

Antifungal activity of a trypsin inhibitor from *Salvia hispanica* L.(chia) seeds against fluconazole-resistant strains of *Candida* spp. and evaluation of its toxicity in vitro

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Abstract

The incidence of *Candida* species resistance to traditional antifungals is increasing globally. This issue significantly impacts patients' lives and raises healthcare expenses, confirming the need for developing novel therapeutic strategies. Recently, a thermostable trypsin inhibitor was isolated from *Salvia hispanica* L. (chia) seeds – named ShTI (MM 11.558 kDa) with an antibacterial effect against *Staphylococcus aureus* species. This work aimed to assess the antifungal effect of ShTI against *Candida* species and its synergism with fluconazole and to evaluate its mode of action. Moreover, preliminary toxicological studies using mouse fibroblast cells were performed. ShTI displayed an anticandidal effect alone against *C. parapsilosis* (ATCC® 22019), *C. krusei* (ATCC® 6258), and six clinical fluconazole-resistant strains of *C. albicans* (2), *C. parapsilosis* (2), and *C. tropicalis* (2) (MIC 50: 4.1 µM and MIC 100: 8.2 µM) and exhibited a synergistic effect when combined with fluconazole against *C. albicans* with complete alteration of the morphological structure of the yeast. The mode of action of ShTI against *C. krusei* (ATCC® 6258™) and *C. albicans* species involves cell membrane damage due to increased membrane permeabilization, overproduction of reactive oxygen species, formation of pseudohyphae, injury of cells and pore formation and consequently cell death. In addition, ShTI (8.65 and 17.3 µM) showed a noncytotoxic and nongenotoxic effect in L929 mouse fibroblast cells. These findings make it plausible to assume that ShTI is a promising antimicrobial candidate, but new assays are required to progress the application of ShTI's potential usage as a novel antifungal.

Introduction

Serious fungal infections have become more prevalent during the past few years in hospital settings, primarily affecting immunocompromised or critically ill patients. Since 2013, the Leading International Fungal Education (LIFE) has estimated that 80% of the world's population, or approximately 5.7 billion people, are affected by fungal illnesses [1, 2]. *Candida spp.* was determined to be the most common pathogenic species among yeast fungal infections, generating both superficial and systemic infections (such as candidemia), a significant factor in human morbidity and mortality in hospital settings [2]. Fluconazole is the largest antifungal drug used in candidemia episodes; however, the indiscriminate use of fluconazole has led to the development of resistant *Candida* species [3]. Resistance to antifungals has a significant impact on patients' quality of life, results in higher healthcare expenses, and demonstrates the need for innovative methods of preventing fungal infection. The alternative is to look for potent new antimicrobials or drug-combination techniques as a means of avoiding resistance mechanisms [4].

Several research groups have proposed protease inhibitors (PIs) as a relevant class of compounds in the quest for novel antifungals. PIs are ubiquitous molecules with a multitude of physiological activities in all species. They have been linked to inducible defense responses against pests and pathogens in plants, which strengthens their potential as antimicrobial agents. Peptide- and protein-based PIs are abundantly expressed in plants; for instance, 1–10% of the total soluble protein content in the seeds of legumes consists of PIs, demonstrating their importance for plant physiology and defense [5]. Plant PIs are a

diversified group of highly stable proteins and peptides that have been investigated for their potential as anticancer, anticoagulant, antihypertensive, antioxidant, and antibacterial agents [6].

Salvia hispanica L. is an herbaceous plant belonging to the *Lamiaceae* family and popularly known as Chia. Currently, chia seeds have been widely used in the food industry due to their high availability and nutritional value. The unique chemical composition corresponds to a high content of polyphenols, lipids, and proteins (15–25% of their weight) [7]. Recently, our research group isolated a trypsin inhibitor from chia seeds named ShTI (MM 11.558 kDa). ShTI exhibited remarkable temperature (100°C for 2 h) and pH stability (2–10). In addition, it demonstrated antibacterial activity, interacting synergistically with oxacillin against a methicillin-resistant *Staphylococcus aureus* strain. According to Souza [8], the antibacterial effect of ShTI involved the overproduction of reactive oxygen species (ROS) and membrane pore formation. Therefore, this inhibitor is an interesting candidate for use as a lead compound in the development of new antimicrobial agents for medical applications.

The study reports the antifungal effect of the trypsin inhibitor from chia seeds, including its potential synergism with fluconazole and its mode of action toward the sensitive and resistant strains of *Candida spp.* Moreover, considering the potential therapeutic application of ShTI in the treatment of microbial infections, preliminary toxicity studies were also conducted.

Materials And Methods

Reagents

Trypsin from bovine pancreas, bovine serum albumin (BSA), Na-benzoyl- D, L-arginine 4-nitroanilide hydrochloride (BApNA), acridine orange, 2,7-dichlorofluorescein diacetate (DCFH-DA), MOPS buffer sodium bicarbonate, propidium iodide, ethidium bromide, fluconazole (FLC), fetal bovine serum (FBS), 3-(4,5-dimethyl-2-thiozoly)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and Sabouraud dextrose agar were purchased from Merck Sharp and Dohme, NJ, USA. The CNBr-activated Sepharose 4B chromatographic matrix was obtained from GE Healthcare. The symmetry C18 column (0.46 × 25 cm, 5 µm particle size, 300 Å pore size) was obtained from Waters Corp. (Milford, MA, USA). RPMI 1640 medium, glutamine, penicillin, and streptomycin were purchased from Gibco (Invitrogen, Carlsbad, CA, USA), and low-melting-point agarose and agarose were obtained from Invitrogen (Carlsbad, CA, USA). All additional chemicals and reagents used were of analytical grade and obtained locally.

Salvia hispanica L. trypsin inhibitor (ShTI) purification

Salvia hispanica L. (chia) seeds were obtained from the local market in Fortaleza, Ce, Brazil, and processed according to Souza [8]. The research was conducted under the authorization to access and use the genetic heritage in Brazil (National Management System of Genetic Heritage and Associated Traditional Knowledge – SISGEN) and received the registration code at SISGEN A879F9E.

The ShTI purification process was carried out as described by Souza [8]. For protein extraction, fat and mucilage-free chia seed flour in 0.05 M Tris-HCl, pH 8.0, with 0.5 M NaCl (1:10 m/v) under stirring (4 h, 4°C). Then, the suspension was filtered, centrifuged ($10,000 \times g$, 30 min, 4°C), and collected for the following procedures: fractionation by heat treatment (30 min, 100°C), affinity matrix chromatography with a trypsin-Sepharose 4B column, and reversed-phase high-performance liquid chromatography. The fractions were monitored at 280 nm, and the ShTI purification was guided by a trypsin inhibitory activity assay using BApNA as substrate, according to Erlanger; Kokowsky; Cohen [9]. The purity and molecular mass were measured by tricine-SDS-PAGE [10]. For antifungal testing, ShTI samples were prepared under sterile conditions, filtered through a 0.45 µm membrane and based on total soluble protein concentrations using BSA as a standard [11].

Yeast strains

Fluconazole-resistant clinical isolate strains of *C. albicans* (strains 1 and 2), *C. parapsilosis* (strains 1 and 2), *C. tropicalis* (strains 1 and 2), and the two ATCC species of *C. parapsilosis* (ATCC® 22019™) and *C. krusei* (ATCC® 6258™) were obtained from the fungi collection of the Laboratory of Bioprospecting of Antimicrobial Molecules (LABIMAN) at the Federal University of Ceará (UFC). The fungal isolates were grown on Sabouraud dextrose agar (35°C, 24 h), and cell suspensions were produced in a sterile 0.9% NaCl solution. A spectrophotometer (530 nm) was used to adjust the cell density until it reached the transmittance corresponding to the 0.5 McFarland scale, which corresponds to $1-5 \times 10^6$ CFU/mL.

Fibroblast Cell Line

The mouse fibroblast cell line L929 was acquired from the Rio de Janeiro cell bank in Rio de Janeiro, Brazil. The cell culture was grown in Dulbecco's Modified Eagle Medium (DMEM) with Earle's salts supplemented with 10% fetal bovine serum, glutamine (2×10^{-3} M), penicillin (100 µg/mL), and streptomycin (100 µg/mL) at 37°C with 5% CO₂.

Anti-candida effect of ShTI

The in vitro antifungal assay of ShTI was performed by applying the broth microdilution test, established by document M27-A3, according to the recommendations from the Clinical & Laboratory Standards Institute – CLSI [12]. Aliquots (0.1 mL) of the yeast cell suspension ($1-5 \times 10^6$ CFU/mL) in RPMI broth (pH 7) were incubated with 0.1 mL of ShTI in a 96-well microtiter plate for 24 hours at 37°C. For the anticandidal assay, ShTI was diluted serially from 0.02 to 8.22 µM final concentrations in distilled water and filtered through 0.22 µm membranes. The minimal protein concentration that produces 50% (MIC 50) or 100% (MIC 100) growth inhibition was described as the minimum inhibitory concentration (MIC) of ShTI. The antifungal fluconazole (FLC) (6×10^{-3} – 5×10^{-2}) and RPMI medium were used as positive and negative controls, respectively. The tests were repeated three times with different samples and visible turbidity compared to controls.

Evaluation of the action mode of ShTI

Assessment of cell membrane integrity

The cell membrane permeabilization test was accomplished by the propidium iodide staining method through fluorescence. *C. krusei* (ATCC® 6258™) and *C. albicans* (strain 1) cells were treated with NaCl (0.15 M), ShTI (2.05 or 4.1 μM), or FLC (5×10^{-2} μM) for 24 h at 37°C in the dark, washed three times with NaCl 0.15 M and centrifuged (2000 $\times g$, 5 min, 25°C). Subsequently, the cell suspension (0.1 mL) was incubated with 1 mM propidium iodide under bundle agitation (75 rpm) for 30 min at 37°C. Posteriorly, plasma membrane permeabilization was observed under a fluorescence microscope (Olympus BX 41; excitation wavelength, 545 nm; emission wavelength, 580–590 nm; $\times 40$ magnification). The presence of red fluorescence indicated membrane permeabilization in the cell suspension [13].

Determination of cellular reactive oxygen species (ROS) production

To assess ROS production, *C. krusei* (ATCC® 6258™) and *C. albicans* (strain 1) were incubated with NaCl (0.15 M), ShTI (2.05 or 4.1 μM), or FLC (5×10^{-2} μM) for 24 h at 37°C, washed three times with NaCl, and centrifuged (2000 $\times g$, 5 min, 25°C). Afterward, 10 μM 2',7'-dichlorofluorescein diacetate (DCFH-DA) was added, and the mixture was incubated for 3 h at 37°C. Then, the cells were visualized under a fluorescence microscope (Olympus System BX 60; excitation wavelength, 488 nm; emission wavelength, 525 nm), where the presence of green fluorescence indicated ROS overproduction [14].

Synergism of ShTI with Fluconazole

To determine the antifungal effect of the combination of ShTI and FLC, a checkerboard titration assay was applied, the fractional inhibitory concentration index (FICI) was calculated, and the synergism between the drugs was obtained. The assay was performed in the broth microdilution test as described previously. ShTI (MIC/32 – MIC/2) and FLC (MIC/32 – MIC/2) were tested against *C. parapsilosis* (ATCC® 22019™), *C. krusei* (ATCC® 6258™), and fluconazole-resistant *C. albicans* strains (strain 1). The experimental procedures were carried out in accordance with the broth dilution method given in the “MIC determination” section. The interaction between ShTI and FLC on fungal growth was determined as follows (FICI) = MIC (FLC combination)/MIC (FLC alone) + MIC (ShTI combination)/MIC (ShTI alone). The drug interaction data were interpreted to conform to $\text{FICI} \leq 0.5$, synergy (SYN); $0.5 < \text{FICI} < 4.0$, indifference (IND); and $\text{FICI} \geq 4.0$, antagonism (ANT) [15].

Scanning electron microscopy analysis

The *C. krusei* (ATCC® 6258™) and *C. albicans* (strain 1) strains were incubated with NaCl (0.15 M) ShTI (2.05 or 4.1 μM), ShTI (1.03 μM + FLC 1×10^{-2} μM), or FLC (5×10^{-2} μM) for 24 h at 37°C in a 96-well microtiter plate. After the cells were centrifuged, they were fixed, dehydrated, transferred to a coverslip and analyzed according to Staniszevska [16]. The morphological analysis of the *Candida* cells was measured using an Inspect™ 50 FEI scanning electron microscope (SEM) (FEI Company, Hillsboro, Oregon, 8 USA) equipped with a low energy detector (Everhart Thornley detector) in the Center of

Toxicology tests in vitro

MTT assay

The MTT assay was carried out on L929 murine fibroblast cell lines (0.3×10^6 cells/mL) to assess the cytotoxic effect of ShTI. The ShTI in 0.1% dimethyl sulfoxide solution (DMSO) was filtered using $0.22 \mu\text{m}$ membranes and incubated for 24 h with L929 cells in 96-well plates in serial dilutions ranging from 0.07 to $17.3 \mu\text{M}$ final concentration. After centrifugation for 15 minutes at 1,500 rpm, the plates were incubated in DMEM containing 0.5 mg/mL MTT. Three hours later, the insoluble dark blue MTT-formazan product formed was dissolved in DMSO. In a microplate reader, the absorbance was measured at 595 nm to quantify the decreased salt in living cells (Spectra Count; Packard, Canada) [17]. DMSO was used in the control sample in place of ShTI. The data were collected from three separate tests, and expressed as the means $\pm$ standard deviations compared by analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).

Cell morphological analysis

To assess the effect on cell morphology, L929 cells were incubated with ShTI 8.65 or $17.3 \mu\text{M}$ (24 h at 37°C). After the cell suspensions were centrifuged (1,500 rpm for 15 min), the precipitate was suspended in PBS and treated with ethidium bromide ($100 \mu\text{g/mL}$) and acridine orange ($100 \mu\text{g/mL}$) solutions and quantified under a fluorescence microscope using a fluorescein filter (40x objective lens). Then, 300 cells from each sample were counted per data point and differentiated into viable, apoptotic, or necrotic cells [18]. Doxorubicin ($0.55 \mu\text{M}$) was used as a positive control. Experiments were conducted in triplicate in three independent experiments, and approximately 300 cells from each sample were counted. The data were analyzed as the means $\pm$ standard deviations compared by analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).

Alkaline comet assay

The comet test was performed according to Singh [19] with minor modifications by Hartmann and Speit [20]. The L929 cells were incubated for 24 h with ShTI ($8.65 \mu\text{M}$ or $17.3 \mu\text{M}$), washed with PBS, and treated with 0.1 mL of 0.15% trypsin. Detached cells were suspended in complete RPMI medium. Then, 0.02 mL of the cells ($\sim 10^6$ cells/mL) were dissolved in 0.75% low melting point agarose and prepared in a microscopic slide previously covered with agarose with a normal melting point (1%) at 4°C for 5 min. Then, the slides were immersed in a lysis solution (1 h, at 4°C), and electrophoresis was performed. The steps were carried out in the absence of light to avoid damage to the DNA. Next, the slides were neutralized, dehydrated, stained with ethidium bromide ($20 \mu\text{g/mL}$), and analyzed by fluorescence microscopy. Then, 300 cells were randomly counted (100 cells/replicate) and classified as follows according to the DNA tail diameter: class 0 - no damage; class 1 - with a tail smaller than the diameter of the head (core); class 2 - with a tail length 1–2– the diameter of the head; class 3 - with a tail greater

than 2 × the diameter of the head; class 4 - headless comets. A damage index (DI) was calculated: $DI = (0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)$. The index ranged from 0 (without damage: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) Collins [20]. The damage frequency (%) was calculated based on the number of cells with the tail versus without. Vehicle and methylmethanesulfonate (MMS) (4×10^{-5} M) were used as a negative control and positive control, respectively. The assay was performed in triplicate, and the data were expressed as the mean ± standard deviation of three independent experiments ($p < 0.05$). Values $*p < 0.05$ were considered significant (one-way ANOVA followed by Tukey's test).

Results

Anti-candida effect of ShTI

ShTI was effective in growth inhibition of fluconazole-resistant strains of *C. albicans* (strains 1 and 2), *C. parapsilosis* (strains 1 and 2), *C. tropicalis* (strains 1 and 2), *C. parapsilosis* (ATCC® 22019) and *C. krusei* (ATCC® 6258™) (Table 1). The results indicated that *C. parapsilosis* (ATCC® 22019) was the most susceptible strain to ShTI treatment, with MIC50 and MIC100 values of 2.05 and 8.2 µM, respectively. The other strain of *C. krusei* (ATCC® 6258™) and fluconazole-resistant clinical strains of *C. albicans* (1 and 2), *C. parapsilosis* (1 and 2), and *C. tropicalis* (1 and 2) exhibited MIC50: 4.1 µM and MIC100: 8.2 µM.

Table 1
Minimum inhibitory concentrations (MIC50 and MIC 100) of ShTI capable of inhibiting fungal growth in the microdilution susceptibility test against *Candida spp.* strains.

<i>Yeast strains</i>	<i>ShTI (µM)</i>	
	MIC50 ^a	MIC100 ^b
<i>C. albicans</i> (strain 1)	4.1	8.2
<i>C. albicans</i> (strain 2)	4.1	8.2
<i>C. parapsilosis</i> (strain 1)	4.1	8.2
<i>C. parapsilosis</i> (strain 2)	4.1	8.2
<i>C.tropicalis</i> (strain 1)	4.1	8.2
<i>C.tropicalis</i> (strain 2)	4.1	8.2
<i>C. parapsilosis</i> (ATCC® 22019™)	2.05	8.2
<i>C. krusei</i> (ATCC® 6258™)	4.1	8.2

^a MIC50 was defined as the lowest concentration that produced a 50% reduction in fungal cell visible growth after 24 h of incubation.

^b MIC100 was defined as the lowest concentration that produced a 100% reduction in fungal cell visible growth after 24 h of incubation.

Evaluation of the mode of action of ShTI

Cell membrane integrity and ROS overproduction

Membrane permeabilization and ROS overproduction were checked by fluorescence microscopy analysis. In the negative control group, no red fluorescence signal was identified in *C. krusei* ATCC® 6258 (Fig. 1B and 2B) and *C. albicans* (strain 1) (Fig. 1J and 2J). In contrast, the cells incubated with ShTI (2.05 µM, Fig. 1D and L) and ShTI (4.1 µM, Fig. 1F and N) exhibited fluorescence signals indicating increased membrane permeabilization and cell membrane damage in both species. Furthermore, DCFH-DA fluorescence was not observed in the *C. krusei* ATCC® 6258 (Fig. 2B) and *C. albicans* (strain 1) control strains (Fig. 2J). However, after exposure to ShTI (2.05 µM and 4.11 µM), the cells of *C. krusei* (Fig. 2D and F, respectively) and *C. albicans* (Fig. 2L, and N, respectively) demonstrated overproduction of ROS, as indicated by fluorescence and the presence of pseudohyphae formation. For both treatments, the cells were incubated with FLC (5×10^{-2} µM) as a positive control (*C. krusei* - Fig. 1H, and 2H and *C. albicans* - Fig. 1P, and 2P).

Evaluation of drug interactions

After obtaining the mode of action of the anticandidal effect of ShTI, we investigated the combination of ShTI and FLC. The results exhibited a synergistic effect of ShTI and FLC against *C. albicans* (FICI = 0.5), reducing the MIC of drugs in 4x (ShTI: 4.1 µM to 1.03 µM and FLC: 5×10^{-2} µM to 10^{-2} µM). However, synergistic activity was not observed in *C. parapsilosis* (ATCC® 22019) (FICI = 1.0) and *C. krusei* (ATCC® 6258™) (FICI = 0.66) (Table 2).

Table 2
Synergistic effect of ShTI and FLC against different *Candida* species.

Yeast Strains ^a	MIC50 ^b (µM)					
	Isolated		Combination		Synergism ^c	
	ShTI	FLC	ShTI	FLC	FICI	Interpretation
<i>C. parapsilosis</i> (ATCC® 22019)	2.05	6×10^{-3}	1.03	3×10^{-3}	1.0	IND
<i>C. krusei</i> (ATCC® 6258™)	4.1	5×10^{-2}	2.05	3×10^{-2}	0.66	IND
<i>C. albicans</i> (strain 1)	4.1	5×10^{-2}	1.03	10^{-2}	0.5	SYN

^a The collection of isolated yeast strains.

^b Minimum inhibitory concentration (MIC50) is the lowest concentration capable of reducing 50% of the growth of fungal cells.

^cThe synergistic effect of ShTI and FLC according to the FICI equation.

Scanning electron microscopy (SEM)

In the morphological analysis, SEM showed a normal structure cell, smooth surface, and intact membrane cell in the control group in *C. krusei* (ATCC® 6258™) and *C. albicans* (strain 1) species (Fig. 3A and 4A, respectively). In contrast, *C. krusei* (ATCC® 6258™) growth inhibition was confirmed through membrane injury cells by pore-forming from up to 1.803 µm, as indicated in the arrow (ShTI: 2.05 µM, Fig. 3B), and alterations of yeast structure with the presence of cells with enlarged hypha-like structure length (elongated structure of 10.15 µm) (ShTI: 4.1 µM; Fig. 3C). The *C. albicans* (strain 1) cells incubated with ShTI (2.05 or 4.1 µM) exhibited a modified yeast-like structure with the formation of pseudohyphae, pore formation with extravasation of intracellular content, and cell death, as indicated by arrows (Fig. 4B and 4C). This effect was more pronounced in the combination of ShTI 1.03 µM + FLC 1×10^{-2} µM, in which there were gross morphologic alterations of the cell structure, contorted appearance and irregular surface, confirming the synergistic effect with a drug concentration 4x lower than the MIC isolated drugs (Fig. 4D). FLC (5×10^{-2} µM), which was used as a positive control in both species (Fig. 3D and 4E).

Toxicology tests in vitro

MTT assay

As expected, in the cytotoxicity assay (MTT test), L929 mouse fibroblast cell lines exhibited viable cells in the control group. Additionally, ShTI treatment at concentrations varying from 0.07 µM to 17.3 µM did not disrupt cellular metabolic activity compared with vehicle treatment (Fig. 5). Cells treated with DMSO were used as a positive control. Considering that the toxic effects are exhibited at higher doses of the drugs and that ShTI did not interfere with the cell viability of L929 cells, in the other assays, we only tested the highest doses of ShTI of 8.65 µM and 17.3 µM.

Cell morphological analysis

In the cell morphologic analysis, the control group exhibited normal cell structure in the cell suspension analyzed. Equivalently, in the ShTI (8.65 µM and 17.3 µM) treatments, the cells exhibited intact morphology, and consequently, we did not identify cell death by apoptosis or necrosis. In contrast, doxorubicin (0.55 µM), a cell antiproliferative drug (negative control), significantly reduced the number of viable L929 cells by apoptosis and necrosis (* $p < 0.05$ compared with vehicle control) (Fig. 6).

Alkaline comet assay

In the alkaline comet assay, used to assess possible DNA damage, the vehicle-treated cells were DNA-damage free. ShTI did not cause any significant damage to the DNA of L929 culture cells at the concentrations tested (8.65 μM and 17.3 μM). In turn, MMS ($4 \times 10^{-5} \text{ M}$), the positive control drug used, caused a high amount of damage to the DNA of the L929 cells (Fig. 8). The data suggested that ShTI ($\text{IC}_{50} > 17.3 \mu\text{M}$) was considered a safe drug in toxicological tests performed against L929 cells lines.

Discussion

Protease inhibitors (PIs) are a diverse group of proteins divided into 82 families and 39 clans according to MEROPS [21]. Overall, 6700 plant protease inhibitors (PPIs) were identified that include proteins ($> 15 \text{ kDa}$) [6], which act to prevent degradation of storage proteins in plants and defend against phytopathogens and are considered promising tools to combat human microbial infections [5]. However, PPIs are rarely explored in the development of therapeutic peptides, even in view of their broad spectrum of action and interesting characteristics for the development of drugs, such as stability and specificity, and are considered safe because they are metabolized into nontoxic amino acids [6].

Recently, a thermostable trypsin inhibitor, named ShTI (11.558 kDa), from chia seeds was isolated by our research group. ShTI was remarkably stable under high temperatures (100°C , 120 min) and a wide range of pH values (2–10). Furthermore, ShTI demonstrated antibacterial activity against *Staphylococcus aureus* alone and in combination with oxacillin via cell membrane pore formation and increased production of reactive oxygen species (ROS) [8]. Despite differences in bacterial and fungal cells, such as cell wall composition and the presence of more specific cytoplasmic organelles in yeasts, numerous studies have found that protease inhibitors act on both types of cells [22, 23]. This inspired us to examine the effects of ShTI on fungal species of interest to human health.

Among fungi, *Candida* species are recognized as the most pathogenic organisms. *C. albicans* is the most commonly isolated species in bloodstream infections (candidemia), and it is characterized as highly adaptable and capable of developing resistance to clinical conventional antifungals [3]. Moreover, nonalbicans species, including *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. krusei*, represent 35–65% of cases of candidemia. Notably, infection by *C. krusei* exhibits high mortality (40–58%) compared to several nonalbicans species and displays a low response to conventional antifungal therapies [24]. Thus, emphasizing the importance of looking for new strategies to solve *Candida* infections.

In this work, it was reported for the first time that a trypsin inhibitor from Chia exhibited antifungal effects against resistant clinical and susceptible strains of *Candida spp.* (MIC_{50} ranging from 2.05 μM to 4.01 μM and MIC_{100} : 8.2 μM). The literature reports others to plant serine protease inhibitors with anticandidal effects. When compared to ShTI, a similar concentration of anticandidal effect was reported in CITI (trypsin inhibitor from *Cassia leiandra*) seeds (MIC_{100} : 8.39 μM) [25], while a lower concentration than those reported in IETI (trypsin inhibitor purified from *Inga edulis*) with MIC_{50} 10.2 μM [26] and a trypsin inhibitor from *Enterolobium timbouva* named EtTI, capable of inhibiting *Candida spp.* species with MIC_{50} values varying from 11.66 to 21.63 μM [27].

The fungal species evaluated include *C. albicans*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis*, the most frequent species found in bloodstream infections associated with high human mortality [28]. In this work, *C. parapsilosis* (ATCC® 22019) was the most susceptible yeast tested in comparison to the other analyzed species. This result confirmed the epidemiological studies that indicate differential susceptibility modes among *Candida* species to drug treatments. According to scientific evidence, *C. parapsilosis* is more resistant to echinocandins (drugs that act on lipids in the cell membrane) and more susceptible to azoles, whereas *C. krusei* responds in the opposite direction and is more resistant to azole drugs [29]. This difference in fungal susceptibility to ShTI may vary due to its different modes of action and the response of the yeast to the treatment, a common characteristic also evidenced in conventional antifungals.

Considering the anticandidal activity of ShTI, its mode of action was characterized by the intrinsically resistant strains of *C. krusei* (ATCC® 6258™) and the fluconazole-resistant clinical strains of *C. albicans* (strain 1) through fluorescence microscopy and scanning electron microscopy (SEM). The results indicated that ShTI disrupted the cell wall and caused damage to the membrane, as confirmed by the uptake of propidium iodide, a marker of cellular damage that only permeates the membrane-compromised cells, culminating in the loss of functional integrity of the membrane and promoting changes in its organization [30].

A serine peptidase N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK) inhibitor was also able to alter the integrity of the cell membrane of clinical isolates from *Candida spp.* by inhibiting the biosynthesis of ergosterol, the main lipid of the composition of the fungal cell membrane [31]. A hypothesis can be raised that the action of drugs based on protease inhibitors, such as TPCK and possibly ShTI, on ergosterol biosynthesis occurs through the inhibition of serine proteases involved in the proteolytic cleavage of the Steroid Regulatory Element Binding Proteins (SREBPs), essential in the regulation of ergosterol biosynthesis genes [32]. Thus, the anticandidal effect of ShTI could be the result of a combination of factors that alter cell homeostasis and cause energetic stress in the mitochondria, resulting in the production of ROS [33]. Therefore, our results confirmed that ShTI treatment induced ROS overproduction in *C. krusei* (ATCC® 6258™) and *C. albicans* (strain 1) cells, as confirmed by the oxidation of DCFH-DA to DCF by intracellular ROS, leading to cell death pathways by apoptosis.

SEM images revealed morphological changes, pseudohyphae formation, perforations in their surface and lysis in *Candida* cells. The change in the surface of planktonic cells caused by ShTI compromises the cellular adhesion of *Candida* cells to substrate, an essential element for the establishment of infection [34] and the formation of microbial biofilms [35]. Therefore, we can infer that ShTI may act in this way to prevent fungal growth. In addition, *Candida* filamentous growth may be an indication that ShTI acts on the expression of yeast shape regulation genes. This effect was observed with another plant protein, soybean toxin (SbTX), capable of inducing the filamentous growth of *C. albicans* cells, in which the transcriptional corepressor (TUP1) gene was involved [36]. Furthermore, the presence of large pores in the membrane was demonstrated with internal content loss with consequent homeostasis imbalance, negatively impacting cell survival and compromising yeast pathogenicity [37].

These results corroborate those of other plant protease inhibitors, such as a trypsin inhibitor purified from *C. leiandra* (MM: 19.48 kDa) that induced oxidative stress in *C. albicans* cells [25], the cysteine protease from *Cassia leiandra* (MM: 16.63 kDa) induced disruption of the cell surface from *C. tropicalis* [38], the synthetic inhibitor bioinspired by the trypsin inhibitor from *Jatropha curcas* (MM: 600–1200 Da) showed an increase in membrane permeabilization in *C. krusei* cells [39], and a Kunitz trypsin inhibitor from *Enterolobium timbouva* (MM: 20 kDa) altered the integrity of the plasma membrane and induced morphological changes in *C. albicans*, *C. buinensis*, and *C. tropicalis* cells [40]. Although the scientific literature reports other studies of plant protease inhibitors against *Candida* species, our study differentially reports this effect against resistant strains of *C. krusei* and resistant clinical isolates of *C. albicans*, with emphasis on the evidenced synergistic effect with fluconazole.

Currently, the most commonly prescribed antifungal drug for *Candida* infections is fluconazole, a triazole antifungal with a high spectrum of action, large bioavailability, and few side effects [41, 42]. Nonetheless, widespread use and prolonged exposure have increased the number of *Candida*-resistant strains [43]. Our results suggest that the combination of ShTI-FLC significantly reduces the required dose of FLC, as confirmed in SEM images, where the *Candida* cells assumed a gross morphological appearance with an irregular and contorted surface. Such characteristics compromise the maintenance of cellular metabolism and consequently its survival and virulence [37] and encourage us to speculate that ShTI is a potential sensitizer of the effect of fluconazole against resistant strains of *C. albicans* (strain 1).

Therefore, we suggest that the drug combination had a species-dependent effect because the ShTI-FLC combination did not exhibit a synergistic effect against *C. parapsilosis* (ATCC® 22019) and *C. krusei* (ATCC® 6258™) strains. This response difference was probably due to the genotypic and phenotypic changes characteristic of the organisms [44]. The literature reported that, based on genotypic analysis, high genetic variability among *Candida* species was confirmed, mainly among infectious species that frequently inhabit humans, such as *C. albicans* [45]. Such genotypic differences may explain why the combination ShTI-FLC effectively inhibited the growth of *C. albicans* cells but was not effective against the nonalbicans cells tested.

Pathogenic fungi such as *Candida* secrete various serine proteases, such as trypsin, in the process of pathogenicity [46]. Therefore, molecules that act by inhibiting the action of these proteases are interesting compounds as new antifungal drugs. A secretory leucocyte proteinase inhibitor (SLPI) (MM: 11.7 kDa) exhibited an antifungal effect against a clinical isolate of *C. albicans* (MIC 18 μ M) and was able to inhibit the proteolytic activity of secreted proteases [47]. Although studies show the action of PIs on fungal proteins, none of the antifungal compounds currently used act by inhibiting serine proteases, which is considered a clinical challenge since proteases are present in human metabolism. However, alternative application routes, such as topical applications, may be considered [46]. In addition, when evaluating the specificity of PI, plant protease inhibitors have a variety of characteristics, such as stability at high temperatures and pH variations, substrate specificity, and high solubility in aqueous solutions, which also allow their oral administration [48].

Based on the promising antimicrobial effect of ShTI, studies of its toxic effects were evaluated to obtain preliminary data on the safety of its application against mammalian cells. The results indicated that ShTI did not significantly alter cell viability in the MTT test and did not exhibit signs of apoptosis, necrosis, or DNA damage in mouse fibroblast cells. The literature has reported that plant protease inhibitors were found to be safe in in vitro toxicity assays [25], and there are several preclinical studies on the pharmacological effects of plant protease inhibitors on human health [49]. In addition, chia seeds are widely used as a food ingredient and have not reported cases of toxicity; on the other hand, beneficial effects such as antioxidant [50], antidiabetic and anticholesterol properties [51] associated with the consumption of seed proteins have been described. Thus, the European Commission, the EFSA Panel on Nutrition, Novel Foods, and Food Allergens (NDA), issued a technical opinion proving the safety of chia as a new food ingredient [52] currently considered one of the most popular seeds in the world [53]. Therefore, it has been suggested that the components present in Chia seeds, such as proteins, are considered safe for human use.

In this work, we started from a preliminary study of the in vitro anti-Candida effect of a trypsin inhibitor from Chia seeds, but new assays will be necessary as a test in vivo model of candidiasis and preclinical toxicity assays to enable the advancement of the application future use of ShTI as a new antimicrobial.

Conclusion

In view of the available data, it is reasonable to say that ShTI has antifungal potential, especially against clinically resistant species of *C. albicans*, and has shown drug safety for use after preliminary toxicology in vitro tests. However, new tests will be needed, such as in vivo models of *Candida* spp. infection and preclinical toxicological assays to ensure the safety of ShTI as a new drug.

Declarations

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Competing Interests

The authors declare no competing interests.

Author Contributions

Francisca Cristiane Nogueira: conceptualization, methodology design, validation, investigation, data curation, formal analysis, writing - original draft and writing - review and editing. Adson Ávila de Souza:

investigation and writing - review and editing. Nadine Monteiro Salgueiro Araújo: investigation and formal analysis. Larissa Alves Lopes de Souza: investigation. Rafael Guimarães Gomes Silva: investigation. Daniele de Oliveira Bezerra de Sousa: funding acquisition. Bruno Cêlho Calvalcanti: methodology design, validation, investigation, data curation, formal analysis from toxicology assays. Manoel Odorico de Moraes Filho: funding acquisition. Lívia Gurgel do Amaral Valente Sá: investigation and formal analysis. Hélio Vitoriano Nobre Júnior: funding acquisition. Hermógenes David de Oliveira: conceptualization, formal analysis, data curation, supervision, funding acquisition, writing - review and editing.

References

1. Bongomin F, Gago S, Oladele RO, Denning DW. Global and multi-national prevalence of fungal diseases-estimate precision. *J Fungi (Basel)*. 2017; 3:57; <https://doi.org/10.3390/jof3040057>
2. Muzahed, Alshery BA, Rabaan AI, El-Masry OS, Acharya S, Alzahrani FM, Mutair AA, Alhumaid S, Al-Tawfiq Ja, Muhammad J, Khan A, Dhama K, Al-Omari A. A 20-year retrospective clinical analysis of *Candida* infections in tertiary center: Single-center experience. *J. Infect. Public Health*. 2022; 15:69-74; <https://doi.org/10.1016/j.jiph.2021.11.014>
3. Costa-De-Oliveira S, Rodrigues AG. *Candida albicans* antifungal resistance and tolerance in bloodstream infections: The Triad Yeast-Host-Antifungal. *Microorganisms*. 2020; 8:1-15; <https://doi.org/10.3390/microorganisms8020154>
4. Pristov KE, Ghannoum MA. Resistance of *Candida* to azoles and echinocandins worldwide, *Clin. Microbiol. Infect*. 2019; 25:792-798; <https://doi.org/10.1016/j.cmi.2019.03.028>
5. Ryan, C. Protease inhibitors in plants: genes for improving defenses against insects and pathogens, *Annu. Rev. Phytopathol*. 1990; 28:425-49; <https://doi.org/10.1146/annurev.py.28.090190.002233>
6. Hellinger R, Gruber CW. Peptide-based protease inhibitors from plants. *Drug Discov. Today*. 2019; 24: 1877-1889; <https://doi.org/10.1016/j.drudis.2019.05.026>. Epub
7. Motyka S, Koc K, Ekiert H, Blicharska E, Czarnek K, Szopa A. The Current State of Knowledge on *Salvia hispanica* and *Salviae hispanicae* semen (Chia Seeds). *Molecules*. 2022; 27(4):1207; <https://doi.org/10.3390/molecules27041207>
8. Souza AA, Lima AM, Sousa DOB, Nogueira FC, Neto JCS, Dias LP, Araujo NMS, Nagano CS, Junior HVN, Silva CR, Sá LGAV, Neto JBA, Barroso FDD, Moraes MEA, Oliveira HD. Chia (*Salvia hispanica* L.) seeds contain a highly stable trypsin inhibitor with potential for bacterial management alone or in drug combination therapy with oxacillin. *Probiotics Antimicrob. Proteins*. 2022; <https://doi.org/10.1007/s12602-022-09979-5>
9. Erlanger BF, Kokowsky N, Cohen W. The preparation and properties of two new chromogenic substrates of trypsin. *Arch Biochem Biophys*. 1961; 95:271-78; [https://doi.org/10.1016/0003-9861\(61\)90145-X](https://doi.org/10.1016/0003-9861(61)90145-X)
10. Schägger H, Von JG. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987;166:368-79; [https://doi.org/10.1016/0003-2697\(87\)90587-2](https://doi.org/10.1016/0003-2697(87)90587-2)

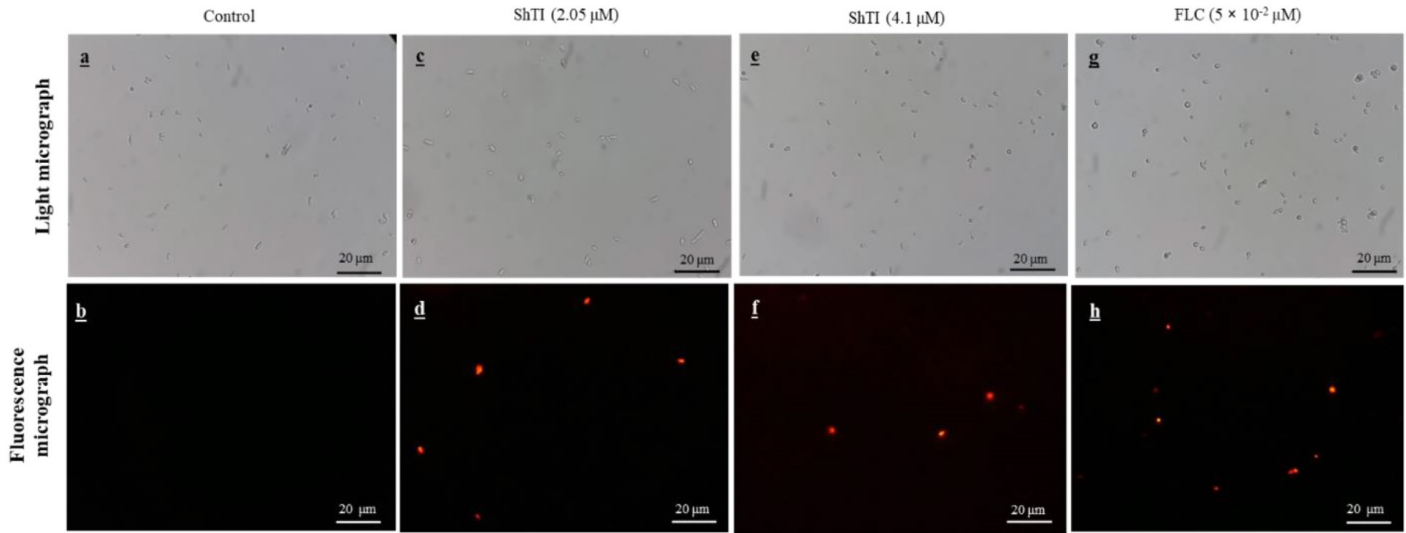
11. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72:248–254
<https://doi.org/10.1006/abio.1976.9999>
12. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A3, 3rd ed Clinical and Laboratory Standards Institute, Wayne, PA. 2008.
13. Regente M, Taveira Gb, Pinedo M, Elizalde MM, Ticchi AJ, Diz MSS, Carvalho AO, De La Canal L, Gomes VM. A sunflower lectin with antifungal properties and putative medical mycology applications, *Curr. Microbiol.* 2014; 69: 88–95; <https://doi.org/10.1007/s00284-014-0558-z>
14. Maurya IK, Pathak S, Sharma M, Sanwal H, Chaudhary P, Tupe S, Deshpande M, Chauhan VS, Prasad R. Antifungal activity of novel synthetic peptides by accumulation of reactive oxygen species (ROS) and disruption of cell wall against *Candida albicans*, *Peptides*. 2011; 32: 1732–1740; <https://doi.org/10.1016/j.peptides.2011.06.003>.
15. Odds FC. Synergy, antagonism, and what the checkerboard puts between them. *J Antimicrob Chemother* 2003; 52(1):1; <https://doi.org/10.1093/jac/dkg301>
16. Staniszewska M, Bondaryk M, Swoboda-Kopec E, Siennicka K, Sygitowicz G.; Kurzatkowski, W. *Candida albicans* morphologies revealed by scanning electron microscopy analysis. *Braz. J. Microbiol.* 2013; 44: 813-21; <https://doi.org/10.1590/S1517-83822013005000056>
17. Mossmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol.* 1983;16:55-63; [https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4)
18. McGahon AJ, Martin SM, Bissonnette RP, Mahboubi A, Shi Y, Mogil RJ, Nishioka WK, Green DR. The end of (cell) line: methods for the study of apoptosis in vitro. *Methods Cell Biol.* 1995;46:153-85; [https://doi.org/10.1016/s0091-679x\(08\)61929-9](https://doi.org/10.1016/s0091-679x(08)61929-9)
19. Singh NP, McCoy MT, Tice RR, Scheider EL. A simple technique for quantification of low levels of DNA damage in individual cells. *Exp. Cell Res.* 1998;175:184-91; [https://doi.org/10.1016/0014-4827\(88\)90265-0](https://doi.org/10.1016/0014-4827(88)90265-0).
20. Hartmann A, Speit, G. The contribution of cytotoxicity to DNA effects in the single cell gel test (comet assay). *Toxicol Lett.* 1997;90:183-88; [https://doi.org/10.1016/s0378-4274\(96\)03847-7](https://doi.org/10.1016/s0378-4274(96)03847-7)
21. Rawlings ND. Peptidase inhibitors in the MEROPS database. *Biochimie.* 2010;92:1463–83; <https://doi.org/10.1016/j.biochi.2010.04.013>
22. Dabhade AR, Mokashe, NU, Patil UK. Purification, characterization, and antimicrobial activity of nontoxic trypsin inhibitor from *Albizia amara* Boiv. *Process Biochem.* 2016;51:659-74; <https://doi.org/10.1016/j.procbio.2016.02.015>
23. Kim MH, Park SC, Kim JY, Lee SY, Lim HT, Cheong H, Hahm KS. Park, Y. Purification, and characterization of a heat-stable serine protease inhibitor from the tubers of new potato variety “Golden Valley”. *Biochem. Biophys. Res. Commun.* 2006; 346: 681-86
<https://doi.org/10.1016/j.bbrc.2006.05.186>.

24. Gómez-Gaviria M, Mora-Montes HM. Current Aspects in the Biology, Pathogeny, and treatment of *Candida krusei*, a Neglected Fungal Pathogen. *Infect Drug Resist*. 2020;13:1673-89
<https://doi.org/10.2147/IDR.S247944>.
25. Araújo NMS, Dias LP, Costa HPS, Sousa DOB, Vasconcelos IM, Morais GAde, Oliveira JTA. C/II, a Kunitz trypsin inhibitor purified from *Cassia leiandra* Benth. seeds, exerts a candidicidal effect on *Candida albicans* by inducing oxidative stress and necrosis. *BBA - Biomembranes* 2019; 1861:1830-32; <https://doi.org/10.1016/j.bbamem.2019.183032>
26. Dib HX, Oliveira DGL, Oliveira CFR, Taveira GB, Mello EO, Verbisk NV, Chang MR, Junior DC, Gomes VM, Macedo MLR. Biochemical characterization of a Kunitz inhibitor from *Inga edulis* seeds with antifungal activity against *Candida* spp. *Arch Microbiol* 2018;201:223-33; <https://doi.org/10.1007/s00203-018-1598-8>
27. Oliveira CFR, Oliveira CT, Taveira GB, Mell EC, Gomes VM, Macedo MLR. Characterization of a Kunitz trypsin inhibitor from *Enterolobium timbouva* with activity against *Candida* species. *Int J Biol Macromol* 2018; 119: 645-53; <https://doi.org/10.1016/j.ijbiomac.2018.07.034>.
28. Bhattacharya S, Sae-Tia S, Fries BC. Candidiasis and Mechanisms of Antifungal Resistance. *Antibiotics* 2020; 9:312; <https://doi.org/10.3390/antibiotics9060312>
29. Ksiezopolska E, Gabaldón T. Evolutionary Emergence of Drug Resistance in *Candida* Opportunistic Pathogens. *Genes* 2018; 9: 461; <https://doi.org/10.3390/genes9090461>
30. Choi H, Lee D. Antifungal activity and pore-forming mechanism of astacidin 1 against *Candida albicans*. *Biochimie*. 2014;105:58-63; <http://dx.doi.org/10.1016/j.biochi.2014.06.014>
31. Souto XM, Ramos LS, Oliveira SSC, Branquinha MH, Santos ALS. The serine peptidase inhibitor N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) affects the cell biology of *Candida haemulonii* species complex. *Fungal Biology*. 2021; 125: 378-388; <https://doi.org/10.1016/j.funbio.2020.12.004>
32. Dhingra S, Cramer RA. Regulation of sterol biosynthesis in the human fungal pathogen *Aspergillus fumigatus*: opportunities for therapeutic development. *Front. Microbiol*. 2017; 8: 92; <https://doi.org/10.3389/fmicb.2017.00092>
33. Taveira GB, Carvalho AO, Rodrigues R, Trindade FG, Da Cunha M, Gomes VM. Thionin-like peptide from *Capsicum annuum* fruits: mechanism of action and synergism with fluconazole against *Candida* species. *BMC Microbiol*. 2016;16:12; <http://dx.doi.org/10.1186/s12866-016-0626-6>
34. Sardi JDCO, Pitangui NDS, Rodriguez-Arellanes G, Taylor ML, Fusco-Almeida AM, Mendes-Giannini MJ. Highlights in pathogenic fungal biofilms. *Rev. Iberoam. De Micol*. 2014; 31:22-29; <https://doi.org/10.1016/j.riam.2013.09.014>
35. Cuéllar-Cruz M, Villagómez-Castro J, Ruiz-Baca E. *Candida* species: new insights into biofilm formation. *Future Microbiol*. 2012; 7: 755-71; <https://doi.org/10.2217/fmb.12.48>.
36. Morais JKS, Bader O, Weig M, Oliveira JTA, Arantes MR, Gomes VM, Cunha M, Oliveira HD, Sousa DOB, Lourencao AL, Vasconcelos IM. Soybean Toxin (SBTX) Impairs Fungal Growth by Interfering with Molecular Transport, Carbohydrate/ Amino Acid Metabolism and Drug/Stress Responses. *Plos one*. 2013; 8: 70425; <http://dx.doi.org/10.1371/journal.pone.0070425>

37. Dadar M, Tiwari R, Karthik K, Chakraborty S, Shahali Y, Dhama K. *Candida albicans* - Biology, molecular characterization, pathogenicity, and advances in diagnosis and control – An update Author links open overlay panel. *Microb. Pathog.* 2018;117:128-138; <https://doi.org/10.1016/j.micpath.2018.02.028>
38. Melo IRS, Dias LP, Araújo NMS, Vasconcelos IM, Martins TF, Morais GA, Gonçalves JFC, Nagano CS, Carneiro RF, Oliveira, JTA. ClCPI, a cysteine protease inhibitor purified from *Cassia leiandra* seeds has antifungal activity against *Candida*. *Int. J. Biol. Macromol.* 2019; 135: 1115-24; <https://doi.org/10.1016/j.ijbiomac.2019.04.174>
39. Souza LAL, Dias LP, Araújo NMS, Carneiro RF, Nagano CS, Teixeira CS, Silva RGG, Oliveira JTA, Sousa DOB. JcTI-Pepl, a synthetic peptide bioinspired in the trypsin inhibitor from *Jatropha curcas*, presents potent inhibitory activity against *C. krusei*, a neglected pathogen. *Biochimie.* 2022; 200: 107-18; <https://doi.org/10.1016/j.biochi.2022.05.014>
40. Oliveira CFR, Oliveira CT, Taveira GB, Mello EO, Gomes VM, Macedo MLR. Characterization of a Kunitz trypsin inhibitor from *Enterolobium timbouva* with activity against *Candida* species. *Int. J. Biol. Macromol.* 2018; 119:645-53; <https://doi.org/10.1016/j.ijbiomac.2018.07.034>
41. Berkow EL, Lockhart SR. Fluconazole resistance in *Candida* species: a current perspective. *Infect. Drug Resist.* 2017;10: 237-45; <http://dx.doi.org/10.2147/IDR.S118892>
42. Shafiei M, Peyton L, Hashemzadeh M, Foroumadi A. History of the development of antifungal azoles: A review on structures, SAR, and mechanism of action. *Bioorg Chem.* 2020;104:104240. <https://doi.org/10.1016/j.bioorg.2020.104240>.
43. Morio F, Jensen RH, Le Pape P, Arendrup MC. Molecular basis of antifungal drug resistance in yeasts. *Int. J. Antimicrob. Agents* 2017; 50: 599–606; <https://doi.org/10.1016/j.ijantimicag.2017.05.012>
44. Wang T, Pan M, Xiao N, Wu J, Wang Q, Cheng T, Yan G, Wu D, Li N, Shao J. In vitro and in vivo analysis of monotherapy and dual therapy with ethyl caffeate and fluconazole on virulence factors of *Candida albicans* and systemic candidiasis. *J. Glob. Antimicrob. Resist.* 2021;27:253-266; <https://doi.org/10.1016/j.jgar.2021.10.005>
45. Merseguet KB, Nishikaku AS, Rodrigues AM, Padovan AC, Ferreira RC, Melo ASA Briones MRS, Colombo AL. Genetic diversity of medically important and emerging *Candida* species causing invasive infection. *BMC Infect Dis.* 2015; 15:57; <https://doi.org/10.1186/s12879-015-0793-3>
46. Burchacka E, Pietra P, Lupicka-Slowik A. Recent advances in fungal serine protease inhibitors. *Biomed Pharmacother.* 2022; 146: 112523; <https://doi.org/10.1016/j.biopha.2021.112523>
47. Curvelo JADR, Barreto ALS, Portela MB, Alviano DS, Hollandino C, Souto-Padron T, Soares RMDA. Effect of the secretory leucocyte proteinase inhibitor (SLPI) on *Candida albicans* biological processes: A therapeutic alternative? *Arco. Oral. Biol.* 2014; 59:928 -37; <https://doi.org/10.1016/j.archoralbio.2014.05.007>
48. Zhao J, Kah YEE. *Protease Inhibitors*. Food Chem., 1st ed., 2019: 253-59; <https://doi.org/10.1016/B978-0-08-100596-5.21749-6>.

49. Srikanth S, Chen Z. Plant protease inhibitors in therapeutics-focus on cancer therapy. *Front. Pharmacol.* 2016; 7: 470; <http://dx.doi.org/10.3389/fphar.2016.00470>
50. Mas AL, Brigante FI, Salvucci E, Pigni NB, Martinez ML, Ribotta P, Wunderlin DA, Baroni MV. Defatted Chia flour as functional ingredient in sweet cookies. How do Processing, simulated gastrointestinal digestion and colonic fermentation affect its antioxidant properties? *Food Chemistry.* 2020; 316:126-279; <https://doi.org/10.1016/j.foodchem.2020.126279>
51. Grancieri M, Martino HSD, Mejia EG. Protein Digests and Pure Peptides from Chia Seed Prevented Adipogenesis and Inflammation by Inhibiting PPAR γ and NF- κ B Pathways in 3T3L-1 Adipocytes. *Nutrients.* 2021; 13: 176; <https://doi.org/10.3390/nu13010176>
52. Turck D, Castenmiller J, Henauw S, Hirsch-Ernst KI, Kearney J, Maciuk A. et al. Safety of Chia seeds (*Salvia hispanica* L.) as a novel food for extended uses pursuant to Regulation (EU) 2015/2283. *EFSA Journal* 2019;17:5657; <https://doi.org/10.2903/j.efsa.2019.5716>
53. Hrncic MK, Iavanovski M, Cor D, Knez Z. Chia Seeds (*Salvia Hispanica* L.): An Overview- Phytochemical Profile, Isolation Methods, and Application. *Molecules.* 2020;18:11; <https://doi.org/10.3390/molecules25010011>

Figures



C. albicans (strain 1)

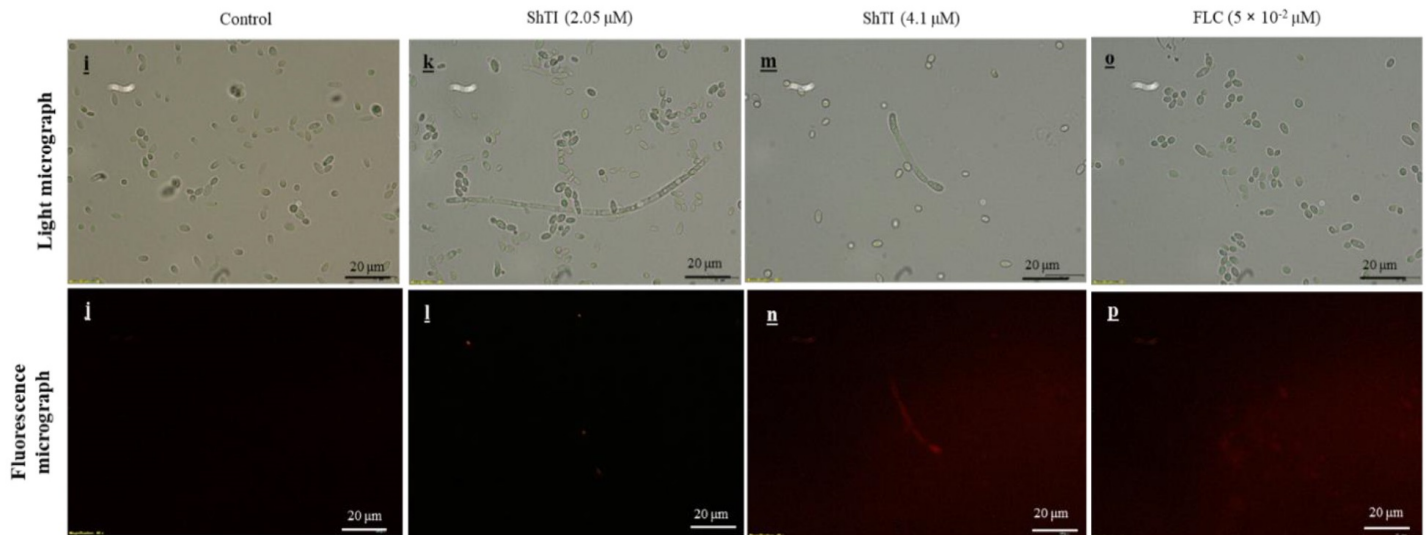
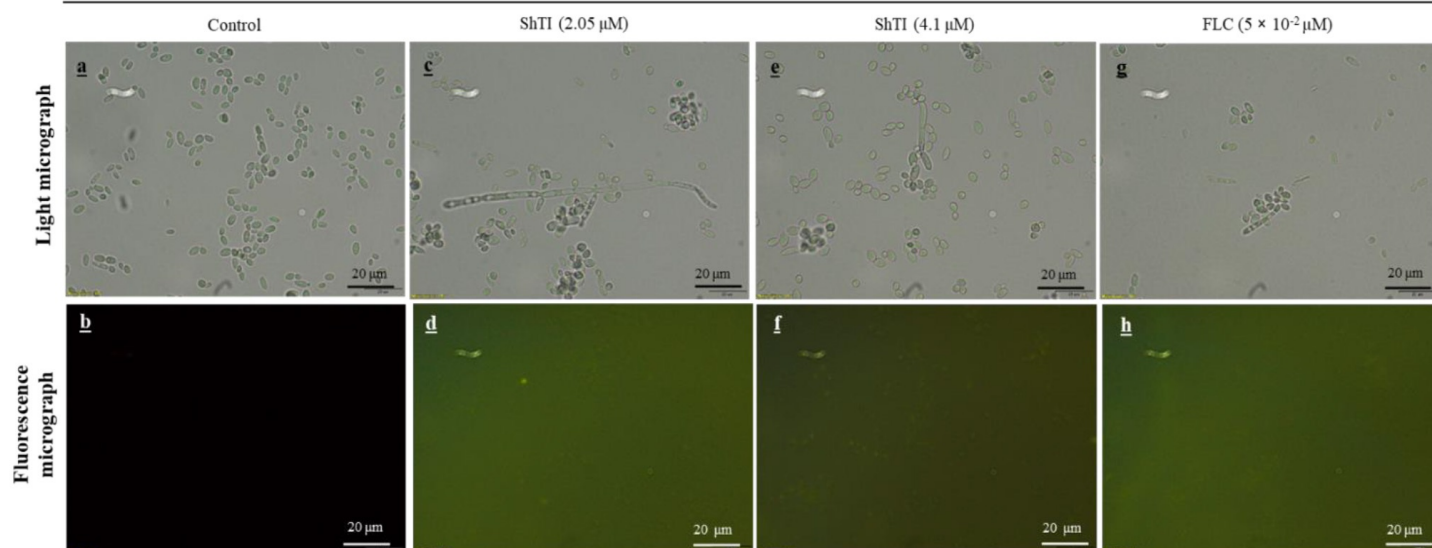


Figure 1

Micrographs of *C. krusei* (ATCC® 6258™) and *C. albicans* (strain 1) cells to assess membrane permeabilization. The cells were treated with 0.15 M NaCl (a, b, i, and j), ShTI 2.05 μM (c, d, k, and l), ShTI 4.1 μM (e, f, m, and n) or FLC 5 × 10⁻² μM (g, h, o, and p). Next, they were incubated with propidium iodide as a fluorescent marker. The images were represented in the light microscope micrographs and fluorescence microscope micrographs (Bars 20 μm).

C. krusei ATCC® 6258



C. albicans (strain 1)

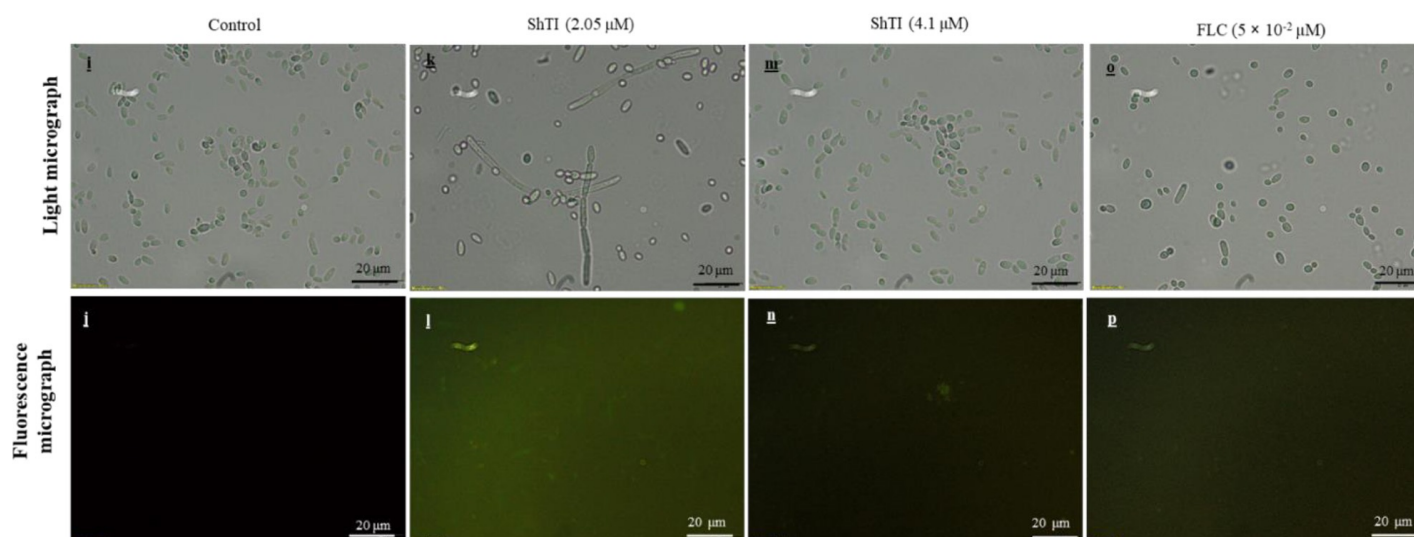


Figure 2

Top panel - Micrographs of *C. krusei* (ATCC® 6258™) and *C. albicans* (strain 1) cells to detect ROS. The cells were treated with 0.15 M NaCl (a, b, i, and j), ShTI 2.05 μ M (c, d, k, and l), ShTI 4.1 μ M (e, f, m, and n) or FLC 5×10^{-2} μ M (g, h, o, and p). Next, cells were exposed to 2',7'-dichlorofluorescein diacetate (DCFH-DA), a fluorescent mark (Bars 20 μ m).

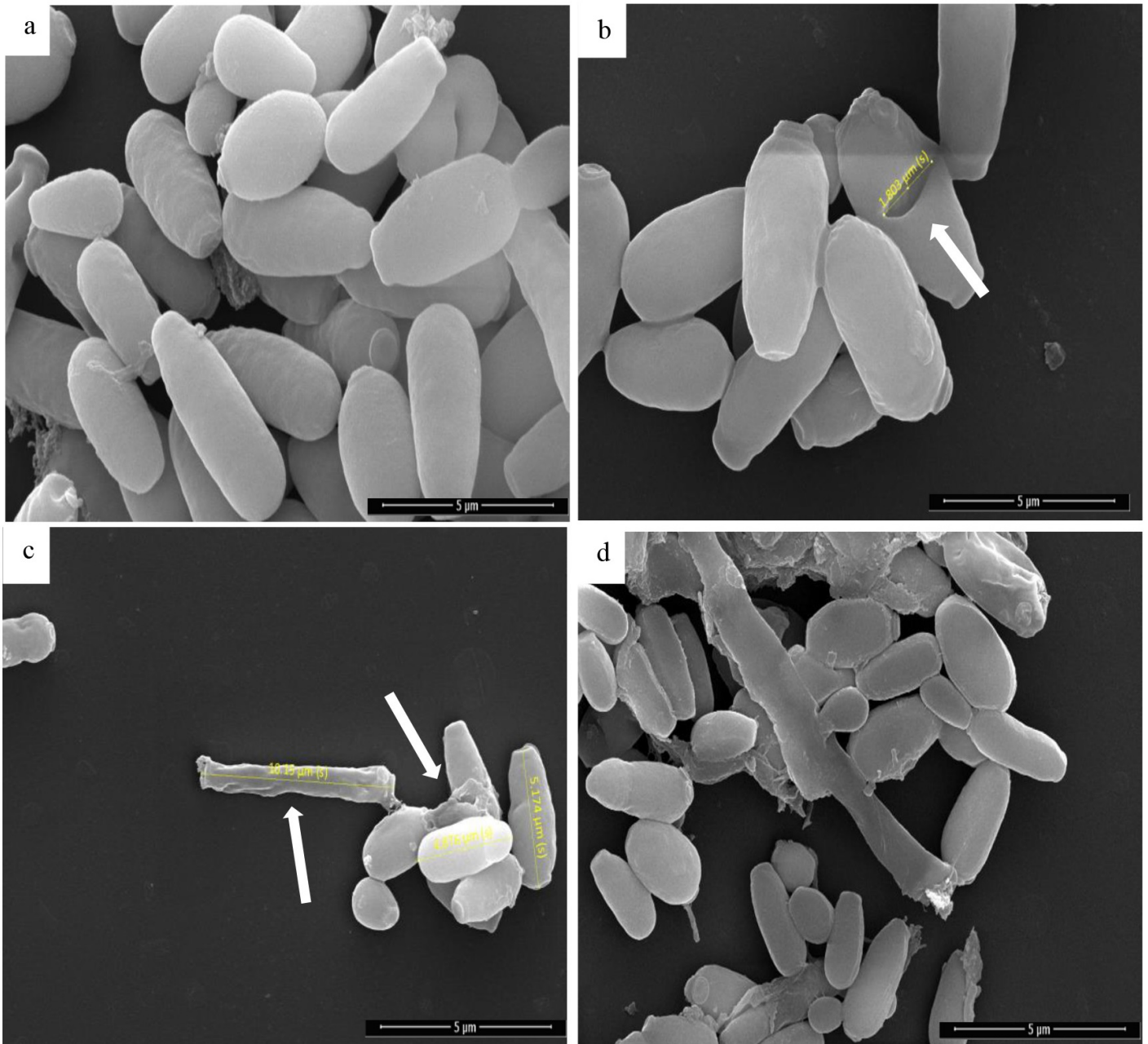


Figure 3

Scanning electron micrographs of *C. krusei* (ATCC® 6258™) cells. In (a), the cells were treated with 0.15 M NaCl. In (b) and (c), the cells were incubated with ShTI (2.05 μM) and (4.11 μM), respectively, and in (d), the cells were treated with FLC (5×10^{-2} μM). The indications in yellow correspond to the pore size of 1.803 μm (b) and the elongated structure from *C. krusei* (c). Data represent typical results of three independent experiments (Bars = 5 μm).

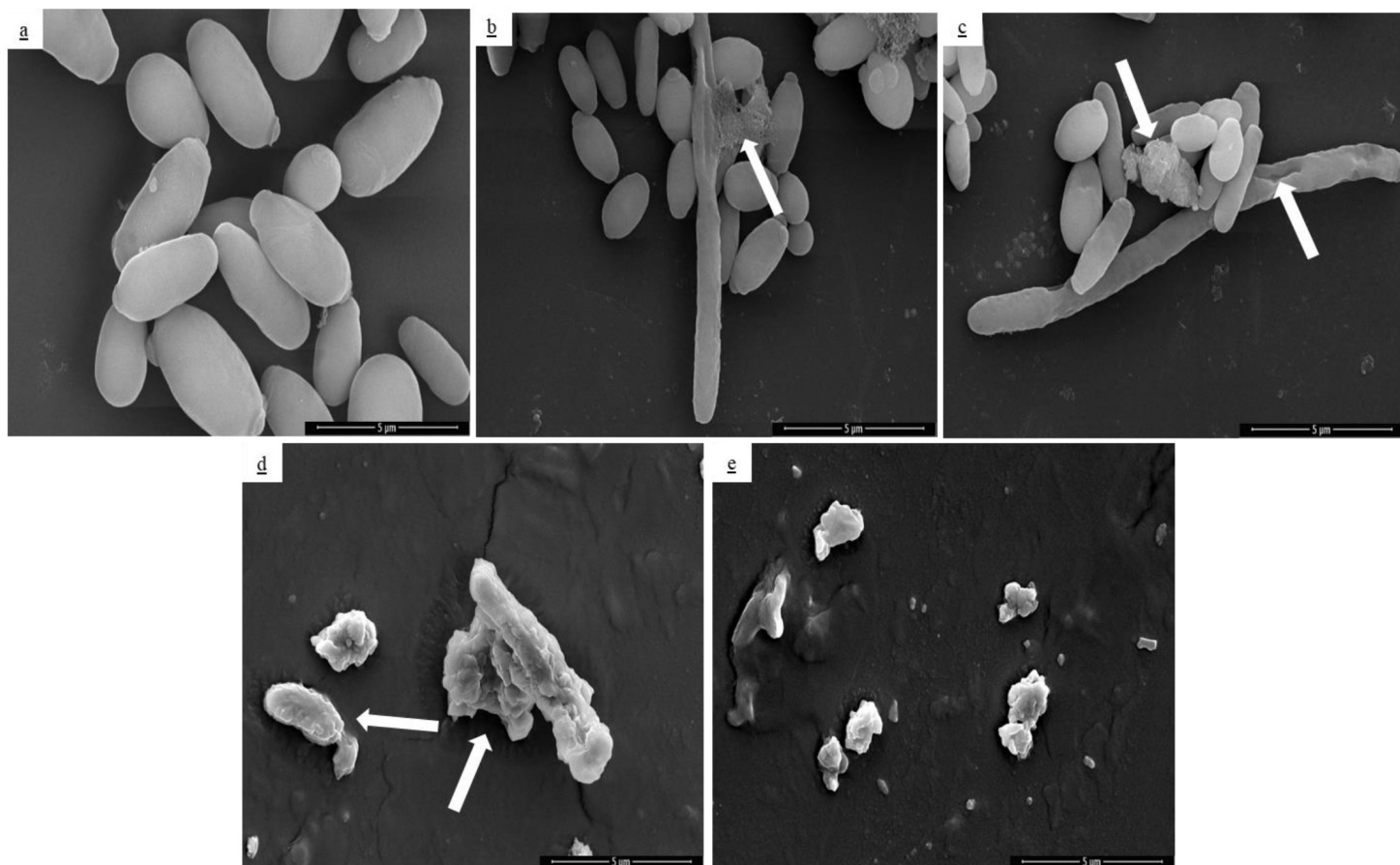


Figure 4

Scanning electron micrographs of *C. albicans* cells. In (a), the cells were treated with 0.15 M NaCl. In (b) and (c), the cells were treated with ShTI at 2.05 μM and 4.11 μM , respectively. (d) The cells were treated with the combination of ShTI (1.03 μM) + FLC (1×10^{-2} μM) and (e) FLC (5×10^{-2} μM). The arrows indicate cell damage. Data represent typical results of three independent experiments (Bars = 5 μm).

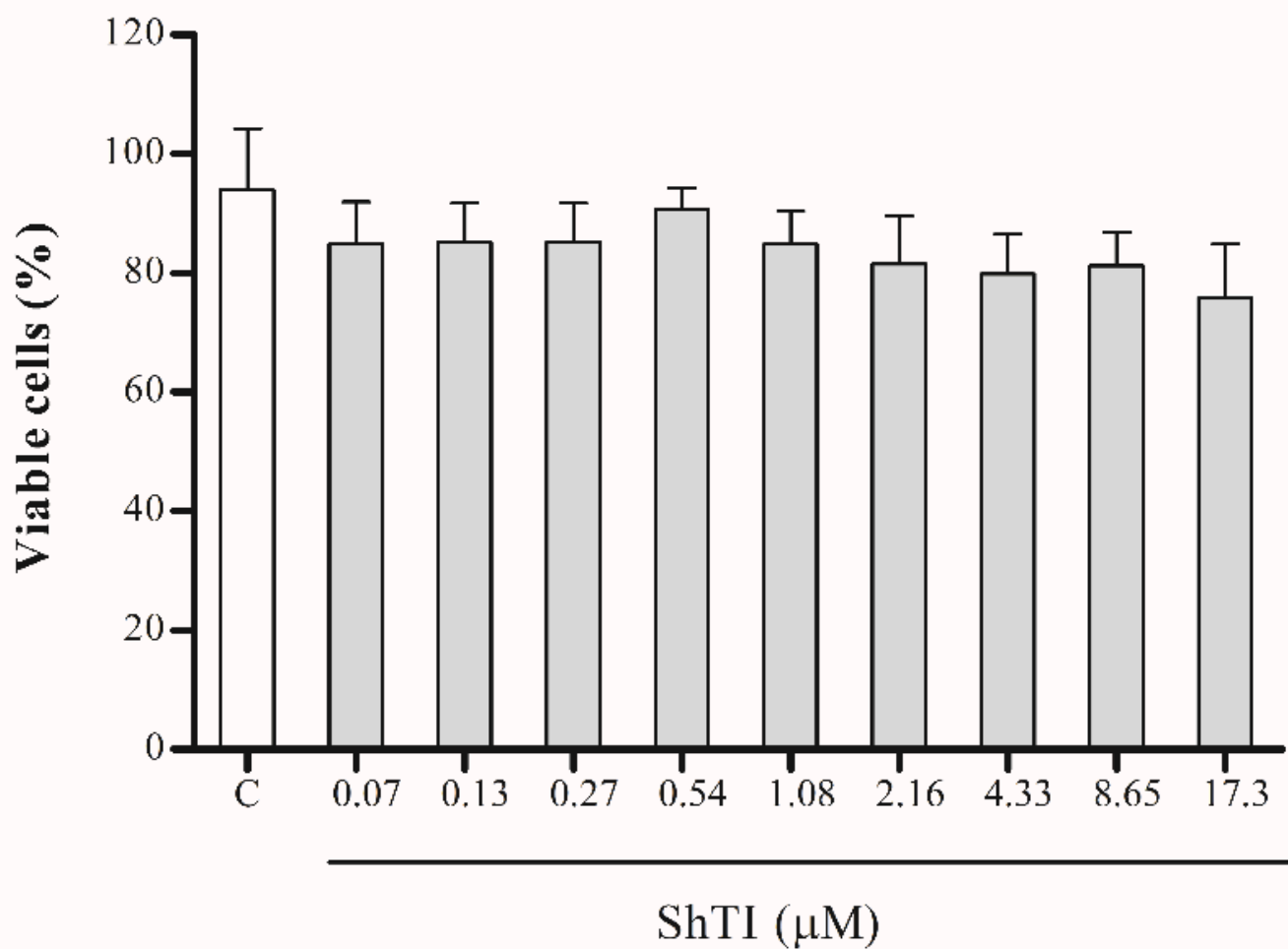


Figure 5

Cell viability assay (MTT test) of ShTI treatments at concentrations varying from 0.07 μM to 17.3 μM in L929 mouse fibroblast cell lines after 3 h of incubation at 595 nm. The positive control was treated with DMSO. Data are expressed as the means $\pm$ standard deviations compared by analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).

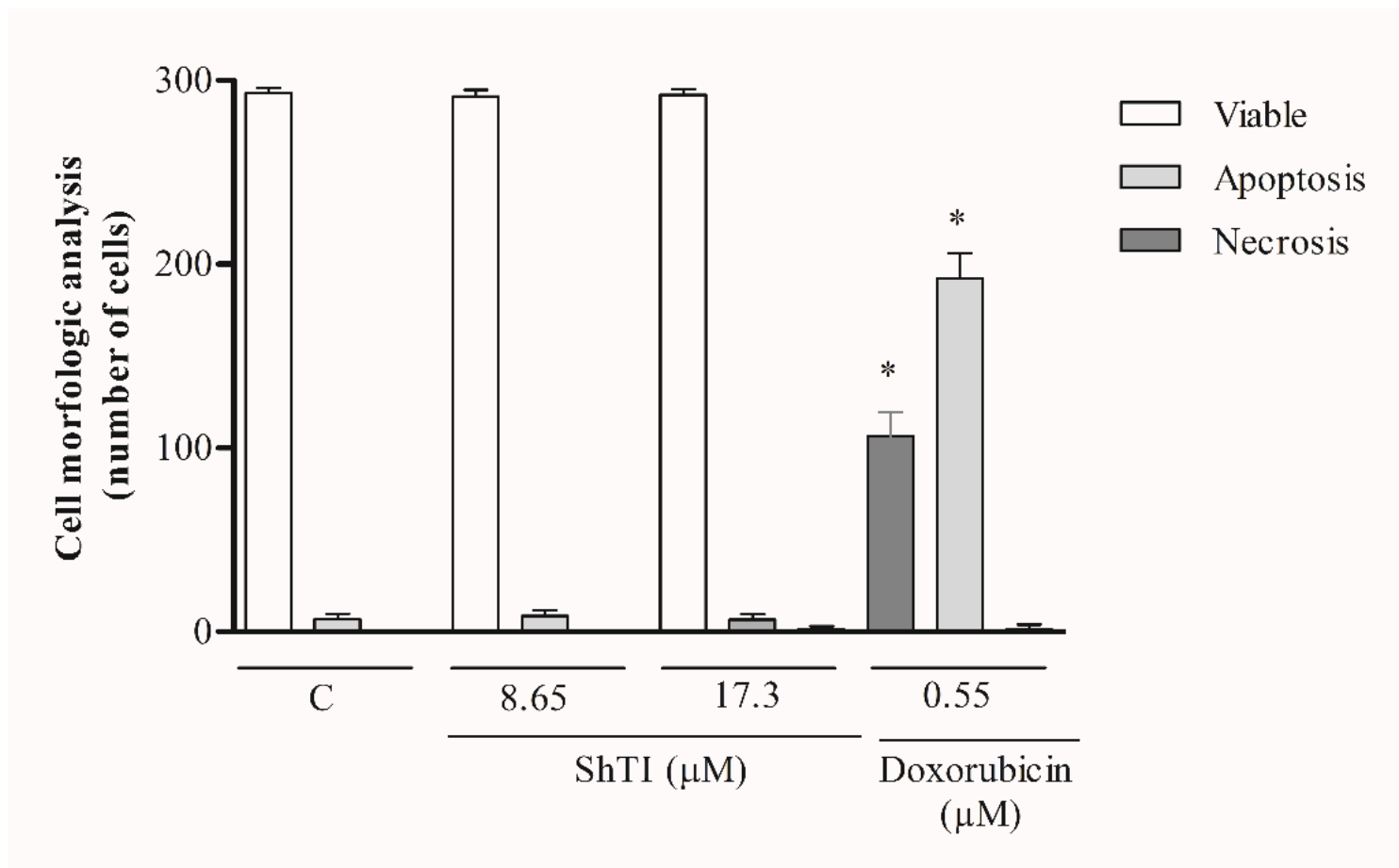


Figure 6

Cell death pattern by apoptosis and necrosis of L929 mouse fibroblast cell lines after 24 h of incubation with ShTI (8.65 and 17.3 μM) in fluorescence microscopy. Doxorubicin (0.55 μM) was used as a positive control. Experiments were conducted in triplicate in three independent experiments, and approximately 300 cells from each sample were counted. The data were analyzed as the means $\pm$ standard deviations compared by analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).

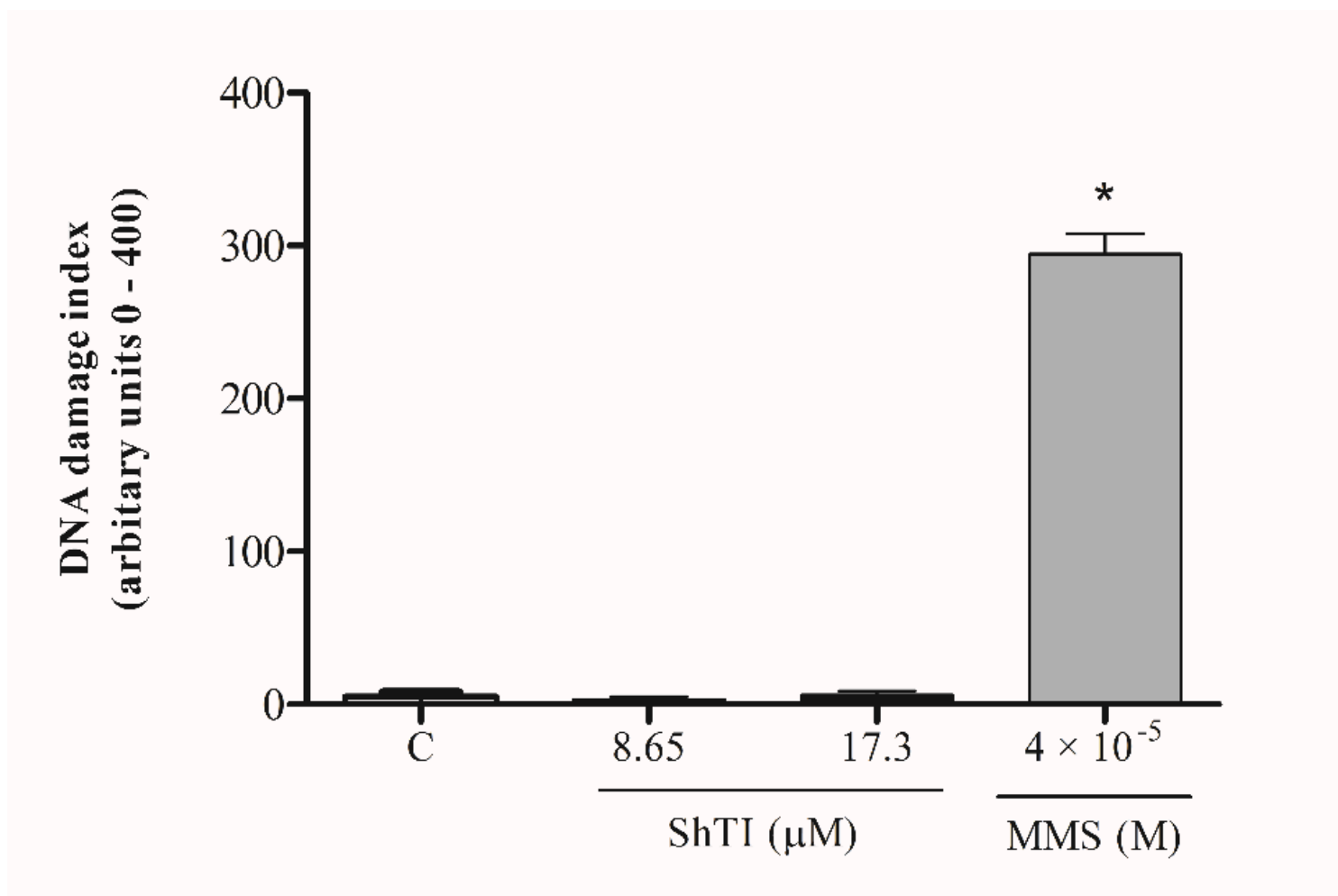


Figure 7

Genotoxicity assay of ShTI (8.65 and 17.3 μM) in L929 culture cells by comet test. Experiments were conducted in triplicate in three independent experiments. The damage frequency (%) was calculated based on the number of cells with the tail versus without. The assay was performed in triplicate, and the vehicle was used as a negative control, while MMS ($4 \times 10^{-5} \text{ M}$) was used as a positive control. Values $*p < 0.05$ were considered significant (one-way ANOVA followed by Tukey's test).