INHIBITORY EFFECTS OF *TRANS*-CINNAMALDEHYDE ON *CANDIDA* SPP. BIOFILM AND PLANKTONIC FORM COMPARED TO NYSTATIN: A COMPARATIVE ANALYSIS

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Trans-Cinnamaldehyde, an α, β-unsaturated aromatic aldehyde, was a derivative of Cinnamonnum obtusifolium - Lauraceae; its bioavailability allows it to be used in the treatment of fungal infections as Candida species causing opportunistic fungal infections are common today. Antibiotic resistance in fungi is often due to their ability to form biofilm. Therefore, new substances with antibiofilm were being investigated as alternative treatment. Representatives of Candida spp. were used to determine the MIC, $MBIC_{50}$, $MBIC_{80}$, $MBIC_{100}$, and MFC of trans-Cinnamaldehyde compared to Nystatin which is used for treatment of Candida infections in Vietnam. The most important antibiofilm effect of trans-Cinnamaldehyde was demonstrated by the CLSI M27-A2 method. Trans-Cinnamaldehyde was given $MBIC_{100}$ higher than PMIC from 10 to 20 times, while Nystatin was given $MBIC_{100}$ higher than PMIC from 5 to 20 times. In addition, PMFC of trans-Cinnamaldehyde was equivalent (Candida albicans and Candida tropicalis) or only twice (Candida glabrata) PMIC. The jump in Candida biofilm inhibitory activity was short, from MBIC50 to $MBIC_{100}$ (no intermediate value, $MBIC_{80}$). Trans-Cinnamaldehyde extract, compared to Nystatin, had a generally similar effect in preventing the growth and loss of Candida spp. both in biofilm and planktonic form and was somewhat more effective against Candida spp. in biofilm form.

Keywords: Cinnamon, *trans*-Cinnamaldehyde, *Nystatin, Candida albicans, Candida glabrata* and *Candida tropicalis*.

I. INTRODUCTION

The prevalence of fungal infections, particularly those caused by *Candida* species, has been a growing concern in recent years. *Candida* biofilms, which are organized communities of fungal cells embedded in a protective extracellular matrix, contribute significantly to the pathogenesis of these infections. Biofilm-associated *Candida* cells exhibit enhanced resistance to conventional antifungal agents, leading to treatment failures and increased morbidity. Therefore, there is an urgent need to explore alternative therapeutic approaches that can effectively target *Candida* biofilms.[1,2]

Trans-Cinnamaldehyde, a natural compound derived from cinnamon bark, has gained attention for its potential antifungal properties.[3] Previous studies have demonstrated the inhibitory effects of *trans*-Cinnamaldehyde on planktonic *Candida* cells, but its efficacy against

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Candida biofilms remains underexplored.[4] In this study, we aimed to evaluate the inhibitory effects of *trans*-Cinnamaldehyde on both *Candida* biofilm and planktonic forms, comparing its effectiveness to that of Nystatin, a commonly used antifungal agent.[5]

The primary objective of this research is to assess the potential of *trans*-Cinnamaldehyde as an alternative therapeutic agent for *Candida* biofilm-related infections. By comparing its inhibitory effects with Nystatin, we can determine its relative efficacy and explore the possibility of using *trans*-Cinnamaldehyde as a novel antifungal treatment option.

To achieve this objective, we employed a comprehensive experimental approach, including minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), and minimum biofilm inhibitory concentration (MBIC) determination.[6] Additionally, we conducted MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays to assess cell viability and employed statistical analysis to evaluate the significance of the results.[7]

The findings of this study have the potential to contribute to the existing literature by providing valuable insights into the efficacy of *trans*-Cinnamaldehyde against *Candida* biofilms. Furthermore, considering the rising concern of antifungal resistance, exploring alternative treatments derived from natural sources, such as *trans*-Cinnamaldehyde, is of utmost importance. By addressing these research gaps, we aim to expand our understanding of potential therapeutic options for *Candida* biofilm-related infections and pave the way for future investigations in this field.

II. MATERIAL AND METHODS

1. Microorganisms, growth media and conditions

An experimental laboratory intervention was designed at the Faculty of Chemical Engineering, Ho Chi Minh City University of Technology, Vietnam. Samples including standard species (ATCC) *Candida albicans* (ATCC 24433), *Candida glabrata* (ATCC 15126), and *Candida tropicalis* (ATCC 13803) were provided by OSI Co., Ltd, Vietnam.

Three strains of yeast *Candida* spp. above were inoculated on activated SDA agar medium, incubated at 37°C for 48h. For each strain of *Candida* spp., take about 5 clusters about 1 mm in diameter were mixed in a test tube in 9 mL of physiological saline containing 0.05% Tween 80. The fungal solution was vortexed for 20-30 seconds. The fungal suspension was adjusted to 0.1 ± 0.02 $OD_{530\ nm}$, equivalent from 1 to 5 x 10^6 CFU/mL.

2. Biological active substances

This study was performed using purified *trans*-Cinnamaldehyde (extracted from Cinnamon Tra My, Vietnam), and Nystatin (purchased from YouMed Co., Ltd, Vietnam). *Trans*-Cinnamaldehyde was dissolved in DMSO (Ho Chi Minh City Institute of Testing, Vietnam), then diluted 100 times in SDB medium supplemented with 1% Tween 80 to reach the maximum test concentration. Nystatin was also dissolved in DMSO, then diluted 100 times in RPMI-1640 medium (Sigma-Aldrich, Germany) supplemented with L-glutamine, without bicarbonate to obtain a concentration twice the maximum test concentration.

3. Minimum inhibitory concentrations (MIC) and minimum fungicidal concentration (MFC) determination

The effect of bioactive substances on the growth of fungi in suspension was investigated by the CLSI M27-A2 method, described in

detail by Wenqian Chang *et al.*, 2021 [8]. *Trans*-Cinnamaldehyde concentrations from 0.01 to 5 mg/mL and Nystatin concentrations from 0.0025 to 0.128 mg/mL were prepared in a suitable growth medium in 96-wells plates (Germany). 100 L of the cell suspension ($OD_{530\ nm}$ = 0.10 ± 0.02) were injected into the respective growth medium containing the biologically active substances in each well. The fungi in the plate were incubated at 37 °C for 48 h. The minimum concentration of biologically active substances that caused a decrease in optical density compared to the control was determined as the minimum inhibitory concentration (MIC) for the growth of the microbial suspension. Each experiment was repeated three times, the average MIC value was taken.

After MIC testing, to determine MFC, 100 L of fluid from wells containing concentrations of MIC, 2 MIC, 4 MIC was spread onto the surface of SDA agar plate. The plates with less than 10 cluster of clusters correspond to the minimum fungicidal concentration (MFC).

4. Minimum biofilm inhibitory concentrations (MBIC) determination

Biofilms were prepared as described in the study by Paldrychova *et al.*, 2019 [9]. In summary, *Candida* spp. biofilms were formed in 96-wells polystyrene plates (TPP, Switzerland) for 48 h. The pre-cultured cells suspension ($OD_{530\ nm}$ = 0.10 ± 0.02) were added to each well and allowed to adhere to the well surface. To determine the minimum biofilm inhibitor concentration (MBIC), substances were added to the well at the beginning (control samples did not have any compounds included). At the end of the culture (at 37°C, 150 rpm), the biofilm cells were gently washed three times with saline and the biofilms were quantified by MTT assay. The minimum concentration of bioactive substances inhibiting biofilm viability (expressed as metabolic activity of biofilm cells compared to control) 50% is $MBIC_{50}$ or 80% is $MBIC_{80}$ or 100% is $MBIC_{100}$. Each experiment was repeated three times, the average value for $MBIC_{50}$, $MBIC_{80}$, $MBIC_{100}$ was calculated respectively.

5. MTT assay

The metabolic activity of biofilm cells was detected using the method described by Vankova *et al.*, 2019 [10] with minor modifications. MTT solution was prepared by 1 mg/mL *N*′-anilino-*N*-[(4,5-dimethyl-1,3-thiazol-2-yl)imino] benzenecarboximidamide (MTT; Sigma-Aldrich, Germany) in PBS. Biofilm cells (prepared as described above) were incubated with 50 L of MTT solution and 60 L of glucose (57.4 mg/mL in PBS; Dong Nam Co., Vietnam) in the dark for 3 hrs at 37°C. The purple formazan crystals formed were dissolved with the diluent (160 mg/mL SDS in 40% dimethylformamide in acetic acid-acidified PBS) with vigorous shaking at 230 rpm. The color intensity of this solution was measured after transferring an amount of 75 L to another 96-wells microtiter plate using a spectrophotometer (Tecan, Switzerland) at 490 nm. Each experiment was repeated three times, the average value was obtained.

6. Statistical analysis

To evaluate microbial growth or biofilm assay data, we used Dixon's Q test to exclude outliers. Arithmetic mean and standard deviation (SD) were calculated for each concentration tested by the mentioned assays as a relative percentage (control sample was 100%). The significance of the difference between control and adjuvant effects was determined by one-way analysis of (ANOVA) with significance $p < 0.05$. Ethics committee approval was not required for this study.

III. RESULTS

1. Formation of *Candida* spp. biofilm on 96-wells plastic board

On the RPMI test medium, after 48 h, biofilms formed at the bottom of all wells of the plastic board, which was observed by microscope (at x4, and x10 objective). The biofilm formed from *Candida albicans* was mainly filamentous, *Candida tropicalis* was composed of yeast and filaments, and *Candida glabrata* was yeast only. (**Figure 1**)

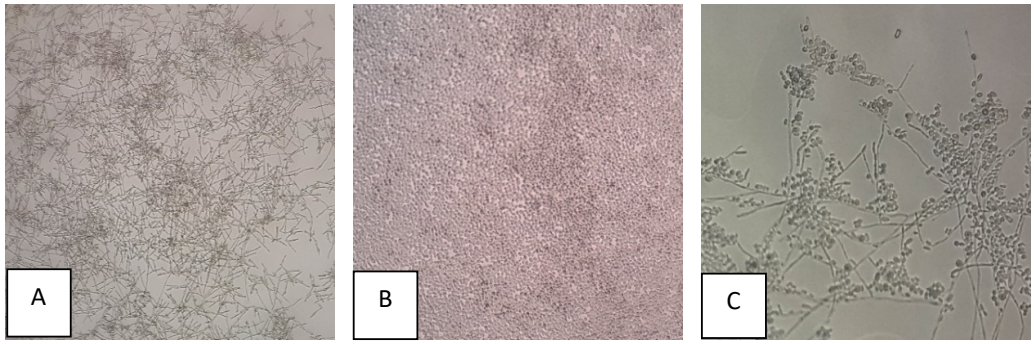

Figure 1. *Candida* spp. biofilm at the bottom of the well after 48 h of culture, observed at x 10 objective. (A) *Candida albicans*. (B) *Candida glabrata*. (C) *Candida tropicalis*.

2. The effect of *trans*-Cinnamaldehyde on *Candida albicans* biofilm

After 48 h of culture, the *trans*-Cinnamaldehyde was exposed to *Candida albicans* biofilm; the difference of biofilms in the wells with *trans*-Cinnamaldehyde compared to the control well without *trans*-Cinnamaldehyde showed that the biofilms affected by *trans*-Cinnamaldehyde was much less dense, and its shape was also altered. (**Figure 2**).

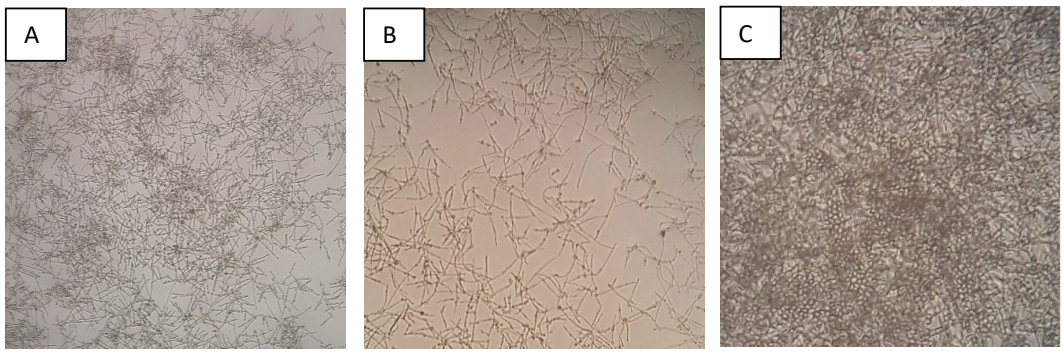

Figure 2. *Candida albicans* biofilm after 48 h tested to *trans*-Cinnamaldehyde, observed at x10 objective. (A) *Candida albicans* before exposure to *trans*-Cinnamaldehyde. (B) *Candida albicans* was affected by *trans*-Cinnamaldehyde after 48 h. (C) *Candida albicans* in the control well after 48 h.

3. Anti-fungal biofilm activity of *trans*-Cinnamaldehyde compared to Nystatin by optical densitometry with MTT assay

The results from **Table 1, Figure 3,** and **Figure 4** show that the degree of inhibition of *Candida* spp. biofilm of Nystatin was highly concentration-dependent to $MBIC_{50}$, $MBIC_{80}$, and $MBIC_{100}$ incrementally. $MBIC_{80}$ was higher than $MBIC_{50}$ from 2.6 to 8 times. $MBIC_{100}$ was higher than $MBIC_{80}$ about 2 times, and it was higher than $MBIC_{50}$ from 5 to 16 times (**Table 1**). Meanwhile, in *trans*-Cinnamaldehyde, there was a clear jump from $MBIC_{50}$ to $MBIC_{100}$ that $MBIC_{100}$ was higher than $MBIC_{50}$ about 2 times (**Table 1**). *Trans*-Cinnamaldehyde had a marked jump from $MBIC_{50}$ to $MBIC_{100}$ (**Figure 3**), while Nystatin gave a gradual jump from $MBIC_{50}$ to $MBIC_{80}$ and then to $MBIC_{100}$ (**Figure 4**).

Table 1. MBIC of *Candida* spp. of *trans*-Cinnamaldehyde and Nystatin

Fungal strains	***trans*-Cinnamaldehyde** (mg/mL)		**Nystatin** (mg/mL)		
	$MBIC_{50}$	**$MBIC_{100}$**	**$MBIC_{50}$**	**$MBIC_{80}$**	**$MBIC_{100}$**
Candida albicans	0.1600	0.3200	0.0015	0.0040	0.008
Candida glabrata	0.1600	0.3200	0.0015	0.0040	0.008
Candia tropicalis	0.3200	0.6300	0.0020	0.0160	0.032

$MBIC_{50}$ minimum biofilm inhibitory concentrations (50%), *$MBIC_{80}$* minimum biofilm inhibitory concentrations (80%), *$MBIC_{100}$* minimum biofilm inhibitory concentration (100%). The results were calculated from 3 experiments.

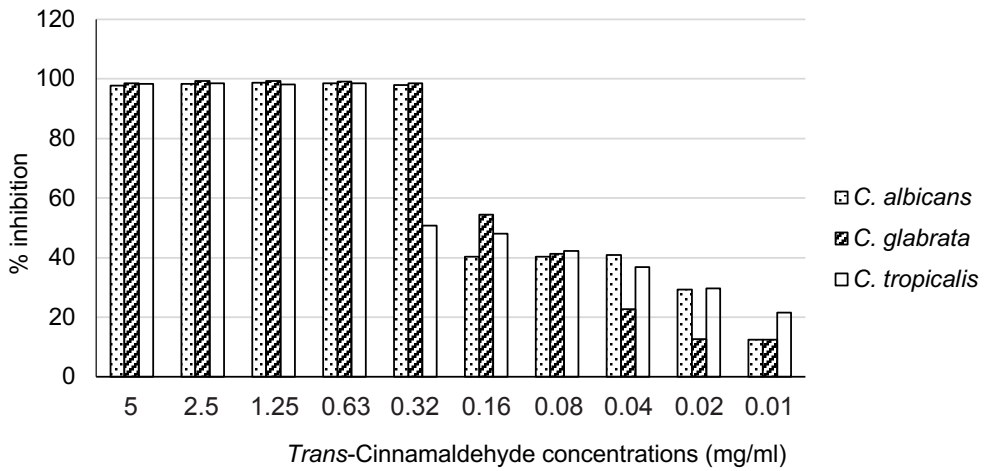

Figure 3. The inhibitory effect of *trans*-Cinnamaldehyde on *Candida* spp. biofilm

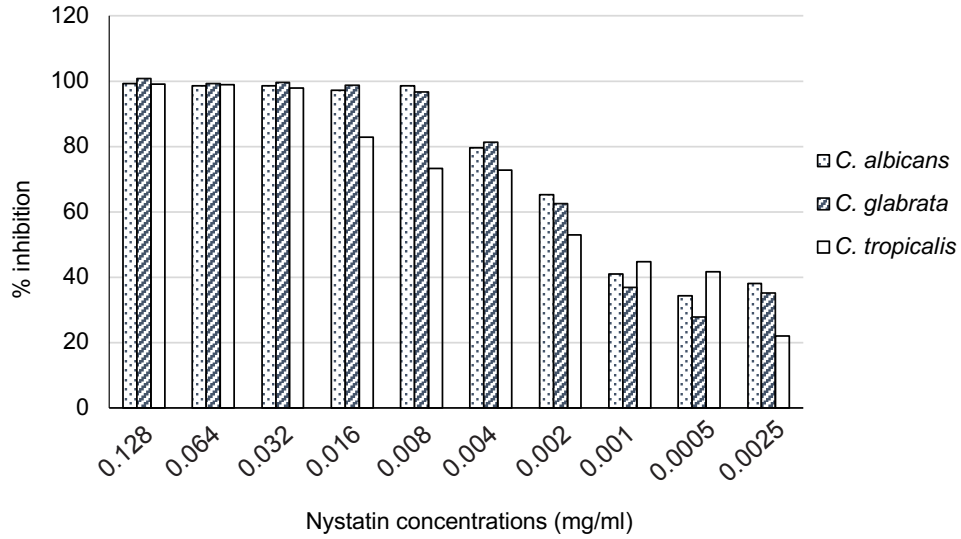

Figure 4. The inhibitory effect of Nystatin on *Candida* spp. biofilm

4. Anti-fungal *Candida* spp. activity on biofilm and planktonic form of *trans*-Cinnamaldehyde compared to Nystatin

The results from **Table 2** show that, in *trans*-Cinnamaldehyde, the PMFC was equivalent to twice the PMIC, thus *trans*-Cinnamaldehyde may be a fungicide. On the other hand, in *trans*-Cinnamaldehyde, $MBIC_{100}$ was higher than PMFC about 10 times. Meanwhile, in Nystatin, $MBIC_{100}$ was higher than PMFC about 2 to 16 times.

Table 2. Comparison of anti-fungal *Candida* spp. activity on biofilm and planktonic form of *trans*-Cinnamaldehyde and Nystatin

Fungal strain	*trans*-Cinnamaldehyde (mg/mL)			Nystatin (mg/mL)		
	PMIC	PMFC	$MBIC_{100}$	PMIC	PMFC	$MBIC_{100}$
Candida albicans	0.0260	0.0300	0.3200	0.0017	0.0040	0.0080
Candida glabrata	0.0150	0.0300	0.3200	0.0008	0.0060	0.0080
Candia tropicalis	0.0330	0.0330	0.6300	0.0017	0.0020	0.0320

PMIC planktonic minimum inhibitory concentrations, *PMFC* planktonic minimum fungicidal concentration, $MBIC_{100}$ minimum biofilm inhibitory concentration (100%). The results were calculated from 3 experiments.

IV. DISCUSSION

Trans-Cinnamaldehyde exhibits antimicrobial activity with a broad spectrum of antifungal activity. This is consistent with the studies of Guynot M. E. *et al.*, 2003[11] and Ooi L. S. *et al.*, 2006.[12] In particular, *trans*-Cinnamaldehyde can affect *Candida* spp. biofilm, this is a valuable property of the extract because the ability of *Candida* species to form resistant biofilms was an important factor in the pathogenicity of this fungus. As in most

microbial biofilm biomass, sessile cells within the *Candida* biofilm were from 20 to 1,000 times less susceptible to antibiotics than planktonic cells.[13] Compared to Nystatin as a fungicide, the minimum inhibitory concentration of *Candida* biofilm of Nystatin was higher than the minimum inhibitory concentration of planktonic *Candida* from 5 to 20 times; and, *trans*-Cinnamaldehyde showed the minimum inhibitory concentration of *Candida* biofilm higher than the minimum inhibitory concentration of planktonic *Candida* from 10 to 20 times.

In addition, the MFC of *trans*-Cinnamaldehyde was equivalent or only twice the MIC. The jump in *Candida* biofilm inhibitory activity was short, from $MBIC_{50}$ to $MBIC_{100}$ (no intermediate value $MBIC_{80}$), leads to *trans*-Cinnamaldehyde may be a fungicide.

The limitations of this study include the relatively small sample size and the use of only three *Candida* spp. to test the effects of *trans*-Cinnamaldehyde. Additionally, toxicity experiments of *trans*-Cinnamaldehyde on cells of other bacterial species were not performed. Therefore, further studies with larger sample size and other bacterial species are needed to evaluate the effect of *trans*-Cinnamaldehyde.

V. CONCLUSION

Although *trans*-Cinnamaldehyde extract compared with Nystatin could significantly inhibit *Candida* spp. biofilm with higher concentrations, given the antifungal effects of this plant, it is hopeful that it could be considered as a potential medicinal plant for the treatment of *Candida* infections, especially in biofilm form. Due to the limitations of the study conducted, further studies with larger sample sizes and a broader range of bacterial species are needed to evaluate the effectiveness of *trans*-Cinnamaldehyde. In addition, studies at the molecular level are also needed to understand the mechanism of action of *trans*-Cinnamaldehyde compared to other antifungal drugs, both in planktonic and biofilm form.

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