Thionin-like peptide from Capsicum annuum fruits: mechanism of action and synergism with fluconazole against Candida species

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Abstract

Background: Thionins are a family of plant antimicrobial peptides (AMPs), which participate in plant defense system against pathogens. Here we describe some aspects of the CaThi thionin-like action mechanism, previously isolated from Capsicum annuum fruits. Thionin-like peptide was submitted to antimicrobial activity assays against Candida species for IC₅₀ determination and synergism with fluconazole evaluation. Viability and plasma membrane permeabilization assays, induction of intracellular ROS production analysis and CaThi localization in yeast cells were also investigated.

Results: CaThi had strong antimicrobial activity against six tested pathogenic Candida species, with IC₅₀ ranging from 10 to 40 μg.mL⁻¹. CaThi antimicrobial activity on Candida species was candidacidal. Moreover, CaThi caused plasma membrane permeabilization in all yeasts tested and induces oxidative stresses only in Candida tropicalis. CaThi was intracellularly localized in C. albicans and C. tropicalis, however localized in nuclei in C. tropicalis, suggesting a possible nuclear target. CaThi performed synergistically with fluconazole inhibiting all tested yeasts, reaching 100 % inhibition in C. parapsilosis. The inhibiting concentrations for the synergic pair ranged from 1.3 to 4.0 times below CaThi IC₅₀ and from zero to 2.0 times below fluconazole IC₅₀.

Conclusion: The results reported herein may ultimately contribute to future efforts aiming to employ this plant-derived AMP as a new therapeutic substance against yeasts.

Keywords: Antimicrobial peptides, Thionin, Synergistic activity, Fluconazole, Candida

Background

Currently, a significant global public health threat is the emergence of pathogenic bacteria, fungi, and yeasts that are resistant to multiple antimicrobial agents. Indeed, few or no effective chemotherapies are available for infections caused by some of these resistant microorganisms [1, 2].

Alternatives to chemotherapies include antimicrobial peptides (AMPs), small molecules produced by all living organisms, which have gained considerable attention because of their potent antimicrobial activity against a broad range of microbes, including viruses, bacteria, protozoa, and fungi [3, 4]. Moreover, some kill microorganisms rapidly, are able to synergize with other AMPs and clinical antibiotics, have low toxicity to mammalian cells, and exert their microbial inhibitory activity at low concentrations. These molecules have multiple targets in the plasma membrane and also in intracellular components, which is thought to make an increase in microbial resistance more difficult [2, 5].

Promising AMPs include plant-derived thionins, a family of basic, low molecular weight (~5 kDa), cysteine-rich peptides. Various family members have high sequence similarity and structure [6–8]. Many of them...
are toxic against yeasts, pathogenic fungi, Gram-positive and Gram-negative bacteria, protozoa, and insects [7–10]. Like other AMPs, thionins’ antimicrobial activity relies on their interaction with phospholipids to cause membrane instability [10].

Infections caused by Candida species have increased substantially over the last 30 years due to the rise of AIDS, ageing population, numbers of immunocompromised patients and the extensive use of indwelling prosthetic devices [1, 11]. Candida albicans is the main cause of candidiasis, however, other Candida species such as C. tropicalis, C. parapsilosis, and C. glabrata are now frequently identified as human pathogens [11–13]. Antifungals, especially fluconazole (FLC), have been used with some success for the treatment of Candida infections; however, there are numerous reports on the emergence of strains resistant to azoles that overexpress multidrug efflux transporters [14, 15].

In a previous report [8], our research team isolated a plant-derived thionin, named CaThi, with strong antimicrobial activity against two pathogenic Candida species, as well as Escherichia coli and Pseudomonas aeruginosa. FLC in combination with AMPs resulting in promising therapeutic results against important human pathogens, such as C. albicans and Cryptococcus neoformans, has been demonstrated [16, 17]. In this work we investigated whether the AMP CaThi could act synergistically with FLC. This synergistic strategy could result in a more efficient response against six Candida strains of clinical importance, avoiding the cytotoxic effects commonly exhibited by thionins against mammalian cells [10] by using low concentrations of this AMP. We were also interested in understanding the mechanism by which plant-derived thionins affect Candida species, which remains partially unknown [10]. These questions are addressed in the present study.

The results reported herein may ultimately contribute to future efforts aiming to develop this plant-derived AMP as a new therapeutic substance against these pathogenic Candida species as well as other yeast infections. Moreover, although the antimicrobial activity of CaThi against Candida species is indeed relevant, our data showed it to be lower than that observed for FLC (Table 1).

Viability assay
CaThi induced viability loss in all yeasts cells tested (Fig. 1a). The most susceptible species to CaThi were C. buinensis, C. parapsilosis and C. albicans with 99.2, 98.9 and 80.3 % of viability loss, respectively, and the less susceptible was C. tropicalis with 47.9 % of viability loss (Fig. 1b). These results indicated that inhibitory effect of CaThi was candididal.

Plasma membrane permeabilization
Candida species cells were tested to determine the membrane permeabilization by Sytox green dye. All yeasts showed Sytox green fluorescence when grown for 24 h in the presence of CaThi IC50. As with other AMPs, it is likely that CaThi acts on the plasma membrane of these Candida species, compromising it structurally and allowing the permeabilization of the labeling dye (Fig. 2). The membrane permeabilization percentage of the treated yeasts with CaThi was assessed (Table 2). A higher number of C. albicans and C. pelliculosa cells presented higher Sytox green fluorescence percentage, suggesting that CaThi is more effective at permeabilizing the membrane of these cells than the other Candida species analyzed.

ROS induction assay
Endogenous production of ROS was analyzed by incubating the yeasts for 24 h with CaThi IC50. Increased ROS production was observed only in C. tropicalis (Fig. 3), suggesting that a CaThi-induced increase in oxidative stress may underlie the growth inhibitory effect on this yeast. Nevertheless, oxidative stresses were not detected for other Candida species, implicating that we could not associate the CaThi role and ROS production with growth inhibition of Candida, at least for the concentrations tested.

Table 1 IC50 (μg.mL−1) of fluconazole and CaThi in different species of Candida respectively

| Yeasts             | Fluconazole | CaThi |
|--------------------|-------------|-------|
| Candida albicans (CE022) | 1.0         | 10.0  |
| Candida tropicalis (CE017) | 1.0         | 10.0  |
| Candida parapsilosis (CE002) | 0.5         | 10.0  |
| Candida pelliculosa (3974) | 5.0         | 40.0  |
| Candida buinensis (3982) | 0.125       | 10.0  |
| Candida mogii (4674)       | 2.5         | 200   |

* represents the concentration of a drug that is required for 50 % inhibition in vitro.
Localization of CaThi in yeast cells

We also investigated whether CaThi was actually internalized in C. albicans and C. tropicalis cells. These yeasts were chosen because they are known to be the most opportunistic pathogens among Candida species. Another important point is that C. tropicalis was the only yeast that presented membrane permeabilization and induction of ROS by CaThi in this work. To perform the test, we used 10 μg.mL⁻¹ of FITC-tagged CaThi to search for intracellular signal fluorescence. We also treated the cells with DAPI for nuclei labeling. Intracellular signal fluorescence of CaThi-FITC was observed in both of these Candida species. However, while CaThi-FITC labeling of C. tropicalis produced a specific and intense spot of fluorescence inside the cells, C. albicans cells showed a more diffuse fluorescence. Overlapping of these CaThi-FITC images with DAPI nuclei labeling.

![Fig. 1 Cell viability loss.](image-url)

**a** Photographs of the Petri dishes showing the viability of yeasts cells after the treatment with IC₅₀ CaThi for 24 h. **b** The table shows the percentage of viability loss of yeasts cells after the treatment with IC₅₀ CaThi for 24 h. CFU = Colony forming unit. (*) Indicates significance by the T test (P < 0.05) among the experiments and their respective controls. The experiments were carried out in triplicate.
indicated a co-localization of these fluorescent signals in *C. tropicalis* but not in *C. albicans* cells (Fig. 4). These data suggest that, at least for *C. tropicalis*, CaThi may have an intracellular target, possibly located in the nucleus.

**Synergism assay**

Given the increase in *Candida* infections, particularly among immunocompromised patients, searches for antifungal therapeutic alternatives are warranted. This concern and the aforementioned data prompted us to investigate whether FLC and CaThi could act synergistically to improve therapeutic results against *Candida* species. The combination of FLC and CaThi showed an increase in inhibitory activity of all of the *Candida* species tested, suggestive of synergistic activity (Table 3). Interestingly, although *C. pelliculosa* had the highest IC$_{50}$ for both substances, when we combined FLC at one-fold below its IC$_{50}$ and CaThi at threefold below its IC$_{50}$, we observed 57 % increase in growth inhibition of this yeast. Similarly, in *C. parapsilosis* cells, when IC$_{50}$ FLC was combined with CaThi threefold below its IC$_{50}$, we obtained 100 % growth inhibition of this yeast. Combined use of FLC and CaThi also strongly inhibited (96 %) *C. tropicalis*, although when used separately the inhibition achieved with these substances did not reach 12 %. Taken together, these data suggest that in combination FLC and CaThi could have an important synergistic action resulting in very effective control of *Candida* species.

**Morphological alterations of CaThi and FLC plus CaThi on yeast growth**

Investigation regarding the possible morphological alterations in yeast cells grown in the presence of FLC, CaThi, or a combination of both substances after the inhibition assays (Fig. 5a) was performed. Optical
Table 2: Fluorescent cell percentage of yeasts treated with CaThi

| Yeasts species   | Sample   | Cell number viewed in DIC | Cell number viewed in fluorescence | % of fluorescence cells |
|------------------|----------|---------------------------|------------------------------------|-------------------------|
| C. albicans      | control  | 62.0 ± 9.3                | 0.6 ± 0.8                          | 0.9                     |
|                  | CaThi    | 20.0 ± 5.0                | 16.6 ± 5.3                         | 83.0                    |
| C. tropicalis    | control  | 41.2 ± 4.2                | 0.8 ± 1.3                          | 1.9                     |
|                  | CaThi    | 9.8 ± 4.9                 | 4.8 ± 4.6                          | 48.9                    |
| C. parapsilosis  | control  | 79.2 ± 12.1               | 0                                   | 0                       |
|                  | CaThi    | 18.2 ± 5.4                | 6.2 ± 1.4                          | 34.0                    |
| C. pelliculosa   | control  | 23.8 ± 3.5                | 0                                   | 0                       |
|                  | CaThi    | 7.2 ± 1.9                 | 6.6 ± 2.3                          | 91.6                    |
| C. buinensis     | control  | 43.6 ± 9.0                | 0.8 ± 1.3                          | 1.8                     |
|                  | CaThi    | 13.6 ± 6.2                | 6.2 ± 1.3                          | 45.5                    |
| C. mogii         | control  | 18.6 ± 3.2                | 0.6 ± 0.8                          | 3.2                     |
|                  | CaThi    | 8.6 ± 2.0                 | 4.4 ± 2.7                          | 51.1                    |

*a* Cells number determination in five random fields of the DIC and fluorescence views of the samples obtained from Plasma membrane permeabilization assay. The total cell number in DIC of each yeast (in control and test) was assumed as 100%.

*b* Indicates significance by the T test (*P* < 0.05) among the experiments and their respective controls.

Fig. 3: Oxidative stress assay. Photomicrography of different yeast cells after reactive oxygen species assay detection by fluorescence microscopy using the fluorescent probe 2',7' dichlorofluorescein diacetate (H$_2$DCFDA). Cells were treated with CaThi for 24 h and then assayed for ROS detection. Control cells were treated only with probe (H$_2$DCFDA). Bars 5 μm.
### Table 3

Inhibition percentage of yeast species treated with CaThi and FLC alone and in combination showing synergism effect in vitro

| Yeasts species | Sample | Concentration (μg.mL⁻¹) | Inhibition (%) | Combination inhibition (%) (CaThi + FLC) |
|----------------|--------|-------------------------|---------------|----------------------------------------|
| *C. albicans*  | CaThi  | 3.5                     | 2.93          | 77.5                                   |
|                | FLC    | 0.5                     | 24.48         |                                        |
| *C. tropicalis*| CaThi  | 3.5                     | 0             | 96.26                                  |
|                | FLC    | 0.5                     | 11.55         |                                        |
| *C. parapsilosis* | CaThi | 3.5                     | 4.0           | 100.0                                  |
|                | FLC    | 0.5                     | 50            |                                        |
| *C. pelliculosa* | CaThi | 15.0                    | 2.63          | 57.45                                  |
|                | FLC    | 2.5                     | 7.8           |                                        |
| *C. buinensis* | CaThi  | 5.0                     | 19.0          | 67.01                                  |
|                | FLC    | 0.06                    | 5.45          |                                        |
| *C. magii*     | CaThi  | 10.0                    | 17.19         | 61.05                                  |
|                | FLC    | 1.0                     | 22.80         |                                        |

*CaThi concentrations ranging 1.3 to 4.0 times below it IC₅₀ and FLC concentrations 2.0 times below it IC₅₀ or at it IC₅₀

*Indicates significance by the ANOVA test (*P < 0.05*) which were calculated by the absorbance values of synergism among the experiments and their respective controls

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**Fig. 4** Localization of CaThi in yeast cells. Photomicrography of *Candida albicans* and *Candida tropicalis* cells incubated for 24 h with 10 μg.mL⁻¹ CaThi-FITC (green fluorescence, open arrows) by fluorescence microscopy. Nuclei were visualized by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) after the CaThi-FITC incubation period (blue fluorescence, filled arrows). Overlap of the DAPI and FITC images (dotted arrows). Bars 20 μm. (*) Indicates the position of digital enlargement
Fig. 5 Effect of CaThi, FLC, and CaThi plus FLC on yeast growth. 

(a) Photomicrography of Candida albicans, Candida tropicalis, Candida parapsilosis, Candida pelliculosa, Candida buinensis, and Candida mogii cells by light microscopy after the growth inhibition assay. Bars 5 μm.

(b) Scanning electron microscopy of Candida pelliculosa and Candida buinensis. Filled arrow (formation of pseudohyphae); open arrow (cell agglomeration); asterisk (amorphous material). Bars 10 μm. Cells grown in the presence of Fluconazole (FLC), CaThi, and FLC plus CaThi.
microscopy analysis revealed that FLC, CaThi, and the combined treatment caused changes in the morphology of cells of *Candida* species. *C. tropicalis, C. parapsilosis, C. pelliculosa,* and *C. mogii* exhibited an apparent difficulty in releasing buds thus leading to the formation of pseudohyphae when grown in the presence of CaThi. Moreover, in the presence of either substance, *C. tropicalis* cells presented hyper branching of pseudohyphae. For *C. albicans* and *C. buinensis*, an intense cellular agglomeration in all treatments was observed. Further, the combination of CaThi and FLC caused a shrunken appearance in *C. albicans* cells.

Scanning Electronic Microscopy (SEM) of *C. pelliculosa* reinforces the optical microscopy observations, corresponding to intense cell agglomeration and pseudohyphae formation in all treatments. For *C. buinensis*, all treatments showed intense cellular agglomeration and apparent difficulty in bud release, but not in the formation of pseudohyphae. For this yeast an amorphous material among cells in all treatments was also observed (Fig. 5b). These results show that CaThi is capable of causing morphological changes similar to FLC, an azole antifungal agent, commonly used in treatment of infections caused by *Candida* species. Importantly, we were able to demonstrate that the combination of these substances potentiates the therapeutic effects against these opportunistic species of *Candida*.

**Discussion**

Plant-derived thionins exhibit toxic effects against a wide range of plant pathogens including bacteria and fungi [18–21]. However, there is a gap regarding the mode of action of plant-derived thionins against human pathogens. Prompted by the considerable increase in the incidence of human infections by *Candida* species, we investigated the potential of CaThi, a plant-derived thionin peptide, as a novel therapeutic drug against six *Candida* strains of clinical interest: *C. albicans, C. tropicalis, C. parapsilosis, C. pelliculosa, C. buinensis,* and *C. mogii*.

The growth inhibition assay was not done with RPMI 1640 medium, which is generally indicated by Clinical and Laboratory Standards Institute guidelines, because it has in its composition a large amount of inorganic salts and it is well known in the literature that the presence of salts, such as sodium chloride and magnesium sulfate, can decrease the inhibitory activity of antimicrobial peptides since it is necessary electrostatic approximation of the peptide with the membrane of microorganisms and the presence of salts disrupts this initial interaction [22–24]. Therefore, as explained above, our growth inhibition assay was done with Sabouraud broth which is a common used medium to growth of fungi, including *Candida albicans*.

Our growth inhibition assays of the six *Candida* species tested revealed that 10 μg.mL⁻¹ was IC₅₀ for CaThi to inhibit the growth of *C. albicans, C. tropicalis, C. parapsilosis,* and *C. buinensis* but 40 μg.mL⁻¹ was necessary to achieve IC₅₀ for *C. pelliculosa* (Table 1), and this inhibitory effect was candidacidal inducing viability loss in all yeast cells tested (Fig. 1). Thi 2.1, a thionin from *Arabidopsis thaliana,* achieved 80 % inhibition of *C. albicans* with 2.5 μg.mL⁻¹ [25]. Although Thi 2.1 showed stronger antimicrobial activity against *C. albicans* than CaThi, these authors did not test it against non-*albicans* species. Thus, whether this thionin would affect other species with similar strength remains unknown.

Sytox green is a dye that only penetrates cells when the plasma membrane is structurally compromised. All yeast species tested showed Sytox green fluorescence (Fig. 2), however CaThi was more effective in plasma membrane permeabilization in *C. albicans* and *C. pelliculosa* (Table 2). Antimicrobial activity against the fungus *Neurospora crassa* by α-Hodothionin, isolated from barley seeds, also occurs via a mechanism involving membrane permeabilization, resulting in the inward flux of Ca²⁺ and K⁺ efflux and consequent potential membrane collapse [26]. Another plant-derived thionin isolated from *Viscum album,* named VtA₃, interacts with the plasma membrane of the fungus *Fusarium solani,* causing its permeabilization and thus inhibiting the growth of this microorganism [27]. Indeed, several studies suggest that most of the biological effects of thionins result from the interaction of these peptides with the target cell membrane. Three mechanisms have been proposed: formation of an ion-selective channel; formation of patches or carpets of peptides; and loss of membrane phospholipids [10].

AMPs have been demonstrated to play a direct role in membrane permeabilization, causing a loss of membrane potential [28]. As cells depend on membrane potential to fulfill their physiological functions, its restoration is mandatory and demands cellular energy. One possible consequence of this process is ROS generation by activated mitochondria [29]. Therefore, we analyzed whether this primary membrane-permeabilizing event in *Candida* species induced by CaThi was followed by oxidative stress. Interestingly, CaThi only induced production of ROS in *C. tropicalis* (Fig. 3). We speculate that CaThi binds to a specific domain of the *C. tropicalis* membrane, which triggers the increase in oxidative stress response through ROS production. However, further studies are needed to establish this. Reports show that increase in ROS production by the target organisms is a recurring mode of action employed by thionins and other AMPs [27, 30, 31]. Indeed, increased death of the fungus *Fusarium solani* subjected to VtA₃ was connected with boosted ROS production by these organisms.

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Psacothea hilaris also provoked in in-
and species. Our study is the first to report increase in ROS production by a plant-derived thionin as an antimicro-
bial mechanism against a human pathogen, C. tropicalis. Therefore, CaThi seems to employ a sophisticated mechanism to inhibit the growth of this opportunistic yeast involving not only membrane permeabilization but also induction of oxidative stress response.

Some AMPs are able to enter the cell, after
the initial cell membrane interaction [33, 34]. Accord-
ingly, the next experiments were designed to analyze
whether CaThi was able to actively enter C. albicans
and C. tropicalis. In the approach used, FITC-tagged CaThi was monitored by fluorescence microscopy. Because CaThi entered C. albicans and C. tropicalis cells, we suggest that a possible intracellular target
for this thionin might be part of a complex mechan-
ism responsible for the death of Candida species.
FITC-tagged CaThi overlapped with DAPI staining, indicating that, in C. tropicalis, this target is nuclear (Fig. 4). Giudici et al. [27] showed that VtA3 entered and accumulated in the fungus F. solani. These authors also demonstrated that this entry was related to the sphingolipid composition of the plasma membrane of this fungus. Our study is the first to show
that a plant-derived thionin (CaThi) is able to enter
human pathogens (C. albicans and C. tropicalis) and
to suggest an intracellular target for it. Our work
opens new perspectives regarding the antimicrobial
mechanism of plant-derived thionins as it suggests
that these peptides’ toxicity may not be restricted to
the plasma membrane.

The real mode of action of AMPs has not been fully elucidated, but much of the described AMPs to date target
the plasma membrane of microorganisms causing pore formation and leading to imbalance in cellular homeostasis [33, 35]. However, some studies showed that not only is permeabilization the cause of a particu-
lar microorganism death, as they may have multiple targets [36] after the interaction with the membrane causing, for example, ROS induction [31, 37]; inhibition of protein synthesis [38, 39]; inhibition of mitochondrial activity [40, 41], and also may trigger signaling cascades
that lead to apoptosis [42, 43]. Thus it is difficult to
identify the most important factor for the candidacidal
effect of CaThi and is technically challenging to characterize the steps leading up to cell death, however
evidence supports that all events described in the
manuscript may have a crucial role in the death of the
tested yeasts.

The continuous emergence of resistance of fungal
strains to conventional antibiotics and antifungals,
especially Candida species, has become an important
medical issue and has spurred the demand for new therapeutic alternatives. This concern prompted us to
investigate whether FLC and CaThi could act synergis-
tically to improve therapeutic results against Candida
species. Here we show that the combination of these
two substances was effective against all Candida species
tested (Table 3), causing drastic morphological changes
in these cells (Fig. 5). Interestingly, we show that the
inhibitory effect of this combination was more effective
for C. albicans, C. parapsilosis, and C. tropicalis, which
are the major yeast species recovered from infections in
immunocompromised patients [13]. The azoles mode
of action occurs by inhibition of the enzyme lanosterol 14
α-demethylase, blocking ergosterol incorporation and
leading to the accumulation of intermediate sterols.
These intermediate sterols do not have the same config-
uration and physical properties of ergosterol, therefore
they cause the plasma membrane to form with altered
properties, changing in fluidity, permeability and impair-
ing nutrient uptake, which ultimately lead to cell toxicity
[44, 45]. In regard to the synergistic effect of FLC and
CaThi, we hypothesize that permeabilization is firstly
caused by CaThi (Fig. 2) facilitating the entrance of FLC
into the cell cytoplasm, triggering structural alterations
in the plasma membrane which feedback positively to
the entrance of more CaThi and FLC. This entrance
creates potential for toxic effects, which were experi-
mentally observed in the lower IC₅₀ used for both
substances in the combinatory treatment (Table 3). Addi-
tionally, secondary toxicity effects were demonstrated,
such as the induction of ROS to C. tropicalis, the CaThi
presence in C. albicans cell cytoplasm and in C. tropica-
lis nuclei. These localizations suggest that CaThi may
have cytoplasmic targets, where interference could
consolidate the inhibitory effect. However, more studies
are necessary to clearly unravel the antimicrobial
mechanism of CaThi against Candida species as well as
the mechanism of synergy between CaThi and FLC.

Conclusions
Investigating a plant-derived thionin mode of action
against opportunistic human pathogenic yeasts is rele-
vant and advisable, whereas most studies involving
plant-derived thionins focus their effects against plant
pathogenic microorganisms as experimental models. In
this report, we demonstrated that CaThi has strong can-
didacidal activity against six pathogenic Candida
species, and it works by permeabilizing the membrane and
inducing oxidative stress response in these yeasts. Addi-
tionally, we present evidence to suggest a nuclear intra-
cellular target for CaThi. Finally, our results show that
FLC and CaThi combined causes dramatic morpho-
logical changes in these yeasts, effective against all
Candida species tested. The combined treatment of CaThi and FLC is a strong candidate for clinical studies aiming to improve therapeutic results against resistant strains of Candida species. Studies involving drug combinations should be reinforced due to the possibility of synergistic effects that increase the toxic effect of the drugs combined when compared to monotherapy. Moreover, drug combinations can broaden the spectrum of antimicrobial activity, minimizing resistant microorganisms selection, increasing security and tolerance using lower drugs doses.

**Methods**

**Biological materials**

Capsicum annuum L. fruits (accession UENF1381) were provided by Laboratório de Melhoramento Genético Vegetal, from Centro de Ciências e Tecnologias Agrone masculas, Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes, Rio de Janeiro, Brazil.

The yeasts Candida albicans (CE022), Candida tropicalis (CE017), and Candida parapsilosis (CE002) were obtained from Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. The yeasts Candida pelliculosa (3974), Candida buinensis (3982), and Candida mogii (4674) were obtained from Micoteca URM from Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil. Yeasts were maintained on Sabouraud agar (1 % peptone, 2 % glucose, and 1.7 % agar-agar) (Merck) in the end of this period, colonies forming units (CFU) were photographed. The same procedure was followed with yeasts treated with CaThi. The experiments were carried out in triplicate, and the results are shown assuming that the control represents 100 % viability. Calculations of the standard deviation and T test were performed with Prism software (version 5.0).

**Viability assay**

To assay the effect of CaThi on the cell viability of yeasts, 1x10^4 cells mL^-1 in Sabouraud broth culture medium and at the corresponding IC_{50} of CaThi values for each yeast were incubated at 30 °C for 24 h in 96-well microplates (Nunc). To determine the control viability, the control cells (without CaThi) were washed once and diluted 1,000-fold in Sabouraud broth culture medium, and an aliquot of 100 μl from this dilution was spread over the surface of a Sabouraud agar medium (contained in a Petri dish) with a Drigalski loop and grown at 30 °C for 48 h. At the end of this period, colonies forming units (CFU) were determined for each yeast species, and the Petri dishes were photographed. The same procedure was followed with yeasts treated with CaThi. The experiments were carried out in triplicate, and the results are shown assuming that the control represents 100 % viability. Calculations of the standard deviation and T test were performed with Prism software (version 5.0).

**Plasma membrane permeabilization assay**

The plasma membrane permeabilization of yeast cells was measured by Sytox green uptake, according to the methodology described by Thevissen et al. [47], with some modifications. Each of the different species of yeasts was incubated with CaThi at the concentration required to inhibit 50 % growth (IC_{50}) of the respective yeast cells for 24 h. After this time, a 100 μl aliquot of each yeast cell suspension was incubated with 0.2 μM of Sytox green in 1.5 mL microcentrifuge tubes for 30 min at 25 °C with constant agitation. Cells were analyzed by DIC optical microscope (Axiovison 4, Zeiss) equipped with a fluorescent filter set for detection of the fluorescein (excitation wavelength, 450−490 nm, emission wavelength 500 nm). To indicate membrane permeabilization, the percentage of fluorescent cells was determined by counting the DIC and fluorescent views.
for each yeast \((n = 5)\). The total cell number in DIC view of each yeast (in control and test) was assumed as 100 %. The experiments were carried out in triplicate. Calculations of \(T^2\) test were performed with Prism software (version 5.0).

**Determining the induction of intracellular ROS in yeast cells**

To evaluate whether the mechanism of action of CaThi involves induction of oxidative stress, the fluorescent probe 2, 7-dichlorofluorescein diacetate (H₂DCFDA) was used to measure intracellular reactive oxygen species (ROS) according to the methodology described by Mello et al. [31]. Each of the different species of yeasts was incubated with the respective IC₅₀ for CaThi in 96-well microplates for 24 h at 30 °C; after this incubation an aliquot of 50 μL of each of yeast cell suspension was incubated with 200 μM of H₂DCFDA in micro centrifuge tubes of 1.5 mL for 1 h at 25 °C with constant agitation at 500 rpm. Cells were analyzed by DIC optical microscope (Axiovision 4, Zeiss) equipped with a fluorescent filter set for detection of the fluorescein (excitation wavelength, 450–490 nm, emission wavelength 500 nm). The experiments were carried out in triplicate.

**CaThi conjugated to FITC localization for optical microscopy**

CaThi at 100 μg was resuspended in 100 μL of 750 mM sodium carbonate-sodium bicarbonate buffer, pH 9.5 containing FITC at 50 μg.mL⁻¹ (previously solubilized in DMSO). This solution was incubated with constant agitation at 500 rpm for 2 h at 30 °C. After this incubation, the sample was submitted to gel filtration chromatography on Sephadex G25 column (Sigma) for elimination of free FITC and recovery CaThi-FITC. The column was equilibrated and run with 20 mM Tris-HCl, pH 8.0 at flow rate of 0.3 mL.min⁻¹. After coupling, 10 μg.mL⁻¹ of CaThi-FITC was incubated with cells of C. albicans and C. tropicalis for 24 h in 96-well microplates. After this time an aliquot of each yeast suspension was removed and incubated with 50 μg.mL⁻¹ of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min for nuclei stain. Cells were analyzed by DIC optical microscope (Axiovision 4, Zeiss) equipped with a fluorescent filter set for detection of the fluorescein (excitation wavelength, 450–490 nm, emission wavelength 500 nm). The entire assay was performed protected from light.

**Synergism assay**

To verify the synergistic activities, we combined FLC with CaThi. Initially yeast cells (1 x 10⁴ cells mL⁻¹) were incubated in Sabouraud broth containing an IC₅₀ concentration or less that for FLC and CaThi concentrations ranging from 1.3 to 4.0 times below the IC₅₀ for the respective yeast and the final volume adjusted to 200 μL in vitro. The assay was performed in 96-well microplates (Nunc) at 30 °C for 24 h. Optical readings at 620 nm were taken at zero hour and every 6 h for the following 24 h. Control cells were: 1) grown in the absence of CaThi and FLC; 2) grown in the presence of FLC; 3) grown in the presence of CaThi. The synergistic activity was deduced comparing optical densities of each control and combined drugs (FLC plus CaThi) considering each yeast strain tested. We define synergism as the combination action of the AMP with other substance that causes an enhanced decrease in the growth of the microorganism, compared with the growth inhibition of the single substances. After synergism, assay cells (controls and tests) were analyzed by DIC optical microscope (Axiovision 4, Zeiss). The data were obtained from experiments performed in triplicate. The data were evaluated using a one-way ANOVA. Mean differences at \(p < 0.05\) were considered to be significant. All statistical analyses were performed using the GraphPad Prism software (version 5.0 for Windows).

**Scanning electron microscopy**

C. pelliculosa and C. buinensis cells were submitted to scanning electron microscopy (SEM) analysis. Yeast cells were grown for 24 h in Sabouraud broth in the presence of FLC (5 μg.mL⁻¹ and 0.125 μg.mL⁻¹, respectively), CaThi (40 μg.mL⁻¹ and 10 μg.mL⁻¹, respectively), and FLC plus CaThi (2.5 μg.mL⁻¹ + 15 μg.mL⁻¹, 0.06 μg.mL⁻¹ + 5 μg.mL⁻¹, respectively) or absence of these drugs, then were fixed for 30 min at 30 °C in a solution containing 2.5 % glutaraldehyde and 4.0 % formaldehyde in 0.1 M cacodylate buffer, pH 7.0. Subsequently, the cells were rinsed three times in 0.1 M cacodylate buffer, pH 7.0; post-fixed for 30 min at 30 °C with 1.0 % osmium tetroxide diluted in 0.1 M cacodylate buffer, pH 7.0; and rinsed again with this same buffer. The yeast cells were gradually dehydrated in alcohol solution (15, 30, 50, 70 to 90 % and finally 100 % alcohol), critical point dried in CO₂, covered with 20 nm gold and observed in a DSEM 962 Zeiss SEM.

**Abbreviations**

AMP: Antimicrobial peptide; FLC: Fluconazole; ROS: Reactive oxygen species; CaThi: Capsicum annuum Thionin; DIC: Differential interference contrast; FITC: Fluorescein isothiocyanate; SEM: Scanning Electron Microscopy.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

Conceived and designed the experiments: GBT, AOC, RR, FGT, MC and VMG. Performed the experiments: GBT and FGT. Analyzed the data: GBT, AOC, FGT, MC and VMG. Contributed reagents/materials/analysis tools: AOC, RR, MC
and VMG. Wrote the paper: GBT, AOC and VMG. All authors read and approved the final manuscript.

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