S. saprophyticus*) and Gram-negative (*E. coli* and *S. enterica*) bacteria was evaluated using the dilution method in a microplate (MIC and MBC). Concentrations ranging from 10 mM to 0.156 mM ICTI were tested. None of the concentrations of ICTI used had a significant effect against bacterial strains.

3.3. Antifungal activity

ICTI was found to exhibit predominantly fungicidal activity with an MIC and MFC of 32.11 µM, when evaluating the growth of four yeast strains (*C. gattii*, *C. albicans* ATCC

90028, *C. glabrata*, and *C. guilliermondii*), as shown in Table 1. The MFC/MIC ratio indicates the fungicidal activity of ICTI. The strains, *C. gattii* AFLP4 and C. *albicans* 90028, were selected for continue with other tests involving the fungicidal activity of the inhibitor, due to the greater pharmaceutical relevance of the two strains.

The combination of ICTI and Amphotericin B showed synergistic effects, according to FICI (≤ 0.5 = synergistic) (Table 2). The synergistic activity of the combination of ICTI and the clinically used antifungal Amphotericin B, against the strains, was quantified by the Checkerboard test at subinhibitory concentrations. This synergistic combination reduced the concentration of ICTI for *C. albicans* by 535-fold and by 4-fold for *C. gattii*. The MIC of Amphotericin B was reduced by 4-fold for *C. albicans*, and 18-fold for *C. gattii*.

The MIC of ICTI in the presence of the osmotic protector, D-sorbitol (0.8 mol.L^{-1}), was unaltered, indicating that the inhibitor has no direct action on the yeast cell wall since it does not interact with free sorbitol in the medium. However, the addition of exogenous ergosterol (1.01 mol.L^{-1}) to the medium increased the MIC of ICTI by 4X, indicating a possible interaction with the plasma membrane (Table 3).

The antibiofilm properties of ICTI were investigated against *C. albicans*. ICTI inhibited 38.5% of *C. albicans* biofilm formation at MIC (Figure 2a) and inhibited biofilm formation by 51.6% at 10XMIC. In the biofilm eradication assay, a 37.2% reduction in biofilm biomass was observed at MIC (Figure 2b) and a 56.9% reduction was seen with 10XMIC. These data were supported by fluorescence microscopy assays, which confirmed the effects of ICTI and Amphotericin B on *C. albicans* (Figure 2e). When analyzing the images, differences were noted between the control biofilm and the treatments with ICTI and Amphotericin B. Differences were observed both in the mass of the biofilm and in the number of viable cells, mainly in the concentration of 10XMIC.

Finally, the acute toxicity of ICTI was evaluated in *G. mellonella* larvae. At the highest concentrations tested (321 µmol.L^{-1}), all larvae treated with ICTI remained alive at the end of 72 h, demonstrating the non-toxicity of ICTI *in vivo*.

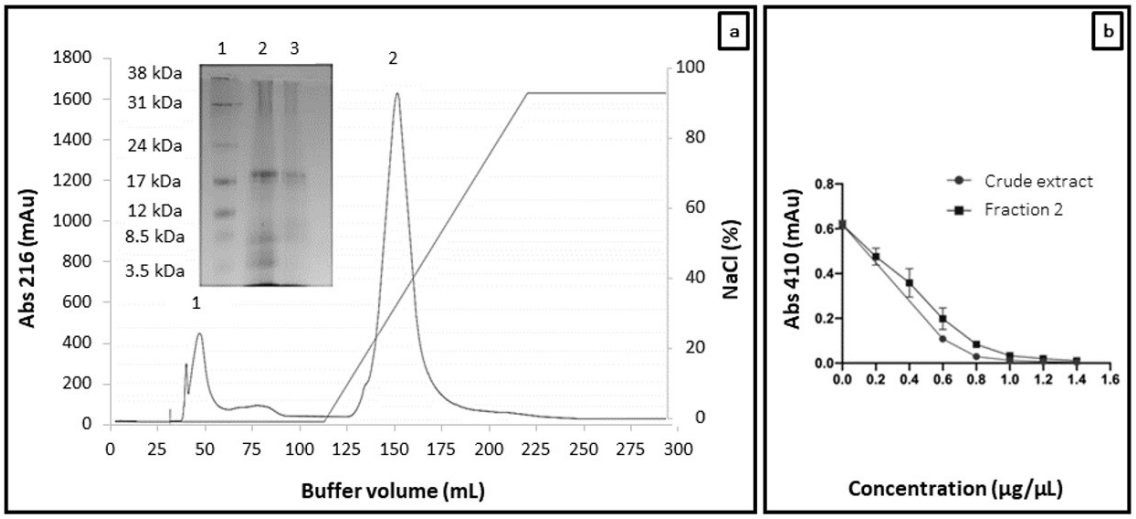

Figure 1. ICTI purification – (a) Ion exchange chromatography (HiPrep^{tm} DEAE-Sepharose column). Insert: SDS-PAGE: lane (1) molar mass markers, (2) Crude Extract, (3) Fraction 2. (b) Bovine trypsin inhibition assay with Crude Extract and fraction 2 (0 - 1.4 µg.µL^{-1}).

Table 1. Antifungal properties of ICTI against pathogenic yeasts.

Yeast	ATCC	ICTI			Amphotericin B		
		MIC	MFC	Ratio	MIC	MFC	Ratio
Cryptococcus gattii	AFLP4	32.11	32.11	1	1.08	1.08	1
Candida albicans	90028	32.11	32.11	1	0.54	0.54	1
Candida glabrata	90030	32.11	32.11	1	0.13	0.54	4.15
Candida guilliermondii	6260	32.11	32.11	1	0.06	0.13	2
Candida albicans	MYA5314	257	257	n.a	0.54	0.54	1
Candida parapsilosis	2209	>514	>514	n.a	0.27	0.54	2
Candida krusei	6258	>514	>514	n.a	0.27	0.54	2
Candida tropicalis	750	>514	>514	n.a	0.54	0.54	1

Footnote: Data in µmol.L^{-1} – ICTI, *Inga cylindrica* trypsin inhibitor; MIC, Minimum Inhibitory Concentration; MFC, Minimum Fungicidal Concentration; Ratio, MFC/MIC; ratios <4 indicates fungicidal activity, n.a, indicates not applicable.

Table 2. Synergistic effect of ICTI, in combination with Amphotericin B, against pathogenic yeasts.

Microorganism	Combination	Individual MIC		Combination MIC		Σ FICI	Activity
		A	B	A	B		
C. albicans	ICTI (A) + Amphotericin B (B)	32.11	0.54	0.06	0.135	0.251	Synergistic
C. gattii	ICTI (A) + Amphotericin B (B)	32.11	1.08	8.02	0.06	0.299	Synergistic

Footnote: Data in µmol.L^{-1}. MIC, Minimum Inhibitory Concentration; FICI, Fractional Inhibitory Concentration Index (MIC (A) in combination/ MIC (A) alone) + (MIC (B) in combination/ MIC (B) alone).

Table 3. Effects of sorbitol (0.8 mol.L^{-1}) and ergosterol (1.01 mol.L^{-1}) on the antifungal activity of ICTI for *C. albicans* and *C gattii*.

Mode of action	*C. albicans*		*C. gattii*	
	Presence	Absence	Presence	Absence
Sorbitol	32.11	32.11	32.11	32.11
Ergosterol	>513.74	32.11	>513.74	32.11

Footnote: Data in µmol.L^{-1}.

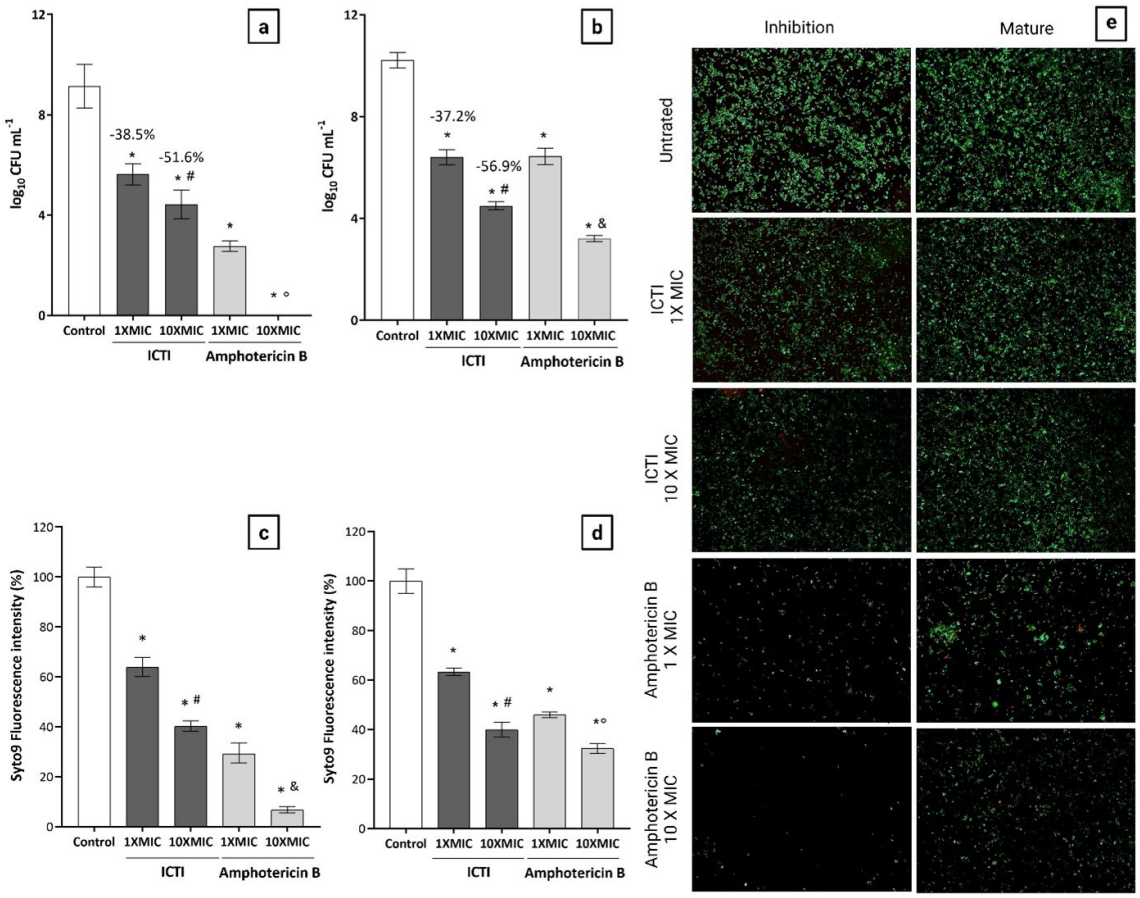

Figure 2. Antibiofilm potential of ICTI in *C. albicans* - (a) Quantitative analysis of the effects of ICTI and Amphotericin B on biofilm formation inhibition, and (b) eradication of mature pre-formed biofilm. Fluorescence emission in % in terms of inhibition of biofilm formation (c) and biofilm eradication (d) from the images obtained by fluorescence microscopy. The quantitative analysis of the effects of ICTI on the viability of biofilm-constituting cells is also shown Fluorescence assays showing the potential of ICTI for inhibiting biofilm formation and eradicating pre-formed biofilms are also shown in comparison with Amphotericin B (e). Biofilms were stained and visualized using SYTO-9 to stain live biofilm cells (green, live cells) and propidium iodide (PI) (red, dead cells). Bars represent means ± standard deviation (ANOVA followed by Tukey´s post-test, * p< 0.0001 in relation to Control, # p<0.0001 in relation to 1xMIC ICTI, ° p<0.001 in relation to 1X MIC Amphotericin B, & p<0.0001 in relation to 1X MIC Amphotericin B).

4. Discussion

Species of the genus *Cryptococcus spp*. and *Candida spp*. are among the most clinically relevant pathogenic yeasts (Pappas et al., 2018, Oliveira et al., 2018). Cryptococcosis is a systemic mycosis caused by inhalation of dehydrated yeast or fungal spores of *Cryptococcus spp*., which enter the lungs and reach the alveoli (Santos et al., 2021). Of the systemic mycoses, this disorder has a higher incidence in immunocompromised patients and is the third most prevalent disease in human immunodeficiency virus (HIV)-positive individuals (Hou et al., 2019). Candidiasis is an opportunistic fungal infection capable of causing skin and mucous infections (oral or vaginal), which can escalate to systemic infections, particularly in immunocompromised patients (Espino et al., 2019; Oliveira et al., 2018).

Currently, the three main classes of drugs used to treat fungal infections are polyenes, azoles and echinocandins. Polyenes, such as Amphotericin B, target ergosterol in the fungal cell membrane; azoles, such as fluconazole, inhibit the Erg11 demethylase enzyme of the ergosterol biosynthesis pathway; and finally, the most recent class of drugs, the echinocandins, such as caspofungin, inhibit the synthesis of the cell wall cross-linking component, β-1,3-glucan (Lohse et al., 2020). However, some microorganisms are already exhibiting resistance to these drugs and the majority are toxic when administered to humans (Zareshahrabadi et al., 2022). Thus, new alternatives are sought for the treatment of these microorganisms.

The use of natural compounds to treat pathogenic microorganisms in humans is increasing, as episodes of microbial resistance against commercial medicines are being reported daily (Dib et al., 2018). Natural compounds are biodegradable, are usually non-toxic to humans and antagonistic to microorganisms, and most importantly, have evolved over millions of years to combat pathogenic yeasts (Chiu et al., 2022). Around 500 million years ago, the first land plants established themselves on Earth and began to interact with microorganisms, developing defense proteins as a form of protection. These proteins include defensins, antimicrobial peptides (AMPs), and PIs, with the latter being found in various parts of plant tissue, including seeds, bulbs, and tubers; Thus, the identification of new PIs from plants has aroused great interest in medicine and biotechnology due to their ability to treat immunological (Karray et al., 2020) and microbiological diseases (Dib et al., 2018; Cotabarren et al., 2020; Herwade et al., 2021).

An evaluation of the antifungal activity of synthetic cowpea defensin, Cp-thionin II, showed that this protein demonstrates fungistatic action (growth inhibition) on the fungal growth of different species, but that these effects are not fungicidal (cell death) (Thery and Arendt, 2018). The fungicidal action of defensins can be influenced by the presence of divalent cations, such as calcium (Ca^{2+}), magnesium (Mg^{2+}) and zinc (Zn^{2+}) and the antifungal activities of some AMPs are also affected by the presence of cations. Cations affect the activities of these proteins by weakening their electrostatic interactions with the negatively charged membrane of microbial cells (Dib et al., 2018). Another obstacle to the use of AMPs is their sensitivity to proteolytic digestion by fungal peptidases, which would make therapeutic use against microbial infections in humans difficult. Therefore, the application of PIs to control fungal infections is pertinent, due to their specific and high-affinity interaction with vital peptidases, associated with the pathogenic mechanism of fungal infection that involves the secretion of peptidases (Dib et al., 2018).

The mechanisms of action of PIs are associated with generating reactive oxygen species (ROS) or disrupting the fungal cell wall and/or membrane integrity, leading to potential cytoplasmic leaks or wall destabilization (Dib et al., 2018; Chiu et al., 2022). PIs can also trigger apoptosis in yeast by blocking some important serine peptidases, such as metacaspases and nuclear mediator of apoptosis (Nma111p) (Dib et al., 2018). ICTI presented promising results in fungicidal activity tests, displaying MIC and CFM values of 32.11 $\mu mol.L^{-1}$ (625 $\mu g.mL^{-1}$). Other trypsin inhibitors from the *Inga* genus have also been shown to be fungicidal agents. IVTI (*Inga vera* trypsin inhibitor) inhibited the growth of *C. buinensis* by 38% at 125 $\mu g.mL^{-1}$ (Bezerra et al., 2016), and ILTI (trypsin inhibitor *Inga laurina*) completely inhibited *C. tropicalis* at 125 $\mu g.mL^{-1}$ and C. *buinensis* at 250 $\mu g.mL^{-1}$ of ILTI (Macedo et al., 2016). IETI (trypsin inhibitor from *Inga edulis*) exhibited inhibitory effects against *C. albicans, C. tropicalis* and *C. buinensis*; notably the latter strain was inhibited by 87% at a concentration of 400 $\mu g.mL^{-1}$ IETI (Dib et al., 2018). In all cases, the authors observed that damage to the fungal membrane occurred after treatment with the inhibitors. This ability to induce cellular damage has been reported for different families of proteins/peptides and plants. These effects include changes in the cell membrane and in the regulation of ion flow, making these molecules ideal models for understanding the functioning of vital proteins that act as ion channels (Bezerra et al., 2016; Dib et al., 2018; Macedo et al., 2016; Oliveira et al., 2018).

ICTI proved to be fungicidal for certain strains of yeast, but ineffective against others; it did not inhibit *Candida tropicalis* but was fungicidal against *C. gattii* at a concentration of 32.2 $\mu mol.L^{-1}$. This may be related to the fact that a variety of peptidases are secreted on the surface of different yeast species, in addition to those present in the extracellular environment, changing the virulence capacity of each strain (Macedo et al., 2016). Furthermore, no other publication reported the action of peptidase inhibitors against planktonic *C. gattii* cells, demonstrating the innovative nature of this study with peptidase inhibitors.

An important virulence characteristic of *C. albicans* is its ability to form biofilms, a cluster of cells protected by an extracellular matrix. *C. albicans* forms biofilms on mucosal surfaces and on implanted medical devices such as catheters, dentures, and heart valves (Lohse et al., 2020). ICTI, when tested against mature *C. albicans* biofilm biomass at a concentration of 32.2 $\mu mol.L^{-1}$, decreased cell viability by about 30%. This result confirms that, due to the protection provided by the polysaccharide matrix, drugs need to be administered at concentrations higher than the MIC value for the treatment of biofilm, compared to planktonic cells. This can lead to side effects in the host, such as damage to the liver and kidneys (Lohse et al., 2020).

Accordingly, compounds with antifungal potential have also been studied to expand the therapeutic arsenal of diseases and reduce the concentrations administered, based on the association of medications with pharmacological synergism (Araújo et al., 2019; Nicola et al., 2019; Lee et al., 2020). Antimicrobial synergy is based on the principle that, in combination with other medicines, such formulations can increase efficacy, reduce toxicity, decrease adverse side effects, increase bioavailability, decrease dose, and reduce concentrations and antimicrobial resistance (Araújo et al., 2019; Tonon 2016). The combination of ICTI and Amphotericin B led to a significant reduction in the MIC concentration of both compounds, indicating that there was an association in the mode of action of the therapy. Tonon (2016) showed that *Coriandrum sativum* essential oil, when associated with Amphotericin B, had synergistic effects on *C. albican*s and additive effects on *C. tropicalis*. A literature search for the synergistic effects of PIs associated with antifungals did not identify any publications with similar innovative results.

As mentioned before, Amphotericin B acts as a fungistatic or fungicidal agent and targets the sterols present in the fungal membrane. In yeasts of the *Candida* and *Cryptococcus* genera, Amphotericin B binds directly to ergosterol, a sterol presents in the yeast membrane (Carolus et al., 2020). Ergosterol is known to maintain cellular function and integrity, and polyene derivatives, such as the antifungal Amphotericin B, bind to ergosterol present in the fungal cell membrane; thus, interfering with permeability and transport functions. Amphotericin B has a selective action on fungal membranes, as opposed to bacteria (Silva et al., 2020), due to the drug's high avidity for ergosterol, the main sterol in the fungal membrane, in contrast to bacterial and animal cells, where cholesterol is the main sterol (Ivanov et al., 2022; Tevyashova et al., 2023).

Compounds that bind to ergosterol cause the formation of pores or transmembrane channels, resulting in the increased permeability of the fungal membrane and causing significant changes in ionic balance. Consequently, there is an extensive loss of a variety of small molecules and electrolytes from the intracellular environment, mainly K^+, which alters the homeostasis of the microorganism (Haro-Reyes et al., 2022; Vanzolini et al., 2023).

The addition of free ergosterol increased the ICTI MIC for both *C. albicans* and *C. gattii*. This can be attributed to a competitive action whereby the exogenous ergosterol, added to the medium, competes for antifungal binding, as opposed to binding to the ergosterol in the fungal cell membrane. This causes an increase in the MIC as more antifungal is needed to outcompete exogenous ergosterol and inhibit fungal growth. This study showed that ICTI did not prevent the growth of bacteria. Two other studies by Bezerra et al. (2016) and Macedo et al. (2016) showed similar results, where the trypsin inhibitors, IVTI and ILIT, displayed fungicidal properties, but did not have bactericidal actions. These findings, coupled with the fact that ICTI had no toxic effect on *G. mellonella* (an invertebrate whose primary membrane sterol is cholesterol), suggest that the antifungal effect of *Inga* species is comparable to that of commercial polyenes such as Amphotericin B. The inhibitor apparently targets ergosterol in the fungal membrane with no effect on animals or bacteria, causing membrane rupture and leading to cell fungal death.

In conclusion, ICTI did not present bactericidal activity under the conditions evaluated, but was active against yeast, with an MIC of 32.11 $\mu mol.L^{-1}$. The combination of ICTI with Amphotericin B showed a synergistic effect, reducing the concentration of ICTI for *C. albicans* by 535 times and by 4 times for *C. gattii*. ICTI also affected the viability of fungal biofilms and promoted the partial eradication of mature forms of biofilm. Based on the results obtained, its suggest that the antifungal activity of ICTI involves binding to ergosterol present in the fungal cell membrane. This specific action is also observed in polyene antifungals, such as Amphotericin B. Given the ability of ICTI to bind ergosterol, membrane damage can occur, promoting extravasation of intracellular contents. ICTI was not toxic when administered to the *G. mellonella* model. Thus, ICTI is a protease inhibitor with antifungal therapeutic potential, exhibiting effectiveness both alone and in combination with Amphotericin B. Moreover, this molecule is non-toxic to non-fungal cells, which supports future trials in other animal models.

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