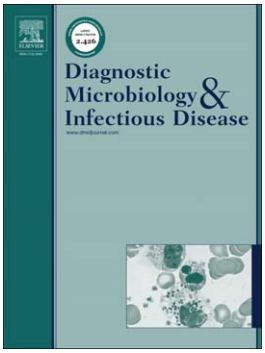

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Synthesis, antifungal activity of caffeic acid derivative esters, and their synergism with fluconazole and nystatin against *Candida* spp.

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Key words: Synergism, *Candida albicans*, Caffeic acid, Antifungal

Abstract

We tested the antifungal potential of caffeic acid and eight of its derivative esters against *C. albicans* ATCC 90028 and nine clinical isolates, and carried out a synergism assay with fluconazole and nystatin. Propyl caffeate (**C3**) showed the best antifungal activity against the tested strains. When in combination, **C3** markedly reduced the MIC of fluconazole and nystatin with synergistic effect up to 64-fold. Finally, **C3** showed a high IC_{50} value and Selective Index (SI) against oral keratinocytes, demonstrating low toxicity against this cell type and selectivity for yeast cells. Further research should confirm its antifungal potential for development of combined therapy to treat *C. albicans* infections.

Introduction

Oral candidiasis is one of the most common opportunistic infections afflicting humans, with *Candida albicans* as the major causative agent of this disease (Garcia-Cuesta *et al*., 2014). The complexity of interactions between *Candida* and other microorganisms in the host, mainly bacteria, suggest that several mechanisms are involved in yeast fitness to the oral cavity. Some studies have shown that *Candida* spp. can co-aggregate with bacteria in dental plaque. This feature may be an important factor for the onset of oral candidiasis as well as fungal colonization of carious cavities and periodontal pockets (Sardi *et al*., 2012; Thurnheer *et al*., 2015). The presence of yeasts in subgingival regions may contribute to the pathogenesis of periodontal disease or increase the chance of candidemia, especially in cases of immunosuppression (Hannula *et al*., 2001; Reynaud *et al*., 2001; Al Mubarak *et al*., 2013). In addition, it has been well documented that systemic diseases such as diabetes and AIDS; physiological conditions such as pregnancy, infancy or old age; nutritional factors; treatment with broad-spectrum antibiotics; use of immunosuppressive drugs and corticosteroids; xerostomia, and use of dentures, may predispose the individual to develop candidiasis (Soll, 2002; Tekeli *et al*., 2004; Manfredi *et al*., 2006).

The current therapy with antifungals has serious drawbacks, in particular due to toxic effects to human cells and adverse effects (Epstein *et al*., 2002; Gabler *et al*., 2008). As the drugs used to treat candidiasis are not always specific and properly prescribed (targeting the causative agent of infection), there has been a significant increase in resistance of *Candida* spp. to traditional antifungal drugs. The increasing microbial resistance rates may also be a result of long-term drug exposure or selection of strains with

intrinsic resistance mechanisms (Ying *et al*., 2013; Fernandez-Ruiz *et al*., 2015; Liao *et al*., 2015; Seifi *et al*., 2015; Freitas *et al*., 2015). Therefore, the development of novel strategies to minimize the toxic effects of current antifungals and improve their effectiveness, has been strongly encouraged.

Natural products have continued to be a rich source of new drugs with clinically significant biological targets. Over the past 34 years, 49% of FDA-approved chemotherapeutic drugs were either natural products or directly derived therefrom (Newman & Cragg, 2016). There is a great interest of the pharmaceutical industry in the discovery of new molecules of natural origin or even their combination with existing drugs, in order to improve efficacy, potency, safety, tolerability, and decrease production costs, side effects and selection of resistant strains (Svetaz *et al*., 2016). A number of studies in the literature have established the value of combined antifungal therapy against resistant strains, in particular standard drugs with naturally-occurring agents (Pippi *et al*., 2015; Han *et al*., 2016).

Caffeic acid (3,4-dihydroxycinnamic acid) is an important phenolic compound commonly found in plants, foods and propolis samples, particularly in the form of caffeic acid phenethyl ester (CAPE) (Paracatu *et al*., 2014; Rzepecka-Stojko *et al*., 2015). It is better known for its pharmacological properties, including antimicrobial, antioxidant, anti-inflammatory and anticancer (Balachandran *et al*., 2012; Kuo *et al*., 2015). Nevertheless, modifications of the caffeic acid structure into esters or amides, for instance, may generate novel analog molecules with enhanced and desired biological activity (Touaibia *et al*., 2011), particularly as antimicrobials (Fu *et al*., 2010).

Herein, we investigated the antifungal potential of caffeic acid and eight of its derivative esters against *C. albicans* ATCC 90028 and nine oral clinical isolates. The most active molecule, propyl caffeate (**C3**), was selected for a synergism assay with fluconazole and nystatin against the *C. albicans* strains, and tested for its toxicity on oral keratinocytes (NOK cells).

Material and Methods

Synthesis of Esters. Caffeic acid (0.2 mM) solution and corresponding alcohols (20 mM) were prepared at 5°C with a solution of *N*, *N*′-dicyclohexylcarbodiimide (DCC, 1.0 mM) in *p*-dioxane (3.0 mL). After the solution was stirred for 48 h, the solvent was removed under reduced pressure. The residue was partitioned three-times with EtOAc and filtered. The

filtrate was serially washed with saturated aqueous citric acid solution (three times), saturated aqueous $NaHCO_3$ (three-times), water (two-times), dried over $MgSO_4$, and evaporated under reduced pressure. The crude products were purified over a silica gel column using an isocratic system of $CHCl_3$–MeOH (98:2). The modifications made in caffeic acid molecule are shown in Table 1.

Microorganisms. *Candida albicans* ATCC 90028 strain and nine highly virulent clinical isolates of *C. albicans* obtained from the oral cavity of patients with diabetics and periodontitis (Sardi *et al*., 2012), were used in this study. This study was approved by the research ethics committee at Piracicaba Dental School, University of Campinas, Piracicaba, SP, Brazil (protocol no. 062/2008).

Determination of Antifungal Activity. The Minimum Inhibitory Concentration (MIC) of caffeic acid and its eight derivatives against *C. albicans* ATCC 90028 was determined using 96-well microplates based on the protocol M27-A3 of the CLSI (2008), with modifications. The esters that showed the lowest MIC values against *C. albicans* ATCC 90028 (C3, C4 and C6) were then tested against nine clinical isolates of *C. albicans*. The synthetic compounds of caffeic acid were diluted in DMSO and tested in concentrations ranging from 250 µg/mL to 0.48 µg/mL (Scorzoni *et al*., 2007). The inoculum was prepared (λ 530 nm, Abs 0.08-0.1) and diluted to 2.5 x 10^3 CFU/mL. The plates were incubated at 35°C for 24 h. The MIC_{100} was determined as the lowest concentration of the compound inhibiting visible fungal growth as indicated by 0.1% resazurin (Sigma-Aldrich, St. Louis, MO, USA). Aliquots from the wells corresponding to the MIC and higher concentrations were sub-cultured on Sabouraud Dextrose Agar (Difco®, Detroit, MI, USA) for determination of the Minimum Fungicidal Concentration (MFC). The MFC was defined as the lowest concentration of the compound causing no visible growth on the agar plate.

Combinatorial Antifungal Activity (Synergism Assay). The ester which showed the best activity against *C. albicans* strains (**C3**) was combined with conventional antifungals commonly used for the treatment of candidiasis, fluconazole and nystatin. Their combinatorial antifungal activity was determined through the checkerboard method using 96-well microplates (Dai *et al*., 2015). A mathematical calculation was used to generate the fractional inhibitory concentration index (FICI), as follows: FICI = (MIC compound 1 in combination / MIC compound 1 alone) + (MIC compound 2 in combination / MIC compound 2 alone). The combinations were classified as synergistic (FICI ≤ 0.5), additive

($0.5 < FICI \leq 1.0$), indifferent ($1.0 < FICI < 4.0$) and antagonistic ($FICI \geq 4.0$) (Soares *et al*., 2014).

Cytotoxic Effects on Oral Keratinocytes. The most active ester derivative (**C3**) as well as fluconazole and nystatin were tested for their cytotoxicity against keratinocytes from the oral mucosa of humans (NOK cells) provided by the Department of Medicine at Harvard Medical School (Dr. Karl Munger). NOK cells were maintained in culture medium for keratinocytes without fetal bovine serum (Gibco, Life Technologies) at 36.5ºC and 5% CO_2. Cells were seeded onto a 96-well plate at a density of 5×10^4 cells/well for 24 h. Then the cells were exposed to the treatments (**C3**, Fluconazole, Nystatin, and their combinations) for 24 h. Cell viability was determined by adding aliquots of 10µL of MTT solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO) to each well. The plates were incubated at 37ºC for 4 h to allow for visualization of precipitated formazan crystals. Aliquots of 100 µL of isopropyl alcohol were added *per* well and absorbance was read using a spectrophotometer at 560 nm (Mosmann *et al*., 1983). Hydrogen peroxide (10%) was used as a positive control for cytotoxicity and untreated cells were considered as the negative control. Based on this cell viability assay, we next determined the half maximal inhibitory concentration (IC_{50}) for the esters, standard-drugs and their combinations. The IC50 was defined as the effective concentration of the compound able to inhibit 50% of the NOK cells. The establishment of this concentration was essential to calculate the selectivity index (SI) (IC50/MIC ratio), which was used as an indicator of potential toxicity against normal cells lines at the effective therapeutic concentration for each strain. Hence, the selectivity index (SI) indicated the relationship between drug toxicity against *C. albicans* and the host cells (Gullo et al., 2012; Mora-Navarro et al., 2016).

Statistical Analysis. All assays were performed in triplicate of independent experiments. The data were analyzed on Graphpad Prism 5.0 (San Diego, CA, USA) by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test, with a significance level of 5%.

Results

Structure-Activity Relationship of Caffeic Acid Derivatives. A screening for antifungal activity of caffeic acid and eight derivative esters against *C. albicans* was carried out.

These esters differ by the number of constituent carbons in the molecule, as shown in Table 1. Caffeic acid and all of its synthesized derivatives showed a fungicidal activity against *C. albicans* ATCC 90028 and presented a structure-activity relationship. As seen in Table 2, the caffeic acid molecule (**C0**) without structural change and the derived **C1** showed MIC and MFC values of 125 µg/mL. The derivatives **C2**, **C5**, **C7** and **C8** showed MIC and MFC values of 31.25 µg/mL. **C3** and **C4** showed strong fungicidal activity against *C. albicans* ATCC 90028, with MIC and MFC values of 15.62 µg/mL, together with **C6** which showed MIC and MFC equal to 7.81 µg/mL. Thus, the compounds **C3**, **C4** and **C6** – which were better than their originating caffeic acid molecule – were further tested against nine oral clinical isolates of *C. albicans.* As shown in Table 3, the compound **C3** demonstrated the best activity when compared with the other caffeic acid derivatives (**C4** and **C6**), with MIC and MFC values ranging between 7.81 and 62.5 µg/mL. The MIC and MFC of **C4** and **C6** ranged between 31.25-125 µg/mL and 7.81-≥250 µg/mL, respectively.

Combinatorial Antifungal Activity with Standard Drugs. Given the strong antifungal activity of **C3** against *C. albicans* strains, we next investigated its combinatorial activity with the standard drugs fluconazole and nystatin. A synergistic activity was observed for the combination between **C3** and fluconazole against most strains, with FICI values ranging from 0.06 to 0.5. Indifferent activity was observed against two clinical isolates, with FICI values close to 2.0. When comparing the MIC values of **C3** and fluconazole alone and combined, we observed a decrease in their MIC and potentiation of fluconazole of 2 to 64-fold (Table 4). The combination of **C3** with nystatin was advantageous (synergistic) against three out of the ten strains when compared with the MIC values of the compounds tested alone, with potentiation of nystatin by 8- to 64-fold. Additive activity was observed against four clinical isolates and *C. albicans* ATCC 90028 strain, with FICI values ranging from 0.53 to 1.0. The additive activity of the combination showed potentiation of nystatin between 32- and 64-fold.

Toxic Effects on Oral Keratinocytes. A viability assay was carried out to evaluate the toxicity of **C3** and standard drugs alone and in combination, against NOK cells. The antifungal compound **C3** showed low toxicity at the tested concentrations, with a high IC_{50} value of 420 µg/mL. Likewise, fluconazole and nystatin showed high IC_{50} values of 320

µg/mL and 400 µg/L, respectively. When combined, both **C3** and the antifungals had their SI value increased, indicating selectivity for yeasts rather than NOK cells (Table 5).

Figure 1 shows the percentage of cell viability of NOK cells treated with the antifungal compounds at different concentrations. The data showed that treatment with **C3**, fluconazole and nystatin maintained over 80% cell viability at all tested concentrations. Hydrogen peroxide, used as a positive control, markedly affected cell viability, with a significant difference when compared to **C3** and standard drugs ($P < 0.05$).

Discussion

Opportunistic infections caused by *Candida* spp. have still been considered a recurrent health issue with high burden worldwide (Rodriguez-Tudela *et al*., 2015; Denning & Gugnani, 2015). Thus, novel therapeutic approaches are much needed to treat *Candida* infections, including the use of naturally-occurring agents. In this study, we demonstrate the antifungal potential of caffeic acid derivatives against *C. albicans* and their successful synergism with antifungals commercially available.

Caffeic acid is commonly found in fruits (Balachandran *et al*., 2012) and propolis samples (Freires *et al*., 2016) used in daily life products and folk medicine. We showed that caffeic acid molecule has fungicidal activity against *C. albicans* ATCC 90028. Nevertheless, the structural modifications performed in this study rendered the caffeic acid molecule much more effective in terms of fungicidal activity, showing a structure-activity relationship. The addition of 2 to 8 carbon atoms in the caffeic acid molecule increased its anti-*Candida* activity. Other studies have also demonstrated the relationship between the number of carbons and the antifungal activity of the molecule (Nihei *et al*., 2003; Soares *et al*., 2014). Among the synthesized compounds, **C3** showed better activity when compared with other caffeic acid derivatives (**C4** and **C6**), showing lower minimum inhibitory and fungicidal concentrations against most strains. Balachandran *et al*. (2012) isolated methyl caffeate from *Solanum torvum* plant and showed antibacterial activity against Gram-negative and Gram-positive bacteria, in addition to antifungal activity against *C. albicans* and *Apergillus flavus*. These findings confirm the antimicrobial potential of caffeic acid derivatives.

Of note, the clinical isolates used in this study showed lower susceptibility to nystatin and fluconazole when compared with the reference strain of *C. albicans* ATCC

90028. This could be due to acquisition of resistance mechanisms as the isolates belonged to diabetic patients. A recent study showed that this group of patients is prone to develop *Candida* infections in cases of poor glycemic control (Zomorodian *et al*., 2016).

Microbial resistance has raised concern over the last decades as the investment in antibiotic discovery is declining considerably over time when compared to high-priced drugs such as chemotherapeutics (Bax & Green, 2015). To make it worse, failure in prescribing the appropriate drug, misuse, and long hospital stay have led to the emergence of azole-resistant isolates, particularly in cases of invasive infections (Liao *et al*., 2015; Sanguinetti, Posteraro & Lass-Flörl, 2015). This opens avenues for the development of combined antifungal therapy, in which drugs with different mechanisms of action (or not) are combined to enhance their antifungal potency and avoid selection of resistant strains. Here, we demonstrated the successful combination of propyl caffeate (**C3**) with fluconazole and nystatin against *C. albicans* strains. A study performed by Lee & Han (2005) demonstrated the synergistic effect of the combination between amphotericin B and berberine (alkaloid) against *C. albicans*. These authors also tested this combination in mice with candidemia and observed that the combined treatment prolonged the lives of mice in 22 days compared to those treated with amphotericin B alone. Other studies have reported the successful combination of essential oils with conventional drugs, including nystatin, fluzonazole and micafugin (Aprotosoaie *et al*., 2008; Rosato *et al*., 2009; Rodrigues *et al*., 2012; Stringaro *et al*., 2014).

With the purpose of future clinical use, we also investigated the effects of **C3** against oral keratinocytes and compared them with those of the standard drugs. NOK cells were chosen for this study as they constitute the main epithelial cell type lining the oral mucosa, which would be highly exposed in case of administration of an oral suspension or a solution for oral candidiasis. Overall, low toxicity was found for **C3**, fluconazole and nystatin, with IC_{50} values higher than their MIC/MFC. These agents also showed a high SI value, which means that if the index is greater than 10.0 the compound exhibits selectivity for killing yeasts rather than the host's cells. The combination between **C3** and fluconazole led to a considerable increase in their SIs, highlighting their selectivity for yeast cells.

Collectively, our findings indicate that the propyl ester modification of caffeic acid molecule (**C3**) has strong fungicidal activity and potentiated the effects of fluconazole and nystatin against *C. albicans* strains, with little effects against oral keratinocytes. Further

studies should focus on the effects of these combinations against *Candida* biofilms and establish **C3** mechanism(s) of action.

The authors declare no conflict of interest.

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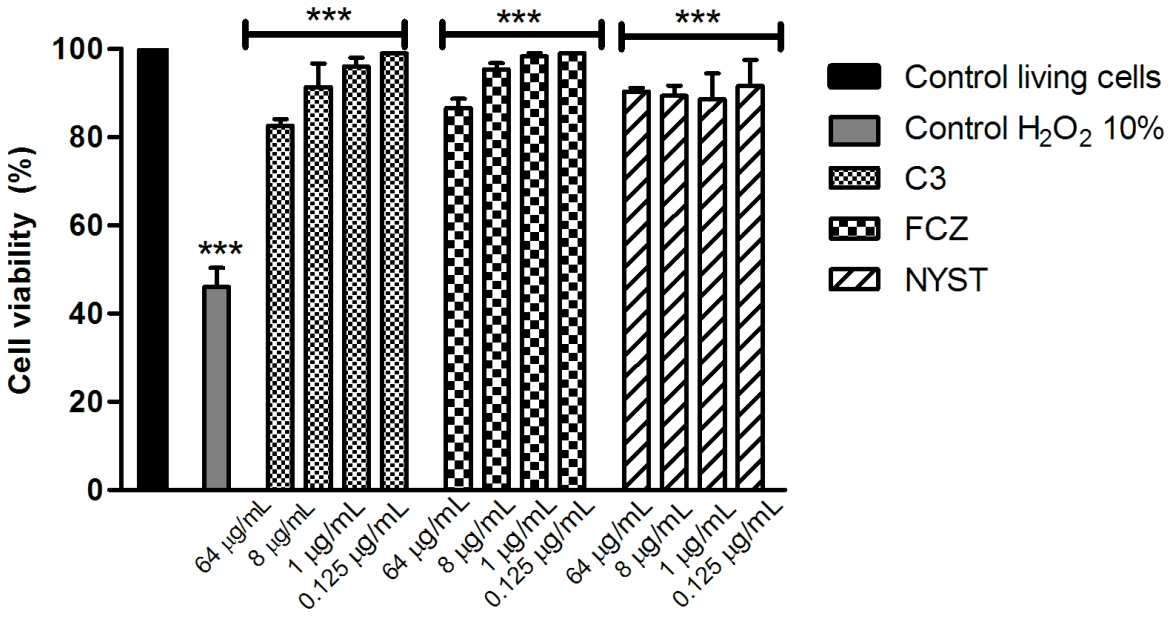
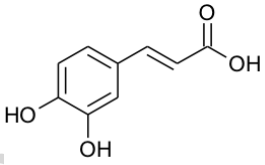
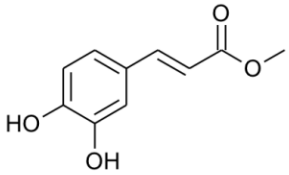
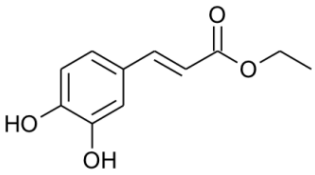
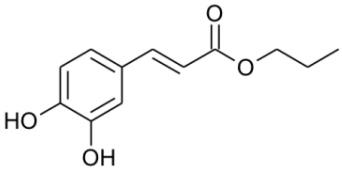
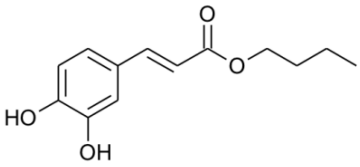
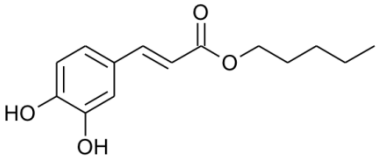
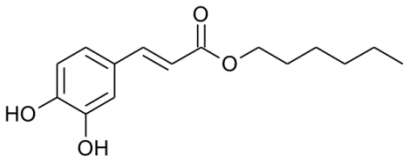
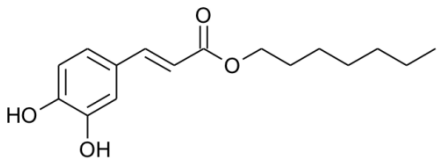


Figure 1

Table 1. Nomenclature, molecular formulas and the chemical structures of the caffeic acid derivative esters tested in this study.

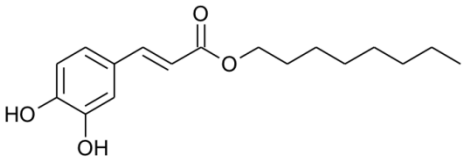
Code	Nomenclature	Molecular Formula	Chemical Structure
C0	Caffeic acid	$C_9H_8O_4$	 Caffeic acid
C1	Methyl caffeate	$C_{10}H_8O_4$	 Methyl caffeate
C2	Ethyl caffeate	$C_{11}H_{12}O_4$	 Ethyl caffeate
C3	Propyl caffeate	$C_{12}H_{14}O_4$	 Propyl caffeate
C4	Butyl caffeate	$C_{13}H_{16}O_4$	 Butyl caffeate
C5	Pentyl caffeate	$C_{14}H_{18}O_4$	 Pentyl caffeate
C6	Hexyl caffeate	$C_{15}H_{20}O_4$	 Hexyl caffeate

C7	Heptyl caffeate	$C_{16}H_{22}O_4$



Heptyl caffeate

C8	Octyl caffeate	$C_{17}H_{24}O_4$



Octyl caffeate

Table 2. Antifungal activity of caffeic acid derivative esters against *Candida albicans* ATCC 90028.

Caffeic Acids Derivates	***Candida albicans* ATCC 90028**	
	MIC (µg/mL)	**MFC (µg/mL)**
C0	125	125
C1	125	125
C2	31.25	31.25
C3	15.62	15.62
C4	15.62	15.62
C5	31.25	31.25
C6	7.81	7.81
C7	31.25	31.25
C8	31.25	31.25
Nystatin	4.0	4.0
Fluconazole	0.5	0.5

Table 3. Antifungal activity of caffeic acid derivative esters (**C3**, **C4** and **C6**) against nine oral clinical isolates of *Candida albicans*. MIC/MFC values are expressed in µg/mL.

C. albicans	**C3**		**C4**		**C6**		**Fluconazole**		**Nystatin**	
clinical isolates	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Ca#22	7.81	7.81	31.25	31.25	7.81	7.81	8.0	8.0	8.0	8.0
Ca#25	31.25	31.25	62.50	62.50	125	125	8.0	8.0	8.0	8.0
Ca#45	15.62	15.62	31.25	31.25	125	125	8.0	8.0	8.0	8.0
Ca#50	31.25	31.25	125	125	>250	>250	2.0	2.0	4.0	4.0
Ca#61	31.25	31.25	62.50	62.50	>250	>250	0.5	0.5	8.0	8.0
Ca#62	7.81	7.81	31.25	31.25	7.81	7.81	8.0	8.0	8.0	8.0
Ca#63	31.25	31.25	62.50	62.50	>250	>250	8.0	8.0	8.0	8.0
Ca#105	31.25	31.25	125	125	>250	>250	8.0	8.0	8.0	8.0
Ca#124	62.50	62.50	125	125	>250	>250	4.0	4.0	8.0	8.0

Table 4. Antifungal effects of propyl caffeate (**C3**) combined with fluconazole and nystatin against *Candida albicans* ATCC 90028 and nine oral clinical isolates. MIC values are expressed in µg/mL.

C. albicans strains	FCZ + C3				NYST + C3			
	FCZ (MIC)	C3 (MIC)	FICI	Effect /Potentiation FCZ	NYST (MIC)	C3 (MIC)	FICI	Effect /Potentiation NYST
ATCC 90028	0.25	0.12	0.50	Synergistic /2X	0.125	7.81	0.53	Additive/32X
Ca#22	0.125	15.62	2.01	Indifferent/64X	0.125	15.62	2.01	Indifferent/64X
Ca#25	0.5	0.12	0.06	Synergistic/16X	4.0	31.25	1.50	Indifferent/2X
Ca#45	1.0	0.12	0.13	Synergistic/8X	0.5	15.62	1.06	Indifferent/16X
Ca#50	0.5	3.90	0.37	Synergistic/4X	0.5	3.90	0.24	Synergistic/8X
Ca#61	0.25	0.12	0.50	Synergistic/2X	0.125	31.25	1.01	Indifferent/64X
Ca#62	0.125	15.62	2.01	Indifferent/64X	0.125	15.62	2.01	Indifferent/64X
Ca#63	1.0	1.95	0.18	Synergistic/8X	0.125	15.62	0.51	Additive /64X
Ca#105	0.5	0.48	0.07	Synergistic/16X	0.25	31.25	1.03	Indifferent/32X
Ca#124	0.5	0.12	0.12	Siyergistic/ 8X	0.25	15.62	0.28	Synergistic/32X

Table 5. IC_{50} value and Selective Index (SI) of propyl caffeate (**C3**), fluconazole (FCZ) and nystatin (NYST) showing the toxicity of the compounds against yeasts in relationship to human oral keratinocytes (NOK cells).

Compound	IC_{50} (µg/mL)	SI (alone)	SI (combination)
C3	420	13.5	26.9 (FCZ) / 13.5 (NYST)
FCZ	320	40	320
NYST	400	50	100

Highlights

- Caffeic acid and eight of its derivative esters were tested for their activity against *Candida* spp.
- Propyl caffeate (**C3**) was the most active compound against reference strain and clinical isolates.
- When combined, **C3** markedly reduced the MIC of fluconazole and nystatin up to 64-fold.
- **C3** showed low toxicity against oral keratinocytes and high selectivity for yeast cells.
- Further research should focus on its combined therapy to treat *C. albicans*-related infections.