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# ATIVIDADE ANTIFÚNGICA, MECANISMO DE AÇÃO, CITOTOXICIDADE E AÇÃO ANTIBIOFILME DA CLORAMINA T SOBRE *Candida* spp.

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# ATIVIDADE ANTIFÚNGICA, MECANISMO DE AÇÃO, CITOTOXICIDADE E AÇÃO ANTIBIOFILME DA CLORAMINA T SOBRE *CANDIDA* SPP

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## DEDICATÓRIA

Aos meus pais, Jamacy e Elda, em retribuição a todo o amor e esforço dispensados para a minha formação moral e intelectual.

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## RESUMO

**Introdução:** Diante das limitações para o uso do hipoclorito de sódio na desinfecção de próteses dentárias e da necessidade do controle da proliferação fúngica nestes sítios, faz-se necessário o estudo de novas substâncias para este fim. Assim como o hipoclorito de sódio, a cloramina T (CAT) é um composto de cloro com ação antibacteriana e alto poder de cloração e oxidação, e sua ação antifúngica merece ser estudada. **Objetivos:** Avaliar a atividade antifúngica, antibiofilme, citotoxicidade e mecanismo de ação da cloramina T trihidrato sobre *Candida* spp. **Materiais e Métodos:** Foram determinadas as concentrações inibitória mínima (CIM) e fungicida mínima (CFM) da CAT sobre 7 cepas de *Candida albicans*, *Candida tropicalis*, *Candida krusei* e *Candida glabrata.* Foi avaliada a cinética de inibição do crescimento de *C. albicans* em diferentes tempos e concentrações e realizado microcultivo de *C. albicans* em ágar fubá para avaliação da possível alteração da micromorfologia frente a diferentes concentrações da substância. O provável mecanismo de ação sobre parede e membrana celular fúngica foi verificado através da determinação da CIM na presença, respectivamente, de sorbitol e ergosterol. A inibição da aderência inicial de células fúngicas, formação e redução do biofilme de *C. albicans* foram avaliados após contato curto (1 min) e prolongado (8 h) com a substância e a formação do biofilme foi mensurada através de leitor de absorbância. Nistatina e hipoclorito de sódio foram utilizados como controles positivos. A citotoxicidade foi avaliada pelo método da hemólise. Foi realizada análise estatística descritiva e inferencial, considerando α=5%. **Resultados:** A $CIM_{75\%}$ encontrada para a CAT foi de 781,3 µg/mL e a relação CFM/CIM sugere uma atividade fungicida frente a maioria das cepas testadas, com provável ação em parede e membrana celulares. A substância mostrou ação imediata e prolongada no teste de cinética e provocou redução da forma filamentosa e inibição de clamidoconídios. No ensaio do biofilme, o hipoclorito de sódio mostrou-se mais eficaz na inibição da aderência inicial das células fúngicas na concentração referente a CIM x 2 ($p \leq 0,05$). A CAT, no entanto, apresentou resultados semelhantes ao hipoclorito de sódio para formação do biofilme maduro ($p>0,05$) e foi mais efetiva na redução do biofilme maduro nos grupos de contato curto na concentração CIM x 2 (24 h) e CIM x 4

(48 h) ($p \leq 0,05$). No teste de citotoxicidade, a CAT provocou hemólise entre 61 e 67,7%. **Conclusão:** A CAT apresenta atividade antifúngica sobre *Candida* spp. e apresenta ação fungicida sobre a maioria das cepas testadas. Sua ação é imediata e prolongada na inibição do crescimento de *C. albicans* e provavelmente ocorre tanto em parede quanto em membrana celular. A CAT causa alterações na micromorfologia de *C. albicans* e possui atividade antibiofilme, sendo efetiva na inibição da aderência inicial das células fúngicas, bem como na formação e redução do biofilme.

**Palavras-chave:** Estomatite sob Prótese; Higienizadores de Dentadura; Cloraminas; Candidíase Bucal; Biofilmes

## ABSTRACT

**Introduction:** Given the limitations to the use of sodium hypochlorite in the disinfection of dental prostheses, and the need for control of fungal proliferation in these sites, it is necessary the study of new substances for this purpose. Even as sodium hypochlorite, chloramine T (CAT) is a chloro compound with antibacterial action and high power of chlorination and oxidation, and their antifungal action needs to be studied. **Objectives:** To evaluate the antifungal activity, mechanism of action, cytotoxicity and anti-biofilm action of chloramine T trihydrate on *Candida* spp. **Materials and Methods:** It was determined the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of CAT on seven strains of *Candida albicans*, *Candida tropicalis*, *Candida krusei* and *Candida glabrata.* It was evaluated the growth kinetics of *C. albicans* by counting colony forming units (CFU) method in different times and substance' concentrations and was performed microculture of *C. albicans* in cornmeal agar plus tween 80 to assess the possible change of its micromorphology with different concentrations of the substance. The possible mechanism of action on fungal cell wall and membrane has been verified by determination of the MIC in the presence, respectively, of sorbitol and ergosterol. The inhibition of initial fungal cell adherence and formation and the reduction in *C. albicans* biofilm were evaluated after short (1 min) and extended (8 h) contact with the substance and biofilme formation was by absorbance reader. Nystatin and sodium hypochlorite were used as positive controls. Cytotoxicity was assessed through the hemolysis method. It was conducted descriptive and inferential statistical analysis, considering $\alpha = 5\%$. **Results:** The $MIC_{75\%}$ found for CAT was 781.3 µg/mL, and the MFC/MIC ratio indicates the presence of fungicidal activity against most of the tested strains, with action probably on cell wall and membrane. The substance demonstrated an immediate and sustained action in the kinetic test and caused reduction of filamentous form and inhibition of chlamydospore. When testing biofilm, sodium hypochlorite was more effective in inhibiting the initial adherence of the yeast cell in MIC x 2 concentration ($p < 0.05$). CAT, however, showed similar results to sodium hypochlorite for inhibition of formation of mature biofilm ($p > 0.05$) and was more effective in reducing the mature biofilm in short contact groups in MIC x 2 (24

h) and MIC x 4 (48h) concentrations (p ≤0.05). In the cytotoxicity assay, CAT led to hemolysis between 61 and 67.7%. **Conclusions:** CAT has antifungal activity against Candida spp. and has fungicidal action on most of the strains tested. Its action is immediate and prolonged in inhibition of *C. albicans* growth and probably occurs both in the wall and in the cell membrane. CAT causes changes in the micromorphology of *C. albicans* and has antibiofilm activity, being effective in inhibiting the initial adherence of the yeast cells as well as in the formation and reduction of biofilm.

**Keywords:** Stomatitis, Denture; Denture Cleansers; Chloramines; Candidiasis, Oral; Biofilms.

## LISTA DE ABREVIATURAS E SIGLAS

**ASD –** Ágar Sabouraud Dextrose

**ATCC -** American Type Culture Collection

**CAT –** Cloramina T Trihidrato

***C. albicans*** – *Candida albicans*

***C. glabrata*** – *Candida glabrata*

***C. krusei*** – *Candida krusei*

***C. tropicalis*** – *Candida tropicalis*

**CBS –** *Central Bureau voor Schimmelcultures,* Coleção Holandesa de Cepas Fúngicas.

**CIM (MIC)** – Concentração Inibitória Mínima (Minimum Inhibitory Concentration).

**CFM (MFC)** – Concentração Fungicida Mínima (Minimum Fungicidal Concentration).

**CSD –** Caldo Sabouraud Dextrose

**DMSO** – Dimetilsulfóxido

**IZ-** Instituto Zimotécnico – Universidade de São Paulo, Piracicaba, Brazil.

**PBS** - Phosphate buffered saline

**SIDA –** Síndrome da Imunodeficiência Adquirida

**SUMÁRIO**

## 1. INTRODUÇÃO

A estomatite protética é uma doença bucal que está associada ao uso de próteses totais, na maioria dos casos, ou parciais removíveis, e caracterizada por inflamação da mucosa subjacente a prótese. Embora seja, em grande parte dos casos, assintomática, alguns indivíduos relatam ardência bucal, desconforto ou gosto ruim diante da ocorrência da doença. O uso contínuo da prótese e a qualidade de sua adaptação, fluxo salivar, hábito de fumar, condições imunológicas, fatores dietéticos e a microbiota oral têm sido mencionados como fatores envolvidos no surgimento e determinação da severidade da doença (GENDREAU; LOEWY, 2011; NAVABI et al., 2013; MARINOSKI; BOKOR-BRATIC; CANKOVIC, 2014; MARTORI et al., 2014).

Estudos têm mostrado que aproximadamente 90% dos casos de estomatite protética são causados por leveduras, principalmente *Candida albicans*, mas têm sido isoladas outras espécies do gênero *Candida* tanto nas próteses quanto nas mucosas de pacientes, como *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida guilliermondii* e *Candida dubliniensis*, as quais também podem estar associadas ao surgimento da doença (FIGUEIRAL et al., 2007; KABAWAT et al., 2014; KILIC et al., 2012).

A presença de leveduras na cavidade bucal de pacientes sadios é esperada e tem sido mencionada cada vez mais na literatura, principalmente em coletas microbiológicas das superfícies das próteses dentárias (FIGUEIRAL et al., 2007). No entanto, sabe-se que a candidíase está bastante relacionada a queda imunológica no hospedeiro e é frequentemente citada em portadores da Síndrome da Imunodeficiência Adquirida (SIDA) e sujeitos submetidos à terapia imunossupressora (BOSCO et al., 2003; GOLECKA et al., 2006).

Dados epidemiológicos contidos no relatório da Pesquisa Nacional de Saúde Bucal – SBBrasil 2010 mostram que na faixa etária de 65 a 74 anos, 76,5% dos brasileiros usa algum tipo de prótese dentária superior e, ainda, a porcentagem de usuários de prótese total foi de 63,1% no país (BRASIL, 2012). Destaca-se, a partir destes dados, a importância de estudar formas de controlar efetivamente o biofilme formado na superfície das próteses dentárias para prevenção da estomatite protética e/ou auxílio no tratamento da doença.

É preconizado na odontologia que o controle do biofilme formado nestas superfícies seja realizado através da remoção mecânica, representada pela escovação das superfícies das próteses dentárias, aliada à desinfecção química, representada pela utilização de um agente antimicrobiano na forma de creme dental ou solução higienizadora (PANZERI et al., 2009; HUH et al., 2014). O agente químico mais indicado tem sido o hipoclorito de sódio (Figura 01), no entanto o mesmo apresenta algumas características indesejáveis, como corrosão de metais e alteração de cor da resina acrílica das próteses e odor e sabor residual desagradáveis (FELTON et al., 2011; AMIN et al., 2014; NEPPELENBROEK et al., 2015).

A cloramina T (CAT – Figura 01) é um composto de cloro que possui comprovada ação antibacteriana (FUURSTED; HJORT; KNUDSEN; 1997; ARNITZ; NAGL; GOTTARDI, 2009). De acordo com a literatura, possui alto poder de cloração e oxidação (GOTTARDI; NAGL, 2005). Na odontologia, a CAT tem sido utilizada como bactericida e desinfetante na composição do gel de Papacárie®, proposto para remoção químico-mecânica de cárie dentária (MIYAGI et al., 2006). Como bochecho, na concentração de 0,25%, a CAT apresentou efeito prolongado na redução na quantidade de micro-organismos salivares (PITTEN; KRAMER, 1999). Também podem ser encontrados na literatura alguns estudos voltados ao desenvolvimento de cremes dentais compostos de CAT indicados para higienização de próteses dentárias (PANZERI et al., 2009; ANDRADE et al., 2012).

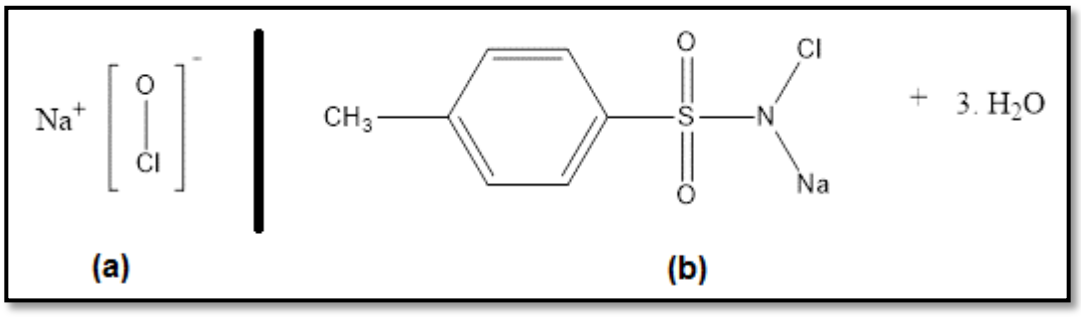


Figura 01. Moléculas de (a) hipoclorito de sódio (NaOCl) e (b) cloramina T trihidrato (C7H7ClNNaO2S).

A realização deste trabalho justifica-se pelo fato de que ainda que a CAT tenha sido avaliada na odontologia quanto a suas propriedades antimicrobianas, os estudos podem ser considerados pontuais e voltados para sua atividade antibacteriana. Poucas informações são encontradas sobre a atividade antifúngica da substância (PANZERI et al., 2009; ANDRADE et al., 2012) e não foram encontrados estudos relevantes acerca da atividade antibiofilme da mesma. Sendo assim, existe a necessidade da realização de estudos a respeito da sua atividade antifúngica frente a uma variedade de cepas consideradas patogênicas e ação antibiofilme, visto que o mesmo representa uma forma de organização e proteção dos micro-organismos e, ainda, no caso da estomatite protética, tem papel essencial no sucesso do tratamento e possibilidades de recidiva da doença (CHANDRA et al., 2001; SALERNO et al., 2011).

Este estudo propôs agregar novos conhecimentos nesta área, através da verificação da atividade antifúngica da CAT por meio da determinação das concentrações inibitória mínima (CIM) e fungicida mínima (CFM) sobre diferentes espécies do gênero *Candida*, estudo da cinética de inibição do crescimento fúngico e efeito sobre a micromorfologia de *C. albicans*, bem como determinação do possível mecanismo de ação da mesma. Em adição a isto, almejando a utilização da solução da substância como agente na desinfecção de próteses, foi realizado o teste de atividade antibiofilme sobre a aderência inicial, formação e redução do biofilme maduro.

A hipótese alternativa prevê que a CAT possui efeito antifúngico e apresente atividade sobre o biofilme de *C. albicans*.

Este trabalho se apresenta na forma de um capítulo e compreende o artigo intitulado “Atividade antifúngica, mecanismo de ação e ação antibiofilme da cloramina T sobre *Candida* spp.”, que será submetido para apreciação no periódico “*Clinical Oral Investigations*”.

## 2. CAPÍTULO 1

O manuscrito a seguir foi submetido para publicação no periódico “Clinical Oral Investigations”, cuja classificação QUALIS/CAPES na área Odontologia é A1 (2015).

**Antifungal and anti-biofilm activity, mechanism of action and cytotoxicity of chloramine T on *Candida* spp.**


**Abstract**

*Objectives:* Given the scarcity of denture disinfection resources, this study aimed to evaluate the antifungal and anti-biofilm activity, mechanism of action and cytotoxicity of chloramine T trihydrate (CAT) on seven strains of *Candida* genus. *Materials and Methods:* The minimum inhibitory (MIC) and minimum fungicidal concentrations (MFC) of CAT were determined. Changes in the growth kinetics of *C. albicans* and possible changes in its micromorphology were evaluated. The possible mechanism of CAT action on the fungal cell wall and cell membrane was verified. The inhibition of initial fungal cell adherence and formation and the reduction in *C. albicans* mature biofilm were evaluated. Cytotoxicity was assessed by the hemolysis method. Nystatin and sodium hypochlorite were used as positive controls. It was conducted descriptive and inferential statistical analysis, considering α = 5%. *Results:* The $MIC_{75\%}$ found for CAT was 781.3 µg/mL. CAT demonstrated an immediate and sustained action in the kinetic test, likely acting on the cell wall and cell membrane simultaneously, and caused changes to the micromorphology of *C. albicans*. The biofilm test produced results similar to those of sodium hypochlorite for inhibition of mature biofilm formation. CAT was more effective in reducing the mature biofilm after successive contacts of 1 min with a concentration of MIC x 2 (24 h) and MIC x 4 (48 h) ($p \leq 0.05$). In the cytotoxicity assay, CAT led to hemolysis between 61 and 67.7%. *Conclusions and Clinical Relevance:* CAT shows antifungal and anti-biofilm activity and has good potential for application as a denture disinfectant agent.



**Keywords:** Stomatitis, Denture; Denture Cleansers; Chloramines; Candidiasis, Oral; Biofilms.

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## 1 Introduction

Denture stomatitis is an inflammation of the oral mucosa that is associated with the use of removable dentures, with the majority of cases relating to the use of complete upper dentures. This disease is strongly associated with the presence of the *Candida* genus but also has the following etiological and/or aggravating factors: frequent trauma, bad denture adaptation, salivary flow deficiency, smoking, poor hygiene, continuous denture use and immune deficiency [1-4].

As oral candidiasis has been associated with the use of dentures, many studies have investigated the most prevalent species of *Candida*, with *Candida albicans* the most cited. However, other species, such as *Candida tropicalis*, *Candida krusei* and *Candida glabrata*, have also been isolated from patients' mucosa and denture bases and are thought to be important in the disease's etiology [3,5,6]. Fungal isolation does not necessarily indicate disease, but dentures can be a favorable site for the proliferation and adherence of microorganisms that are likely to promote the emergence of disease under the adverse conditions mentioned above.

Given the substantial number of denture users, there is a need to control sites that may encourage the presence and maintenance of fungal species in the oral cavity. Mechanical denture cleaning is the most commonly used method, but the scientific community recognizes the need to use chemicals adjunctively in denture hygiene, and studies have been developed in this regard [7-9].

Sodium hypochlorite has been indicated as a denture cleaning solution [7,10] but has undesirable characteristics, such as an unpleasant residual odor and flavor, risk of mucosal burns, metal corrosion and acrylic resin color change [7,11,12]. These limitations support the need to study compounds that effectively inhibit and reduce denture biofilm but that have fewer side effects.

Chloramine T (CAT) is an active chlorine compound with the chemical formula $C_7H_7ClNNaO_2S$. This synthetic compound has recognized antimicrobial activity, which is cited as being the result of its chlorination/oxidation power [13,14]. The stability [15] and antiseptic capacity [16,17] of CAT have been studied for many years. In dentistry, CAT has been used as a bactericide and disinfectant [18,19]. There are also studies in the literature that aim to develop CAT-based dental creams suitable for the cleaning of dentures [20,21]. However, as there is a

lack of investigations focusing on the antifungal activity and mechanism of action of CAT, it is important to conduct studies with a greater diversity of *Candida* species and that evaluate the mode of action of the compound and its action on biofilm.

Given the antimicrobial potential offered by CAT and the need to study chemicals with antifungal abilities for application as aids in mechanical denture cleaning, this study aimed to investigate the antifungal activity, mechanism of action and anti-biofilm action of CAT on *Candida* genus strains.

## 2 Materials and Methods

### 2.1 Microorganisms

The strains of *Candida* spp. used for the microbiological tests were obtained from American Type Culture Collection (ATCC): *C. albicans* ATCC 60193, *C. tropicalis* ATCC 750 and *C. krusei* ATCC 3413; from the Dutch collection Central Bureau voor Schimmelcultures (CBS): *C. albicans* CBS 562, *C. tropicalis* CBS 94 and *C. krusei* CBS 73; and from the IZ collection (Instituto Zimotécnico ESALQ - USP): *C. glabrata* IZ 07.

### 2.2 Determination of minimum inhibitory concentration (MIC)

The MIC was determined using the microdilution technique in Sabouraud's dextrose broth (SDB, KASVI, Curitiba, Brazil) according to the protocol proposed by the Clinical and Laboratory Standards Institute (CLSI) [22]. The antifungal activity of chloramine T trihydrate (CAS 7080-50-4, INLAB, São Paulo, Brazil) was evaluated to an initial concentration of 25,000 µg/mL against the fungal strains of the *Candida genus* aforementioned.

An initial volume of 100 µL of SDB was initially plated in each well of a microtiter plate. A volume of 100 µL of CAT solution was then placed into the first well of each column and serially diluted by withdrawing an aliquot of 100 µL from the most concentrated well and placing it into the next well. At the end of each column, the last aliquot withdrawn was discarded.

A volume of 100 µL of fungal strain inoculum prepared in accordance with CLSI standards [22] in SDB at a final concentration equivalent to $2.5 \times 10^3$ CFU/mL was then added to each well. The plates were incubated for 24 h at 37°C, and then 50 µL of TCT dye (2,3,5-triphenyl tetrazolium chloride), to confirm the presence of viable microorganisms, was added to each plate well and then incubated again for 24 h. For the reading, wells with red staining were considered to contain viable microorganisms. The MIC was taken to be the lowest concentration of the compound that visibly inhibited fungal growth.

The tests were performed in triplicate, and the MIC was calculated according to the results mode. The positive controls used for this test were nystatin (Sigma-Aldrich, São Paulo, Brazil), with an initial concentration of 12.5 µg/mL, and sodium hypochlorite (Sigma-Aldrich, São Paulo, Brazil), initially at 2,500 µg/mL. Feasibility control of the strains, sterility control of the

culture medium and a DMSO control (1%, dimethyl sulfoxide, Sigma-Aldrich, São Paulo, Brazil), which was used for the preparation of the nystatin solution, were performed at the same time as the test.

### 2.3 Determination of minimum fungicidal concentration (MFC)

Based on the results obtained for the MIC, 50 µL of the contents of the wells at the MIC and the two concentrations immediately higher (MIC x 2 and MIC x 4) was seeded in Petri dishes containing Sabouraud's dextrose agar (SDA, KASVI, Curitiba, Brazil). The plates were incubated at 37°C for 24 h. The reading was performed by visual observation of fungal growth in the culture medium. The MFC was taken to be the lowest concentration capable of inhibiting visible growth in the subculture [22].

The MFC/MIC ratio was calculated to determine whether the compound had fungistatic (MFC/MIC ≥ 4) or fungicidal (MFC/MIC < 4) activity [23].

### 2.4 Evaluation of fungal growth kinetics

The study of the effect of each compound on the growth and multiplication of *C. albicans* ATCC 60193 fungal cells was performed using the colony forming units (CFU) counting method [24-25]. The evaluation times set for this test corresponded to T0 (initial), T1 (1 hour after the initiation), T2 (2 h), T4 (4 h), T6 (6 h), T8 (8 h), T12 (12 h) and T24 (24 h after the start of the test).

The test was performed in a 96-well plate using the protocol proposed in the microdilution technique [22] and CAT was added in concentrations of the MIC, MIC x 2 and MIC x 4. Nystatin and sodium hypochlorite were used as positive controls. Growth control of the tested strain and sterility control of the culture medium were performed concurrently.

For the evaluation of fungal growth kinetics, after homogenization, 10 µL of the contents of the wells was seeded at predefined time intervals into Petri dishes containing SDA, and these were incubated at 37°C for 24 h for a subsequent CFU count. After incubation, the count was performed, and the values were transformed into log CFU/mL and presented in the form of a fungal cell death curve.

The data were statistically analyzed for normality (Shapiro-Wilk's test), and the analysis of variance statistical test with Tukey's post-test ($p \leq 0.05$) was applied to compare the compounds and growth effects at each time point to evaluate effective fungal growth inhibition. Statistical analyses were performed using the IBM SPSS Statistical Software (version 20.0).

2.5 Verification of the possible mechanism of action of the compounds on the fungal cell wall - sorbitol test

The microdilution technique [22] was performed in the presence of sorbitol (INLAB, São Paulo, Brazil), an osmotic protector, to verify the possible mechanism of action of CAT on the cell wall of *C. albicans* (ATCC 60193 and CBS 562). The technique used was similar to that performed when determining the MIC; however, the inoculum used was prepared with sorbitol to a final concentration of 0.8 M. The plates were incubated at 37°C, and readings were performed after 24 and 48 h of incubation.

The positive controls used in this test were caspofungin diacetate (Sigma-Aldrich, São Paulo, Brazil), with an initial concentration of 5 µg/mL [26,27], and sodium hypochlorite, initially at 2,500 µg/mL. The MIC in the presence of sorbitol was taken as the lowest concentration of the compound that visibly inhibited fungal growth [25,28,29].

2.6 Verification of the possible mechanism of action of the compounds on the fungal cell wall - ergosterol test

The test in the presence of exogenous ergosterol (Sigma-Aldrich, São Paulo, Brazil) at concentrations of 100, 200 and 400 µg/mL was performed using the microdilution technique [22]. The inocula of the *C. albicans* strains (ATCC 60193 and CBS 562) were prepared separately by means of SDB culture plus ergosterol at the three tested concentrations. Each concentration was tested in triplicate. The plates were incubated at 37°C, and readings were taken after 48 h [29].

The positive controls used in this test were nystatin [30,31] and sodium hypochlorite, with respective initial concentrations of 12.5 and 2,500 µg/mL. A control was also prepared with 96% ethanol and Tween 80, which were used in the preparation of ergosterol solutions. The MIC in the presence of ergosterol was taken as the lowest concentration of the compound that visibly inhibited fungal growth.

2.7 Evaluation of the effect of the compound on fungal micromorphology

Microculture in cornmeal agar (CA) plus Tween 80 [32,33] was used to evaluate possible changes in the fungal micromorphology of *C. albicans* CBS 562 caused by CAT at $CIM_{75\%}$ and higher concentrations.

In the test, 1 mL of CA from the liquid phase containing the compound at the concentration to be tested was placed on a glass slide. After solidification of the medium, two parallel grooves were made with sterile disposable straps containing the tested strain, and a glass coverslip was placed over the medium. This assembly was placed in a moist chamber

formed by sterile Petri dishes and filter paper soaked in sterile distilled water to maintain system moisture and prevent drying of the medium. The system was incubated at 37°C for a period of 48 h.

Evaluation of fungal micromorphology was performed using light microscopy at 40x magnification for observation of the formation of characteristic structures such as blastoconidia, pseudohyphae and chlamydospores. Nystatin and sodium hypochlorite were used as positive controls in the test.

### 2.8 Evaluation of the antifungal activity of CAT on *C. albicans* biofilm

The tests evaluated CAT's anti-biofilm activity at concentrations of the MIC, MIC x 2 and MIC x 4 at three different time points: the initial adherence of fungal cells and the formation and reduction of *C. albicans* mature biofilm. To this end, groups were named according to the time of addition of the compound, contact time with the fungal cells and time for reading of biofilm formation (Table 1).

The tests were performed on *C. albicans* ATCC 60193 monospecies biofilm, and the compound's contact times with the fungal cells were selected to simulate two forms of clinical application: a short contact period of 1 min every 8 h, simulating immersion of the denture into the compound during the time devoted to daily oral hygiene, and prolonged contact for 8 consecutive hours, simulating soaking dentures in the compound overnight.

The tests were performed in triplicate. Sodium hypochlorite was used as a positive control at concentrations of the MIC, MIC x 2 and MIC x 4 in all groups. Sterility and growth controls were performed following the same methodology for each group.

#### 2.8.1 Evaluation of the compound's anti-biofilm activity on initial adherence to fungal cells

In a 96-well flat-bottom microtiter plate, 100 µL of inoculum prepared in SDB and containing 2.5 x $10^5$ CFU/mL was transferred to each well of the plate with the aid of a pipettor. A volume of 100 µL of the compound at concentrations corresponding to the MIC, MIC x 2 and MIC x 4, as determined in advance, was then added to the wells corresponding to group 1. The plate was incubated for 2 h at 37°C, which allowed the yeast to stay adhered to the bottom of the wells.

After the adherence stage and the suspension of the fungal strain with the compound, each well was aspirated, and the plate was washed twice with 200 µL of phosphate buffered saline (PBS) to remove the non-adhered cells. After washing, 100 µL of SDB medium was transferred into each well, and the plate was incubated again at 37°C for a period of 48 h.

**Table 1** Characterization of groups formed to evaluate the anti-biofilm activity of the compound according to the time point of addition of the compound, contact time with the fungal cells and elapsed time for biofilm measurement

| Group | Description |
|---|---|
| Group 1 - Evaluation of the compound's anti-biofilm activity on *initial adherence* to fungal cells | Compound added before initial adherence. Contact time: 2 consecutive hours. Time reading: 48 h. |
| Group 2 - Evaluation of compound's anti-biofilm activity on the *formation* of *C. albicans* mature biofilm | Compound added after initial adherence (2 h). Contact time: 1 min. Time reading: 48 h. |
| Group 3 - Evaluation of compound's anti-biofilm activity on the *formation* of *C. albicans* mature biofilm | Compound added after initial adherence (2 h). Contact time: 8 consecutive hours. Time reading: 48 h. |
| Group 4 - Evaluation of compound's anti-biofilm activity on the *reduction* of *C. albicans* mature biofilm | Compound added after formation of mature biofilm (48 h). Contact time: 3 x 1 min. Time reading: 24 h. |
| Group 5 - Evaluation of compound's anti-biofilm activity on the *reduction* of *C. albicans* mature biofilm | Compound added after formation of mature biofilm (48 h). Contact time: 6 x 1 min. Time reading: 48 h. |
| Group 6 - Evaluation of compound's anti-biofilm activity on the *reduction* of *C. albicans* mature biofilm | Compound added after formation of mature biofilm (48 h). Contact time: 8 h. Time reading: 24 h. |
| Group 7 - Evaluation of compound's anti-biofilm activity on the *reduction* of *C. albicans* mature biofilm | Compound added after formation of mature biofilm (48 h). Contact time: 2 x 8 h. Time reading: 48 h. |

To perform the reading and quantification of the formed biofilm, after incubation, the wells were washed twice with 200 µL of PBS and air dried for 45 min. A volume of 100 µL of 0.4% crystal violet aqueous solution was added to each well and remained in contact with the biofilm for 45 min. After incorporation of the dye, the wells were washed three times with 200 µL of sterile distilled water and immediately decolorized with 200 µL of 95% ethanol. Forty-five minutes after this last procedure, 100 µL of the decolorized solution was transferred to a well in a new plate, and the amount of crystal violet was measured at 600 nm in an absorbance reader (GloMax-Multi, PROMEGA) [34].

Due to the absence of criteria in the literature for calculating biofilm inhibition, absorbance values obtained in the wells were used to calculate the percentage inhibition (% inhibition) of biofilm considering the yeast growth group as with 100% of biofilm formation. These values were categorized into scores for further statistical analysis: a score of 1 reflected

% inhibition ≤ 25%, a score of 2 indicated 25% < % inhibition ≤ 50%, a score of 3 indicated 50% < % inhibition ≤ 75%, and a score of 4 indicated 75% < % inhibition ≤ 100%.

The data were statistically analyzed using the Mann-Whitney statistical test ($p \leq 0.05$), and the scores for each compound in a given group were compared for each concentration. The Kruskal-Wallis statistical test ($p \leq 0.05$) was performed for comparisons between concentrations of the same compound in each group.

2.8.2 Evaluation of compound's anti-biofilm activity on the formation of *C. albicans* mature biofilm

For initial adherence of the fungal cells, 100 µL of inoculum prepared in SDB and containing 2.5 x $10^5$ CFU/mL was transferred to each well of a 96-well flat-bottom microtiter plate with the aid of a pipettor. The plate was then incubated for 2 h at 37°C in an incubator.

After the adherence stage, the fungal strain suspension was aspirated from each well, and the plate was washed twice with 200 µL of PBS to remove the non-adhered cells. After washing, 100 µL of SDB medium was transferred into each well. A total of 100 µL of each compound, at concentrations corresponding to the MIC, MIC x 2 and MIC x 4, was then added to the group 2 and 3 wells.

For group 2 wells, compounds remained in the wells for 1 min. The wells were then washed again twice with 200 µL of PBS, and finally, 100 µL of SDB was added to each well, and the plate was incubated for 48 h at 37°C for later reading.

For group 3 wells, the compounds remained in the wells for 8 consecutive hours. The wells were then again washed twice with 200 µL of PBS, and finally, 100 µL of SDB was added to each well, and the plate was incubated for 48 h at 37°C for later reading.

After incubation, the steps for reading and quantification of the formed biofilm were followed as described in the previous section. Percentage inhibition was calculated based on the values of the experimental groups and the growth control values and categorized and analyzed as described above.

2. 8. 3 Evaluation of the compound's anti-biofilm activity on the reduction of *C. albicans* mature biofilm

For this test, the initial fungal cell adherence procedures were the same as described in the previous section.

After the adherence stage, the fungal strain suspension was aspirated from each well, and the plate was washed twice with 200 µL of PBS to remove the non-adhered cells. After washing, 100 µL of SDB medium was transferred into each well. The plates were then incubated for 48 h in an incubator at 37°C for formation of the mature biofilm.

After 48 h from the start of mature biofilm formation, the biofilm reduction test was performed with the addition of the compound at concentrations of the MIC, MIC x 2 and MIC x 4. Initially, the culture medium was aspirated from the wells to remove planktonic cells. The wells were then washed twice in 200 µL of PBS. After washing, 100 µL of SDB medium was transferred into each well.

A volume of 100 µL of the compound at the tested concentrations was then added to the wells corresponding to groups 4, 5, 6 and 7.

For groups 4 and 5, the compounds remained in the wells for 1 min. The wells were then again washed twice with 200 µL of PBS, and finally, 100 µL of SDB was added to each well, and the plate was incubated at 37°C. The compounds were added to the wells every 8 h according to the aforementioned procedure.

For group 4, the reading was taken 24 h after the first addition of the compound, totaling 3 applications of 1 min. For group 5, the reading was taken after 48 h, and there were 6 applications in total.

For groups 6 and 7, the compound remained in the wells for 8 consecutive hours. After this period, the wells were washed twice with 200 µL of PBS, and finally, 100 µL of SDB was added to each well, and the plate was returned to the incubator.

For group 6, the reading was performed 24 h after the addition of the compound, representing a total contact time of the test compounds with the yeast of 8 h.

For Group 7, 100 µL of the compound was again placed in the wells 24 h after the start of the experiment and remained in contact with the biofilm for 8 h, following the same washing procedure and addition of the culture medium after completion of the contact time. The plate was returned to the incubator until 48 h from the start of the test period, and the reading was performed after 16 h of exposure of the biofilm to the compound for two alternating periods.

The controls and biofilm quantification procedures were performed in the same manner as described for the aforementioned test.

## 2.9 Evaluation of cytotoxicity through the hemolysis method

Peripheral blood samples were collected by venipuncture into heparinized tubes from five healthy individuals aged between 18 and 40 years, without hemoglobin or erythrocyte disorders and with no history of these diseases among first-degree relatives. Following total blood collection, samples were centrifuged at 1200-1500 rpm for 15 minutes, and plasma, platelets and white blood cells were separated [35]. The erythrocytes were washed three times in 15 ml of phosphate buffered saline (PBS) and then diluted to a 2% solution in PBS. Initially, 50 µl of different concentrations of CAT (ranging from 25,000 to 781.25 µg/ml based on its previously determined $MIC_{75\%}$) were dispensed into the wells of a 96-well U-bottom microdilution plate. Then 50 µl of the red blood cells suspension were added to the wells at a ratio of 1:1 in relation to CAT. One hour after incubation with the red blood cells, 70 µl of the supernatant from

each well were transferred to a 96-well flat-bottom microdilution plate to measure the hemolytic activity based on absorbance (Multi-GloMax) using a 560 nm wavelength filter. The assay was performed in duplicate, and the positive and negative controls consisted of distilled water and saline solution, respectively [36]. Sodium hypochlorite at 0.5% was also tested as a positive control. The percent of hemolysis was calculated using the formula: % hemolysis = (AS - AN / AP - AN) x 100 where, AS, AN, and AP correspond to the absorbance of the test substance, negative control and positive control, respectively. The research proposal has been reviewed and approved by the human research ethics committee from the Center of Health Sciences of the Federal University of Paraiba (CAAE: 43914615.0.0000.5188).

## 3 Results

### 3.1 Determination of the MIC and MFC

Table 2 shows the data describing the antifungal activity of CAT, sodium hypochlorite and nystatin on the *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* strains tested. The MIC values found varied between 195.3 µg/mL for *C. albicans* CBS 562 and 1,562.5 µg/mL for *C. tropicalis* CBS 94 and *C. glabrata* IZ 07. The concentration of 781.3 µg/mL can be defined as the concentration able to inhibit 75% of the tested strains ($MIC_{75\%}$). For the MFC, the concentrations ranged between 390.6 and 3,125 µg/mL, which were observed for the two tested species *C. krusei* and *C. glabrata,* respectively.

The solvent (DMSO) that was used in the preparation of the nystatin solution showed no antifungal activity. The MFC/MIC ratio indicates the fungicidal activity for all concentrations of the compounds, except for the activity of CAT against *C. albicans* CBS 562, which was considered fungistatic.

**Table 2** Antifungal activity of chloramine T (CAT) and the positive controls sodium hypochlorite (NaOCl) and nystatin on the *Candida* genus strains

| Strain | Compound | MIC (µg/mL) | MFC (µg/mL) | MFC/MIC | Antifungal activity |
|---|---|---|---|---|---|
| *Candida albicans* ATCC 60193 | CAT | 781.3 | 1,562.5 | 2 | Fungicidal |
| | NaOCl | 625.0 | 625.0 | 1 | Fungicidal |
| | Nystatin | 0.391 | 0.781 | 2 | Fungicidal |
| *Candida albicans* CBS 562 | CAT | 195.3 | 781.3 | 4 | Fungistatic |
| | NaOCl | 312.5 | 625.0 | 2 | Fungicidal |
| | Nystatin | 0.391 | 0.781 | 2 | Fungicidal |
| *Candida tropicalis* ATCC 750 | CAT | 781.3 | 781.3 | 1 | Fungicidal |
| | NaOCl | 625.0 | 625.0 | 1 | Fungicidal |
| | Nystatin | 0.391 | 0.391 | 1 | Fungicidal |
| *Candida tropicalis* CBS 94 | CAT | 1,562.5 | 1,562.5 | 1 | Fungicidal |
| | NaOCl | 1,250.0 | 1,250.0 | 1 | Fungicidal |
| | Nystatin | 0.391 | 0.391 | 1 | Fungicidal |
| *Candida krusei* ATCC 3413 | CAT | 390.6 | 390.6 | 1 | Fungicidal |
| | NaOCl | 312.5 | 312.5 | 1 | Fungicidal |
| | Nystatin | 1.563 | 1.563 | 1 | Fungicidal |
| *Candida krusei* CBS 73 | CAT | 390.6 | 390.6 | 1 | Fungicidal |
| | NaOCl | 312.5 | 312.5 | 1 | Fungicidal |
| | Nystatin | 0.781 | 0.781 | 1 | Fungicidal |
| *Candida glabrata* IZ 07 | CAT | 1,562.5 | 3,125.0 | 2 | Fungicidal |
| | NaOCl | 1,250.0 | 1,250.0 | 1 | Fungicidal |
| | Nystatin | 0.391 | 0.781 | 1 | Fungicidal |

### 3.2 *C. albicans* growth kinetics

The kinetics test identified differences in the behavior of *C. albicans* from the first contact with the compounds. At the initial time (T0) at the MIC concentration (Fig 1), CAT was effective at inhibiting fungal cells, with a greater inhibition than nystatin ($p \leq 0.05$). At a concentration of MIC x 2 (Fig 2), all compounds significantly reduced the number of CFU/mL,

with no statistical difference between them ($p > 0.05$). At the MIC x 4 concentration (Fig 3), all compounds were effective at reducing the number of CFU/mL, but CAT and sodium hypochlorite showed greater inhibition than nystatin ($p \leq 0.05$).

After 1 h, there was no inhibition by the compounds at the MIC concentration, and after 2 h of contact, no compound at any tested concentration was able to significantly inhibit fungal cells. In the period between 4 and 8 h of contact of the compounds with fungal cells, all compounds were equally capable of reducing fungal growth ($p \leq 0.05$) at all tested concentrations. At the time corresponding to 12 h, CAT at the MIC concentration was less effective than nystatin and sodium hypochlorite, although CAT did significantly inhibit fungal growth. After 24 h, none of the compounds at the MIC concentrations was able to inhibit fungal cell growth.

The CAT concentrations of MIC x 2 and MIC x 4 caused effective inhibition at all studied times ($p \leq 0.05$) except after 2 h.

Figures 01, 02 and 03 present fungal behavior in response to the action of CAT, nystatin and sodium hypochlorite at MIC, MIC x 2 and MIC x 4 concentrations, respectively.

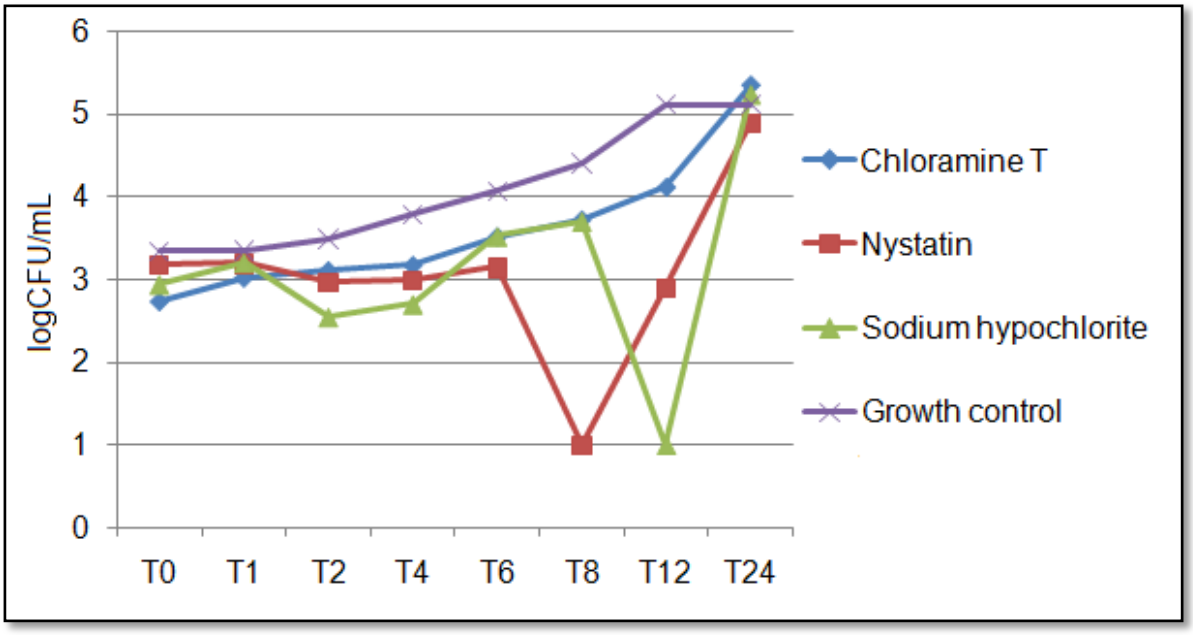


**Fig 1** *Candida albicans* ATCC 60193 fungal growth kinetics for chloramine T, sodium hypochlorite and nystatin at the MIC concentrations (chloramine T: 781.3 µg/mL; nystatin: 0.39 µg/mL; sodium hypochlorite: 625 µg/mL)

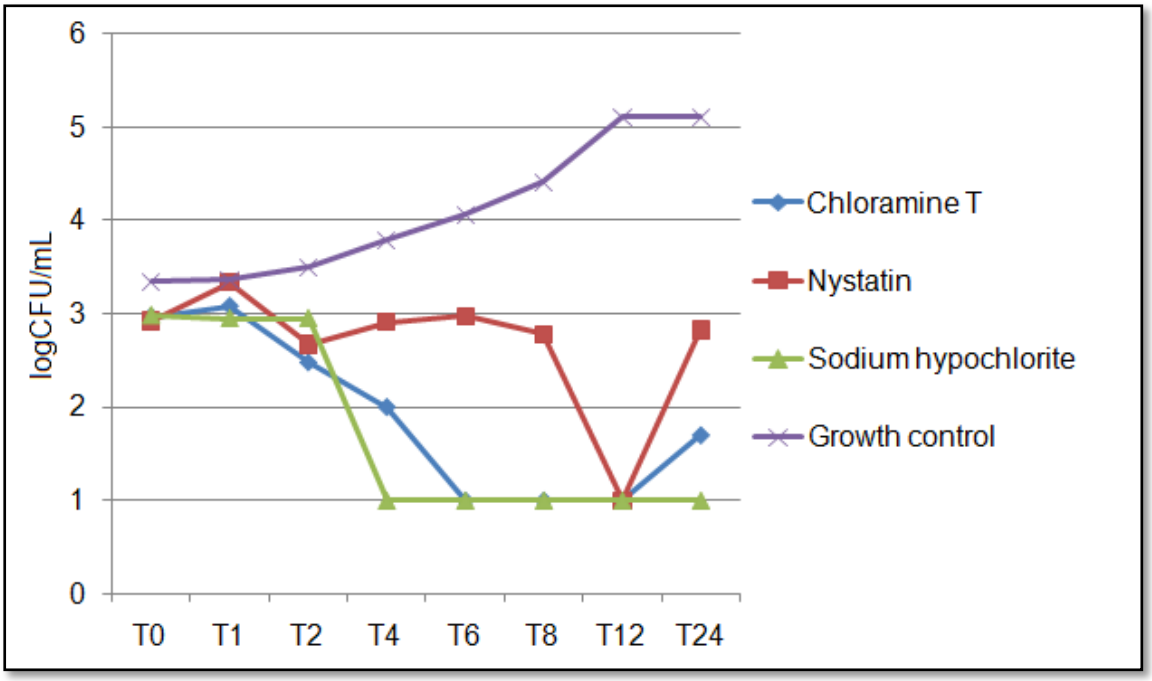


**Fig 2** *Candida albicans* ATCC 60193 fungal growth kinetics for chloramine T, sodium hypochlorite and nystatin at MIC x 2 concentrations (chloramine T: 1,562.2 µg/mL; nystatin: 0.78 µg/mL; sodium hypochlorite: 1,250 µg/mL)

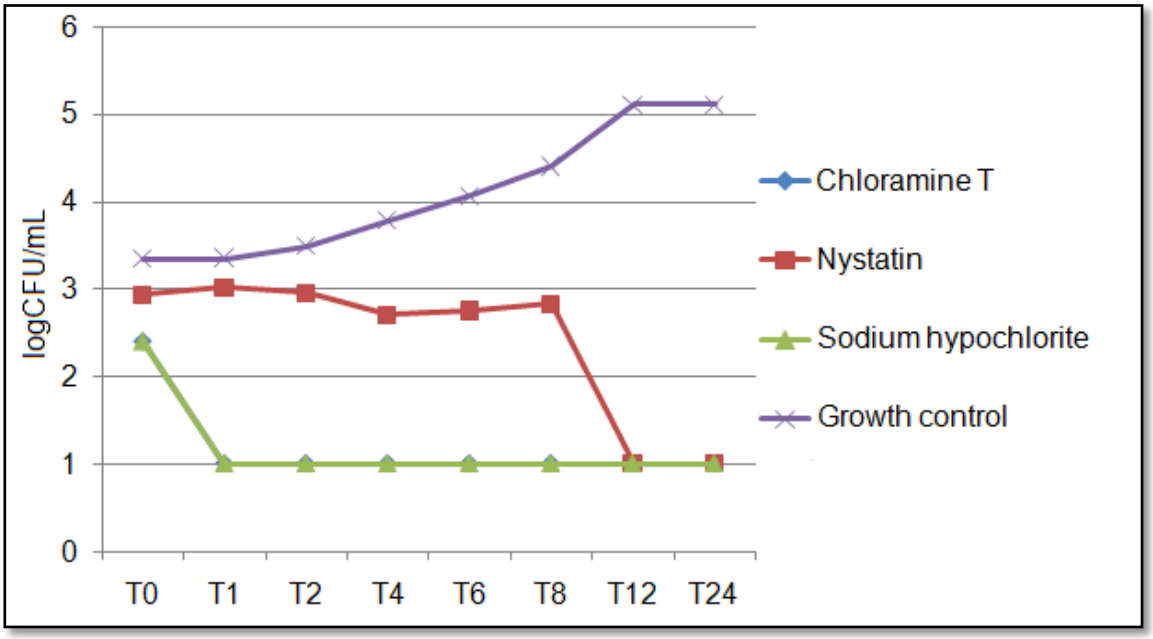


**Fig 3** *Candida albicans* ATCC 60193 fungal growth kinetics for chloramine T, sodium hypochlorite and nystatin at MIC x 4 concentrations (chloramine T: 3,125 µg/mL; nystatin: 1.56 µg/mL; sodium hypochlorite: 2,500 µg/mL)

3.3 CAT mechanism of action

The presence of sorbitol and exogenous ergosterol resulted in an increase in the MIC of CAT on the *C. albicans* strains studied. This result indicates that CAT likely acts on both the fungal cell wall and the cell membrane. The same is presumable to sodium hypochlorite, which also showed an increase of CIM in the presence of sorbitol and ergosterol. Table 3 shows the results obtained in the presence of sorbitol, and Figure 04 illustrates the behavior of the compounds in the presence of exogenous ergosterol at different concentrations.

**Table 3** Effect of chloramine T (CAT), sodium hypochlorite (NaOCl) and caspofungin on the fungal cell wall of *Candida albicans* in the presence and absence of 0.8 M sorbitol

| Strain | Compound | MIC (µg/mL) without sorbitol | MIC (µg/mL) with sorbitol |
|---|---|---|---|
| *Candida albicans* ATCC 60193 | CAT | 781.3 | 6,250.0 |
| | NaOCl | 625.0 | 2,500.0 |
| | Caspofungin | < 0.039 | > 5 |
| *Candida albicans* CBS 562 | CAT | 195.3 | 3,125 |
| | NaOCl | 312.5 | 2,500.0 |
| | Caspofungin | < 0.039 | 5.0 |

3.4 Effect of CAT on *C. albicans* micromorphology

Microscopic evaluation of *C. albicans CBS 562* microculture identified the presence of abundant pseudohyphae, blastoconidia and chlamydospores. In the presence of CAT and nystatin, there was a reduction in the filamentous form that was directly proportional to the increase in concentration. For sodium hypochlorite, concentrations of 1,250 and 2,500 µg/mL caused total inhibition of the formation of pseudohyphae and chlamydospores, with only blastoconidia present. Figure 05 shows images, which were obtained by light microscopy at 40x magnification, of the fungal strain microculture in the presence of the tested compounds and a growth control.

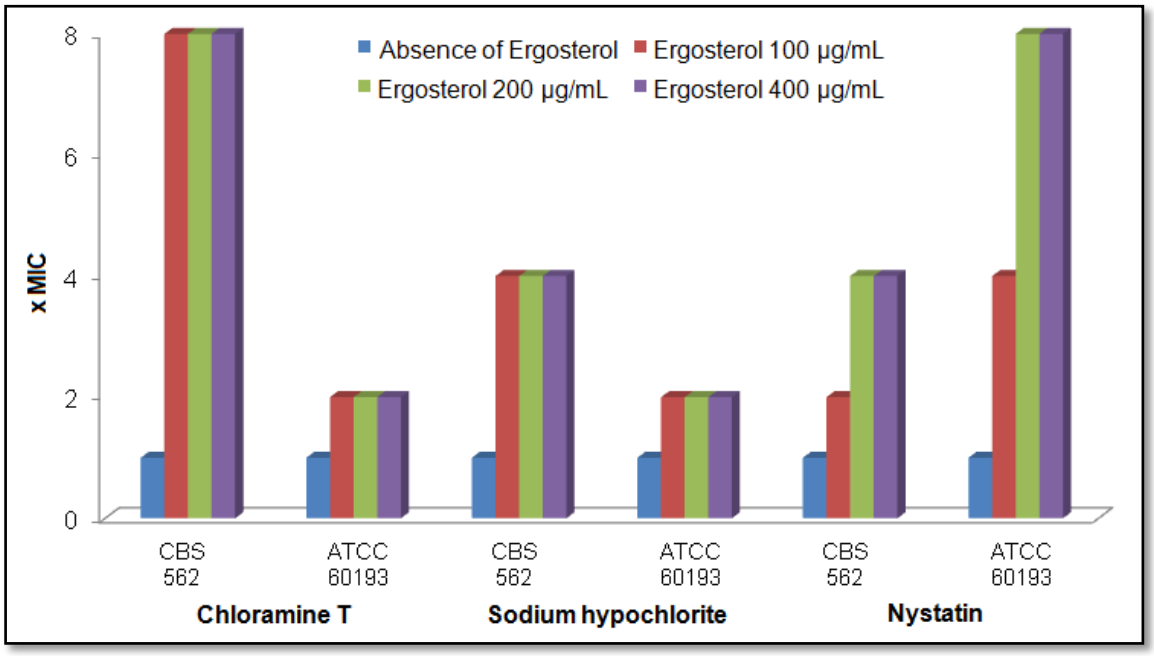


**Fig 4** Effect of chloramine T, sodium hypochlorite and nystatin on *Candida albicans* (ATCC 60193 and CBS 562) fungal cell membrane in the absence and presence of exogenous ergosterol at concentrations of 100, 200 and 400 µg/mL. Values (x MIC) are presented as a function of the MIC of each compound in the absence of exogenous ergosterol

3.5 Effect of CAT on the adherence, formation and reduction of *C. albicans* mature biofilm

In the anti-biofilm activity tests, sodium hypochlorite was better at reducing the C. *albicans* adherence at MIC x 2 ($p \leq 0.05$), but CAT demonstrated activity similar ($p > 0.05$) to that of sodium hypochlorite at all concentrations for the groups evaluating the inhibition of the formation of C. *albicans* biofilm (Table 04). However, CAT was more effective ($p \leq 0.05$) at reducing the *C. albicans* mature biofilm than sodium hypochlorite at MIC x 2 in group 4 and at MIC x 4 in group 5 (Table 05). In the groups with long contact times of the compound with mature biofilm (8 h), there was no difference between CAT and sodium hypochlorite at the concentrations tested. There was also no statistically significant difference between the concentrations of each compound in the groups studied.

Tables 04 and 05 show the inhibition categories (% inhibition) of the compounds for the different groups.

3.6 Cytotoxicity of CAT on human erythrocytes

All tested concentrations of CAT in the range 25,000 to 762.25 µg/ml demonstrated hemolytic activity between 61 and 67.7%. Hypochlorite, used as a control, caused complete destruction of the erythrocytes immediately after being added to the wells, which was considered as parameter of 100% hemolytic activity. Saline (0.9% NaCl), used as a negative control, did not cause hemolysis as expected.

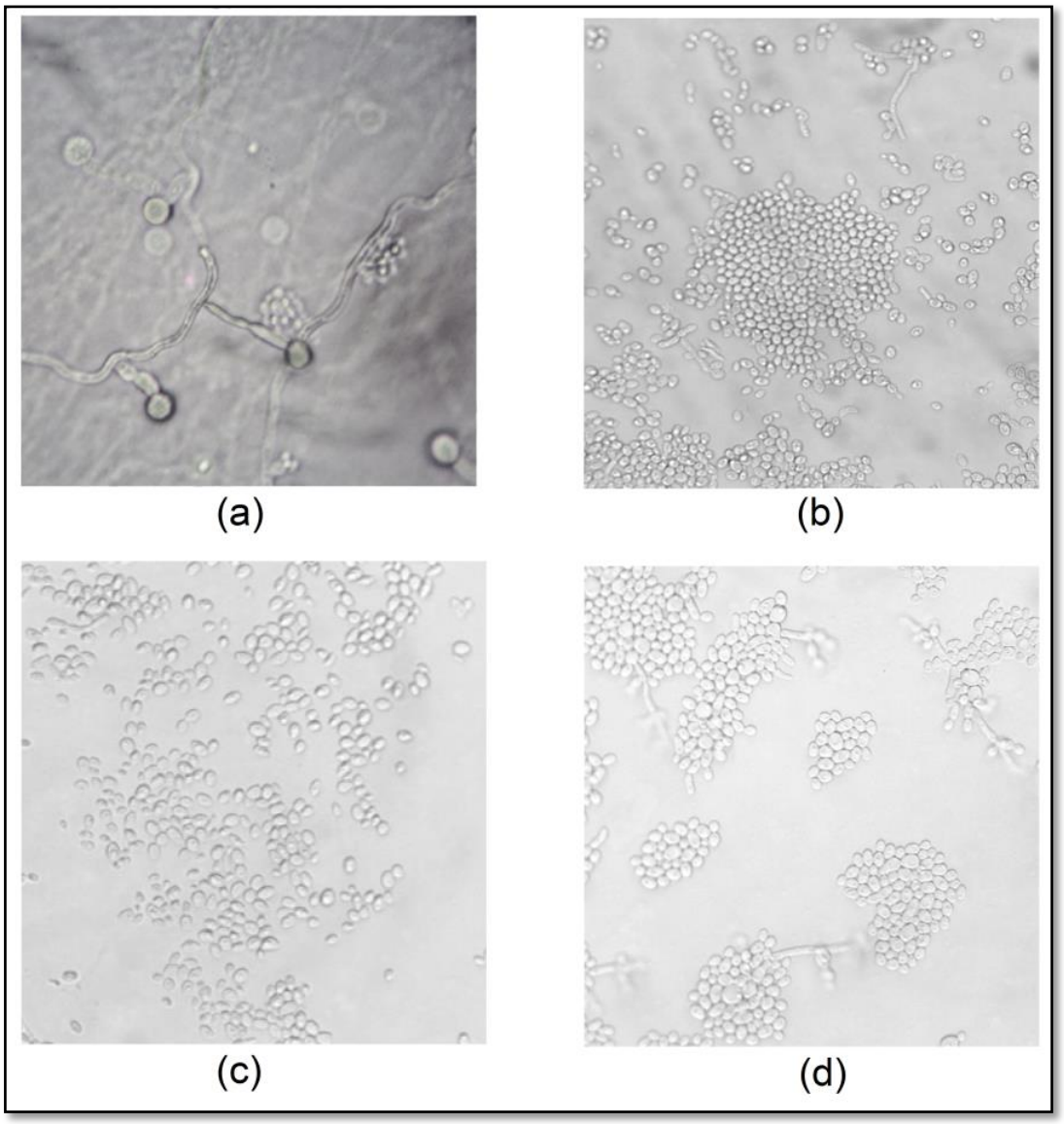


**Fig 5** *Candida albicans* (CBS 562) micromorphology (40x magnification) in the absence (growth control) (a) and presence of chloramine T (b), sodium hypochlorite (c) and nystatin (d) at concentration MIC x 2.

**Table 4** Categories of percentage values for inhibition of initial fungal cell adherence and the formation of *Candida albicans* ATCC 60193 mature biofilm in the groups, as characterized by different contact times, after 48 h of incubation

| | Inhibition of initial adherence | | Inhibition of mature biofilm formation | | | |
|---|---|---|---|---|---|---|
| | Group 1 (Contact time: 2 h) | | Group 2 (Contact time: 1 min) | | Group 3 (Contact time: 8 h) | |
| Concentration | Chloramine T | Sodium hypochlorite | Chloramine T | Sodium hypochlorite | Chloramine T | Sodium hypochlorite |
| MIC | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | % inhibition ≤ 25%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ |
| MIC x 2 | % inhibition ≤ 25%$^{Aa}$ | 50% < % inhibition ≤ 75%$^{Ba}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ |
| MIC x 4 | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ |

Different uppercase letters on the lines represent a significant difference between the compounds in each group. Equal lowercase letters in each column represent similarity between the concentrations of each compound.

**Table 5** Categories of percentage values for the reduction of *Candida albicans* ATCC 60193 mature biofilm in the groups, as characterized by different contact times.

| | Group 4 (Contact time: 3 x 1 min) | | Group 5 (Contact time: 6 x 1 min) | | Group 6 (Contact time: 8 h) | | Group 7 (Contact time: 2 x 8 h). | |
|---|---|---|---|---|---|---|---|---|
| Concentration | Chloramine T | Sodium hypochlorite | Chloramine T | Sodium hypochlorite | Chloramine T | Sodium hypochlorite | Chloramine T | Sodium hypochlorite |
| MIC | 50% < % inhibition ≤ 75%$^{Aa}$ | % inhibition ≤ 25%$^{Aa}$ | % inhibition ≤ 25%$^{Aa}$ | % inhibition ≤ 25%$^{Aa}$ | 50% < % inhibition ≤ 75%$^{Aa}$ | 50% < % inhibition ≤ 75%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ |
| MIC x 2 | 50% < % inhibition ≤ 75%$^{Aa}$ | % inhibition ≤ 25% $^{Ba}$ | % inhibition ≤ 25%$^{Aa}$ | % inhibition ≤ 25%$^{Aa}$ | 50% < % inhibition ≤ 75%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ |
| MIC x 4 | 50% < % inhibition ≤ 75%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | % inhibition ≤ 25%$^{Ba}$ | 50% < % inhibition ≤ 75%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ | 25% < % inhibition ≤ 50%$^{Aa}$ |

Different uppercase letters on the lines represent a significant difference between the compounds in each group. Equal lowercase letters in each column represent similarity between the concentrations of each compound.

## 4. Discussion

Although CAT has been studied for many years and it has been approached in several ways, there is still a lack of studies regarding its use for dentistry and directed toward its antimicrobial activity and cytotoxicity [20,21]. This is the first study to evaluate the antifungal activity of CAT on *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* and the effect on *C. albicans* growth kinetics and micromorphology. In addition, the mechanism of action and anti-biofilm activity of CAT were ascertained.

The $MIC_{75\%}$ of CAT for the tested strains was 781.3 µg/mL standing out the value 195.3 µg/mL found for the strain of *C. albicans* CBS 562, and the MFC/MIC ratio reflects the fungicidal activity of the compound for most of the strains, except for *C. albicans* CBS 562. The variation in MIC can be explained by the different genetic profile of the strains and possible acquired resistance mechanisms. Regarding the difference between fungistatic and fungicidal activity, the literature cites this as concentration dependent in most cases, with the same compound able to act in both manners [25,37].

There were not found in the literature studies to compare the MIC results front of genus *Candida* strains. However, a study carried out to assess the fungicidal activity of CAT at a concentration of 100 µg/ml on a clinical strain of *Aspergillus fumigatus*, did not find positive results for the substance [38]. This result does not mean that CAT has no action on the tested strain and cannot be compared to those obtained in the present study due to the methodological difference to determine the antifungal activity and the discrepancy between the concentrations tested, whereas 100 µg/mL is below the MIC values obtained for the strains of *Candida* spp.

When tested against bacterial strains (*Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter cloacae*, *Staphylococcus epidermidis*, and *Serratia marcescens*), the literature shows CAT MIC values ranging between 500 and 2.000 µg/mL, values found approximate to this study [39].

A previous study of *C. albicans* death kinetics in response to CAT noted that a concentration of 1,000 µg/mL caused a 2 $\log_{10}$ reduction in CFU/mL after 30 min of contact, and a concentration of 100 µg/mL achieved this reduction in 1 h [13]. In the present study, the lowest concentration tested, 781.3 µg/mL, was able to significantly reduce ($p \leq 0.05$) the number of fungal cells immediately after addition of CAT (T0), but this concentration was not able to reduce the $\log_{10}$ level over time. The concentration of 1,562.5 µg/mL (MIC x 2) decreased the CFU/mL after 6 h of contact, and MIC x 4 (3.125 µg/mL) resulted in complete removal of fungal cells after 1 h.

Some chemical characteristics of CAT, such as its efficient chlorination and oxidation of microorganisms [40] and its high stability and ability to maintain chlorine levels for a prolonged time [15], explain, respectively, the rapid antifungal effect at T0 and the prolonged action of CAT that caused significant inhibition of fungal growth up to 12 h at all concentrations tested.

According to the literature, active chlorine compounds act primarily through the chlorination of the extracellular protein matrix of microorganisms, forming a chlorine layer.

However, a moderate chlorine layer is not able to decrease cell viability, and therefore, penetration of the compound into the cell is important, as it is followed by destruction of vital components by oxidation [40].

Studies claim that penetration ability is related to molecular size and polarity. However, it was found that although CAT is considerably larger than other active chlorine compounds and it presents as an anion [13], CAT has highly reactive compounds and a high capacity for chlorine layer formation that has an immediate destructive impact on the microbial surface [40]. The results of the present study are supported by these findings because the MIC of CAT increased in the presence of both sorbitol and exogenous ergosterol, suggesting a simultaneous action on the cell membrane and cell wall.

The study of *C. albicans* micromorphology in the presence of antifungal agents is important because fungal form is one of several factors that affect its virulence and pathogenicity. The formation of pseudohyphae and hyphae causes tissue invasion by enabling the cell to exert a mechanical force favorable for tissue penetration. Chlamydospores are considered to be resistance structures [41,42]. The CA culture medium used for *C. albicans* microculture in the present study facilitates the expression of virulence forms and allows us to evaluate possible changes caused by the presence of the compounds.

At all concentrations tested, CAT reduced the formation of pseudohyphae and led to an absence of chlamydospores. Sodium hypochlorite at concentrations of 1,250 and 2,500 µg/mL completely inhibited the presence of filaments and chlamydospores. This result is important because it complements the others presented in the present study and because it may be inferred that CAT, at the concentrations tested, promotes the general maintenance of fungal cells in the form of blastoconidia, which are considered less virulent.

Taking into account the adherence and penetration capacity of *C. albicans* on the acrylic resin of dentures and the fact that hyphae lead to the formation of a thicker biofilm more resistant to removal [43], the results presented here are even more important for the indication of CAT as a solution for denture disinfection, underscoring the potential of this compound in this area.

Whereas biofilms are the most common organization of microorganisms, as they provide greater protection and resistance, testing on *C. albicans* biofilm is essential to corroborate the information obtained in terms of planktonic cells and to approximate the real world as much as possible. In denture stomatitis, the biofilm formed on the denture merits attention because disease can recur after treatment of the mucosa when there is maintenance of the biofilm adhering to the material [43,45].

In the present study, it was decided to conduct tests on a *C. albicans* mono-species biofilm because it is the most prevalent in clinical isolates of denture stomatitis [3,5,6]. Due to the absence in the literature of a protocol for denture disinfection, the groups were based on an attempt to simulate two ways of using the compounds: applied for 1 min, 3 times a day, at the time dedicated to oral hygiene or contact for 8 consecutive hours, simulating overnight disinfection. In view of the lack of a protocol for categorizing the values of biofilm formation inhibition, a particular

methodology was suggested, which can be used in future studies for the purpose of standardization for presentation and analysis of this type of data.

The results obtained showed that CAT has similar anti-biofilm activity to sodium hypochlorite regarding inhibition of the initial adherence of the fungal cells and the formation of mature biofilm. The tested concentrations did not differ significantly. In the groups testing its ability to reduce *C. albicans* mature biofilm, CAT was more effective than the hypochlorite at the MIC x 2 concentration in group 4, simulating 3 x 1 min contacts over 24 h, and at MIC x 4 concentration for groups 5 and 6, simulating 6 x 1 min contacts over 48 h. In both cases, CAT decreased the biofilm by 50 to 75%. In the groups with contact for 8 consecutive hours, there were no significant differences between the compounds.

The results of Panzeri et al. [20] differ from those found in the present study because in their study, a CAT-based dentifrice at a concentration of 10,000 µg/mL was not able to significantly reduce the number of CFU/mL of *C. albicans* present in denture biofilms after 2 min of daily brushing for 21 days. In the same way, a clinical trial with dentifrices containing the CAT 2.000 and 10,000 µg/mL [21], used three times daily for 7 days, found that they were not able to significantly remove the biofilm of dental prosthesis. The differences in the formulation and the dosage may have affected the activity of the active compound and may thus explain the difference between these results.

Substances indicated for denture disinfection can be harmful and thus should not be in contact with the patient's mucosal tissues, that is why it is recommended to thoroughly wash the denture after disinfection. However, it is critical to study the potential cytotoxic effects of a given substance before indicating it for clinical use. With this purpose, we evaluated the cytotoxicity of CAT using the hemolysis method, which can be considered a preliminary, although meaningful, assay to evaluate the characteristics of test substances.

All CAT concentrations tested, either higher or lower than its MIC, showed hemolytic activity between 61 and 67.7%, suggesting that such effect is not concentration dependent. Sodium hypochlorite caused complete hemolysis immediately after contact with the erythrocytes, confirming its high cytotoxicity. According to the literature [46], CAT did not cause visible cellular effects on a human cell line of squamous cell carcinoma at concentrations between 1 and 10 µg/ml after 30 min exposure. At higher concentrations (100, 1,000 and 10,000 µg/ml), it led to changes in cell morphology after 30 min and complete fragmentation after 24 h.

The present work represents an initial study of the antifungal activity of CAT on species involved in oral candidiasis associated with dentures. Given the scarcity of studies on CAT in dentistry and the methodological differences that make it difficult to compare data, this is a breakthrough in the study of CAT. The results of the tests performed provide important knowledge about the inhibition of fungal growth kinetics, the mode of action of CAT, changes in fungal micromorphology and anti-biofilm activity. It is important to emphasize that chloramine T showed a better antibiofilm activity in short contact times (1 min) than sodium hypochlorite, which is one of the widely used substances to disinfect dentures.

The results demonstrate the good potential for the use of CAT for denture disinfection. It is suggested, therefore, that further tests be performed toward the evaluation of cytotoxicity, the verification of anti-biofilm activity using a multi-species model and the investigation of the effect of CAT on the properties of denture components.

## 5 Conclusion

CAT offers antifungal activity against *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* and has a fungicidal action on most of the tested strains; It is able to significantly reduce the growth of *C. albicans* immediately after initial contact and has prolonged inhibitory activity, likely acting on the cell wall and cell membrane simultaneously. CAT changes *C. albicans* micromorphology, causing reductions in the filamentous form and the inhibition of chlamydospores and also has *C. albicans* anti-biofilm activity and is effective at inhibiting the initial adherence of fungal cells and biofilm formation and at reducing mature biofilm. It has high hemolytic activity, however lower than that of sodium hypochlorite.

## Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The research proposal has been reviewed and approved by the human research ethics committee from the Center of Health Sciences of the Federal University of Paraiba (CAAE: 43914615.0.0000.5188).

## Conflict of Interest

The authors declare that they have no conflict of interest.

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## 3. CONSIDERAÇÕES GERAIS

Este trabalho representa um avanço no estudo da CAT, que é utilizada há muito tempo devido a suas propriedades antissépitcas (DAKIN; COHEN; KENYON, 1916; SWEET; MACYNSC, 1985; NAGL et al., 2003) e apresenta um potencial para uso na odontologia. Tem caráter inovador, pois foi avaliada a atividade antifúngica da CAT frente cepas de *Candida* de importância clínica na área, especialmente para a candidose bucal, associada ou não ao uso de prótese, nomeadamente: *C. albicans*, *C. tropicalis*, *C. krusei* e *C. glabrata.* Adicionalmente, foi avaliada a cinética de inibição do crescimento e possíveis alterações na micromorfologia de *C. albicans*, provável mecanismo de ação da substância e atividade antibiofilme.

Nos testes para determinação da CIM, os valores de CAT variaram entre 195,3 e 1562,5 µg/mL correspondentes a *C. albicans* CBS 562, apresentando o menor valor, *C. tropicalis* CBS 94 e *C. glabrata* IZ 07, responsáveis pelo maior valor. Entretanto, o valor de $CIM_{75\%}$ da CAT foi calculado em 781,3 µg/mL. A relação CFM/CIM reflete uma atividade fungicida da substância para a maioria das cepas, exceto para *C. albicans* CBS 562. As diferenças encontradas são esperadas devido à variedade das espécies estudadas e a diferença de origens, assim como a possibilidade de desenvolvimento prévio de mecanismos de resistência.

Não foram encontrados na literatura estudos para comparação dos resultados de CIM da CAT frente a cepas do gênero *Candida*. No entanto, os valores encontrados neste estudo aproximam-se da faixa encontrada para bactérias, em estudo realizado por outros autores (FUURSTED; HJORT; KNUDSEN, 1997), que apresentaram valores entre 500 e 2.000 µg/mL para 9 espécies bacterianas diferentes. A falta de estudos na literatura utilizando cepas fúngicas de interesse para a odontologia justifica a realização deste estudo e o avanço na investigação da CAT.

No teste para avaliação da cinética de inibição do crescimento de *C. albicans*, a CAT mostrou redução imediata (T0) significativa ($p \leq 0,05$) da quantidade de células fúngicas viáveis, em todas as concentrações testadas, além de efeito prolongado, provocando inibição ($p \leq 0,05$) do crescimento até o

tempo de 24 h, nas concentrações de 1.562,5 e 3.125 µg/mL, e até 12 h, na menor concentração testada, correspondente a 781,3 µg/mL. Estes resultados corroboram achados anteriores (ARNITZ; NAGL; GOTTARDI, 2009), onde a CAT a uma concentração de 1.000 µg/mL provocou redução de 2 $\log_{10}$ de UFC/mL após 30 min de contato, e uma concentração de 100 µg/mL alcançou esta redução em 1h.

A ação imediata e prolongada da CAT pode ser explicada, respectivamente, pela eficiente cloração e oxidação dos micro-organismos (GOTTARDI; NAGL, 2005) e pela grande estabilidade da molécula e capacidade de manutenção dos níveis de cloro por um maior tempo (AUSTIN; TAYLOR, 1918), citadas na literatura.

A literatura cita, ainda, que os compostos de cloro ativo atuam através da cloração da matriz proteica externa do micro-organismo, formando uma camada de cloro, seguida da penetração da substância no interior da célula, que leva a destruição de componentes vitais por oxidação. Foi verificado que a CAT possui compostos altamente reativos e grande capacidade de formação da camada de cloro que exerce um imediato impacto destrutivo na superfície microbiana (GOTTARDI; NAGL, 2005), o que suporta os achados deste estudo, visto que a CIM da CAT aumentou na presença tanto de sorbitol quanto de ergosterol exógeno, sugerindo a ação simultânea em membrana e parede celulares.

Uma das limitações dos estudos *in vitro* sobre células planctônicas é o fato de que as cepas encontram-se, majoritariamente, na forma de blastoconídios, considerada menos virulenta (TORTORANO et al., 2005). O estudo das possíveis alterações da micromorfologia fúngica através do microcultivo de *C. albicans* em ágar fubá acrescido de tween 80, que favorece a expressão das formas de virulência, como pseudo-hifas e clamidoconídios, torna-se importante para avaliar o comportamento fúngico na presença da substância, pois a forma fúngica é um dos diversos fatores relacionados a sua virulência e patogenicidade (ERNST, 2000; GOW; BROWN; ODDS, 2002).

Em todas as concentrações testadas, a CAT provocou redução na formação de pseudo-hifas e ausência da expressão de clamidoconídios. Já o hipoclorito de sódio, nas concentrações de 1.250 e 2.500 µg/mL, foi capaz de inibir completamente a expressão de filamentos e clamidoconídios. Este resultado

é relevante, pois complementa os demais apresentados neste estudo, onde é possível inferir que a CAT, nas concentrações testadas, promove, de forma geral, manutenção das células fúngicas na forma de blastoconídios, considerada menos virulenta.

Considerando, ainda, que a forma de biofilme é a organização mais comum dos micro-organismos, que lhes confere maior proteção e resistência, e que as próteses dentárias representam possíveis sítios para aderência dos mesmos, com possibilidade de formação de um biofilme que precisa ser considerado no tratamento e prevenção da estomatite protética (SALERNO et al., 2011; CHANDRA et al., 2001), optou-se por avaliar a atividade antibiofilme da CAT sobre um biofilme mono-espécie de *C. albicans*, escolhida por ser a mais prevalente nos isolados clínicos da estomatite protética (MARINOSKI; BOKOR-BRATIC; CANKOVIC, 2014; KILIC et al., 2012; KABAWAT et al., 2014). Devido à ausência na literatura de um protocolo voltado para desinfecção de próteses, os grupos foram baseados na tentativa de simular duas formas de uso das substâncias: aplicação por 1 min, 3 vezes ao dia, no momento dedicado a higienização bucal; ou contato por 8 h consecutivas, simulando desinfecção noturna.

A CAT e o hipoclorito de sódio apresentaram resultados similares ($p \geq 0,05$) quanto à inibição da aderência inicial das células fúngicas e da formação do biofilme maduro. Para a redução do biofilme maduro de *C. albicans,* a CAT mostrou-se mais eficaz que o hipoclorito de sódio ($p \leq 0,05$) na concentração referente a CIM x 2, no grupo referente a 3 contatos de 1 minuto, e na concentração CIM x 4, no grupo de 6 contatos de 1 minuto. Em ambos os casos, a CAT apresentou redução do biofilme entre 50 e 75%. Nos grupos de contato por 8 h consecutivas, também não houve diferença estatística entre as substâncias.

Os resultados deste estudo diferem dos encontrados por autores que testaram a ação de dentifrícios à base de CAT, a uma concentração de 10.000 µg/mL, em esquema de uso de 2 min durante 21 dias (PANZERI et al., 2009) e concentrações de 2.000 e 10.000 µg/mL, utilizados 3 vezes ao dia durante 7 dias (ANDRADE et al., 2012), os quais não encontraram, respectivamente, redução significativa do número de UFC/mL de *C. albicans* presentes em biofilmes de próteses dentárias e remoção significativa do biofilme de próteses dentárias de 30 pacientes. Como os dentifrícios apresentam uma complexa composição, as

diferenças na formulação e forma farmacêuticas podem explicar as diferenças encontradas e impedem uma comparação efetiva dos resultados.

Todas as concentrações testadas para a CAT, superiores e inferiores a CIM, apresentaram valores de hemólise entre 61 e 67,7%. Este resultado sugere que a capacidade hemolítica da CAT não varia com a concentração. O hipoclorito de sódio causou hemólise total imediatamente após o contato com as hemácias, confirmando sua alta citotoxicidade. Na literatura (NAGL et al., 2003), a CAT quando avaliada quanto a citotoxicidade sobre células de linhagem humana (carcinoma epidermóide) não foi capaz de causar danos celulares visíveis em concentrações entre 1 e 10 µg/mL após 30 min de contato. Em concentrações superiores, correspondentes a 100, 1.000 e 10.000 µg/mL, causou alterações na forma da célula após 30 min e completa fragmentação após 24 h.

Por ser um estudo *in vitro*, este trabalho apresenta limitações, como o uso de células planctônicas e biofilme mono-espécie para determinação da atividade antifúngica e antibiofilme. No entanto, essas etapas são justificadas pelo fato da necessidade da realização de estudos iniciais acerca da atividade antifúngica da CAT que fundamente estudos posteriores.

A partir dos resultados encontrados e considerando a ausência de estudos prévios com maior aprofundamento na avaliação da atividade antifúngica, mecanismo de ação e atividade antibiofilme da CAT, este estudo representa um avanço na área e confirma o potencial de utilização da CAT na odontologia, mais especificamente na desinfecção de próteses dentárias. Sugere-se a realização de estudos futuros direcionados à avaliação da citotoxicidade, verificação de atividade antibiofilme utilizando modelo multiespécie e investigação do efeito da substância sobre as propriedades dos componentes das próteses dentárias.

## 4. CONCLUSÃO

A CAT possui atividade antifúngica sobre *C. albicans*, *C. tropicalis*, *C. krusei* e *C. glabrata*, apresentando ação fungicida sobre a maioria das cepas testadas; apresenta ação imediata e prolongada na redução do crescimento de *C. albicans*; induz a expressão da *C. albicans* na forma de blastoconídios, reduzindo o desenvolvimento de pseudo-hifas e inibindo a formação de clamidoconídios; apresenta atividade antibiofilme sobre *C. albicans*, sendo capaz de inibir a aderência inicial e formação do biofilme maduro e reduzir o biofilme maduro pré-existente. Apresenta alta capacidade hemolítica, entretanto inferior à do hipoclorito de sódio.

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## APÊNDICE 1

**TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

Prezado (a) Senhor (a),

Esta pesquisa é sobre o estudo da Cloramina T, que possui comprovada ação desinfetante e bactericida, a respeito da sua atividade antifúngica e estudo da citotoxicidade em células humanas. A pesquisa está sendo desenvolvida pelas pesquisadoras Gabriela Lacet Silva Ferreira e Larissa Rangel Peixoto, alunas do Programa de Pós Graduação em Odontologia da Universidade Federal da Paraíba, sob a orientação do Prof. Ricardo Dias de Castro.

Os objetivos do estudo são avaliar *in vitro* a atividade antifúngica da Cloramina T e determinar a citotoxicidade desta substância, através do ensaio de hemólise.

A finalidade deste trabalho é fornecer subsídios para pesquisas posteriores e trazer benefícios clínicos futuros, através da formulação de possíveis substâncias com aplicabilidade no tratamento da candidíase oral. Dessa forma, sua participação será bastante benéfica para a aquisição de informações essenciais para o conhecimento da atividade da substância em estudo.

Solicitamos a sua colaboração para coleta de sangue, como também sua autorização para apresentar os resultados deste estudo em eventos da área de saúde e publicar em revista científica. Por ocasião da publicação dos resultados, seu nome será mantido em sigilo. Informamos que essa pesquisa oferece riscos mínimos de desconforto durante a coleta e constrangimento, entretanto a equipe irá tomar medidas para que isto seja minimizado.

Esclarecemos que sua participação no estudo é voluntária e, portanto, o(a) senhor(a) não é obrigado(a) a fornecer as informações e/ou colaborar com as atividades solicitadas pelo Pesquisador(a). Caso decida não participar do estudo, ou resolver a qualquer momento desistir do mesmo, não sofrerá nenhum dano.

Os pesquisadores estarão a sua disposição para qualquer esclarecimento que considere necessário em qualquer etapa da pesquisa.

Diante do exposto, declaro que fui devidamente esclarecido(a) e dou o meu consentimento para participar da pesquisa e para publicação dos resultados. Estou ciente que receberei uma cópia desse documento.

_____ _____

Assinatura do Participante da Pesquisa Assinatura da Testemunha

**Contato do Pesquisador (a) Responsável:**

Caso necessite de maiores informações sobre o presente estudo, favor ligar para a pesquisadora Gabriela Lacet Silva Ferreira.

Endereço (Setor de Trabalho): Programa de Pós Graduação em Odontologia - Cidade Universitária, João Pessoa, Paraíba, Brasil. CEP: 58051-900

Telefone: (83) 8834-8943 - E-mail: gabriela_lacet@hotmail.com

Comitê de Ética em Pesquisa do Centro de Ciências da Saúde da Universidade Federal da Paraíba Campus I - Cidade Universitária - 1º Andar – CEP– João Pessoa/PB – Telefone: (83) 3216-7791 – E-mail: eticaccsufpb@hotmail.com

_____ _____

Assinatura do Pesquisador Responsável Assinatura do Pesquisador Participante

**ANEXO 1**

UNIVERSIDADE FEDERAL DA PARAÍBA
CENTRO DE CIÊNCIAS DA SAÚDE
COMITÊ DE ÉTICA EM PESQUISA

# CERTIDÃO

Certifico que o Comitê de Ética em Pesquisa do Centro de Ciências da Saúde da Universidade Federal da Paraíba – CEP/CCS aprovou por unanimidade na 4ª Reunião realizada no dia 21/05/2015, o Projeto de pesquisa intitulado: **"ATIVIDADE ANTIFÚNGICA, CITOTOXIDADE E MECANISMO DE AÇÃO DA CLORAMINA T SOBRE CEPAS DO GÊNERO *CÂNDIDA* E SUA AÇÃO SOBRE PROPRIEDADES FÍSICAS DA RESINA ACRÍLICA"**, da pesquisadora Gabriela Lacet Silva Ferreira. Protocolo 0193/15. CAAE: 43914615.0.0000.5188.

Outrossim, informo que a autorização para posterior publicação fica condicionada à apresentação do resumo do estudo proposto à apreciação do Comitê.

Andrea M. C. Lima
Andrea Márcia da C. Lima
Mat. SIAPE 1117510
Secretária do CEP-CCS-UFPB

**ANEXO 2**

Normas para submissão do artigo ao periódico “Clinical Oral Investigations”.

## Instructions for Authors

### Types of papers

Papers may be submitted for the following sections:

Original articles

Invited reviews

Short communications

Letters to the editor

It is the general policy of this journal not to accept case reports and pilot studies.

### Editorial Procedure

If you have any questions please contact:

Prof. Dr. G. Schmalz

University of Regensburg

Department of Conservative Dentistry and Periodontology

Franz-Josef-Strauß-Allee 11

93053 Regensburg

Germany

e-mail: gottfried.schmalz@klinik.uni-regensburg.de

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**Manuscript Submission**

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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**Title Page**

The title page should include:

The name(s) of the author(s)

A concise and informative title

The affiliation(s) and address(es) of the author(s)

The e-mail address, telephone and fax numbers of the corresponding author

## Abstract

Please provide a structured abstract of 150 to 250 words which should be divided into the following sections:

Objectives (stating the main purposes and research question)

Materials and Methods

Results

Conclusions

Clinical Relevance

These headings must appear in the abstract.

## Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

## **Text**

### Text Formatting

Manuscripts should be submitted in Word.

Use a normal, plain font (e.g., 10-point Times Roman) for text.

Use italics for emphasis.

Use the automatic page numbering function to number the pages.

Do not use field functions.

Use tab stops or other commands for indents, not the space bar.

Use the table function, not spreadsheets, to make tables.

Use the equation editor or MathType for equations.

Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

LaTeX macro package (zip, 182 kB)

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

**References**

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].

2. This result was later contradicted by Becker and Seligman [5].

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Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

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Journal article

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Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

ISSN.org LTWA

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For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

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All tables are to be numbered using Arabic numerals.

Tables should always be cited in text in consecutive numerical order.

For each table, please supply a table caption (title) explaining the components of the table.

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Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.