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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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ABSTRACT

We tested the antifungal potential of caffeic acid and 8 of its derivative esters against *Candidaalbicans* ATCC 90028 and 9 clinical isolatesand 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 indexagainst 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.

1. 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 coaggregate 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 (Al Mubarak et al., 2013; Hannula et al., 2001; Reynaud et al., 2001). 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 (Manfredi et al., 2006; Soll, 2002; Tekeli et al., 2004).

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 (Fernandez-Ruiz et al., 2015; Freitas et al., 2015; Liao et al., 2015; Seifi et al., 2015; Ying et al., 2013). 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 Food and Drug Administration–approved chemotherapeutic drugs were either natural products or directly derived therefrom (Newman and 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,to improve efficacy, potency, safety, tolerabilityand 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

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against resistant strains, in particular standard drugs with naturallyoccurring agents (Han et al., 2016; Pippi et al., 2015).

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(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 8 of its derivative esters against *C. albicans* ATCC 90028 and 9 oral clinical isolates. The most active molecule, propyl caffeate (C3), was selected for a synergism assay with fluconazole and nystatin against the *C. albicans* strainsand tested for its toxicity on oral keratinocytes (NOK cells).

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

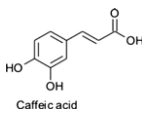
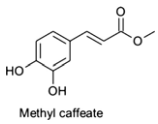
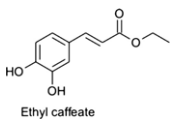
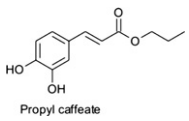
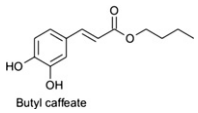
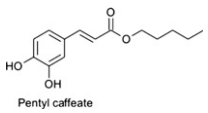
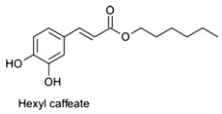
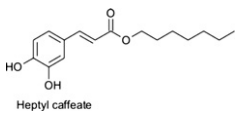
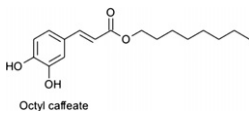
Code	Nomenclature	Molecular formula	Chemical structure
C0	Caffeic acid	$C_9H_8O_4$	 Caffeic acid
C1	Methyl caffeate	$C_{10}H_8O_4$	 Methyl caffeate
C2	Ethyl caffeate	$C_{11}H_{12}O_4$	 Ethyl caffeate
C3	Propyl caffeate	$C_{12}H_{14}O_4$	 Propyl caffeate
C4	Butyl caffeate	$C_{13}H_{16}O_4$	 Butyl caffeate
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 *C.albicans* ATCC 90028.

Caffeic acid derivates	*C.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

2. Materials and methods

2.1. Synthesis of esters

Caffeic acid (0.2 mmol/L) solution and corresponding alcohols (20 mmol/L) were prepared at 5 °C with a solution of *N*,*N*′-dicyclohexylcarbodiimide (1.0 mmol/L) 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 3times with EtOAc and filtered. The filtrate was serially washed with saturated aqueous citric acid solution (3 times), saturated aqueous $NaHCO_3$ (3times), water (2times), 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.

2.2. Microorganisms

C.albicans ATCC 90028 strain and 9 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).

2.3. Determination of antifungal activity

TheMICof caffeic acid and its 8 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 9 clinical isolates of *C. albicans*. The synthetic compounds of caffeic acid were diluted in DMSO and tested in concentrations ranging from 250to 0.48 μg/mL (Scorzoni et al., 2007). The inoculum was prepared (λ 530 nm, Abs 0.08–0.1) and diluted to 2.5×10^3 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, StLouis, MO, USA). Aliquots from the wells corresponding to the MIC and higher concentrations were subcultured 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.

2.4. 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

Table 3
Antifungal activity of caffeic acid derivative esters (C3, C4, and C6) against 9 oral clinical isolates of *C.albicans*.

C. albicans clinical isolates	C3		C4		C6		Fluconazole		Nystatin	
	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

MIC/MFC values are expressed in micrograms per milliliter.

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 ≤ 1.0), indifferent (1.0 < FICI < 4.0), and antagonistic (FICI ≥4.0) (Soares et al., 2014).

2.5. 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 (DrKarl Munger). NOK cells were maintained in culture medium for keratinocytes without fetal bovine serum (Gibco; Life Technologies) at 36.5 °C 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 10 μL of MTT solution (5 mg/mL) (Sigma-Aldrich) to each well. The plates were incubated at 37 °C 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, standarddrugs, and their combinations. The IC_{50} 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) (IC_{50}/MIC ratio), which was used as an indicator of potential toxicity against normal cells lines at the effective therapeutic concentration for each strain. Hence, theSIindicated the relationship between drug toxicity against *C. albicans* and the host cells (Gullo et al., 2012; Mora-Navarro et al., 2016).

2.6. 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 1-way analysis of variancefollowed by Bonferroni's multiple comparison test, with a significance level of 5%.

3. Results

3.1. 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 9 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 from 7.81 to 62.5 μg/mL. The MIC and MFC of C4 and C6 ranged from 31.25 to 125 μg/mL and 7.81 to ≥250 μg/mL, respectively.

Table 4
Antifungal effects of propyl caffeate (C3) combined with fluconazole and nystatin against *C.albicans* ATCC 90028 and 9 oral clinical isolates.

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/2×	0.125	7.81	0.53	Additive/32×
Ca#22	0.125	15.62	2.01	Indifferent/64×	0.125	15.62	2.01	Indifferent/64×
Ca#25	0.5	0.12	0.06	Synergistic/16×	4.0	31.25	1.50	Indifferent/2×
Ca#45	1.0	0.12	0.13	Synergistic/8×	0.5	15.62	1.06	Indifferent/16×
Ca#50	0.5	3.90	0.37	Synergistic/4×	0.5	3.90	0.24	Synergistic/8×
Ca#61	0.25	0.12	0.50	Synergistic/2×	0.125	31.25	1.01	Indifferent/64×
Ca#62	0.125	15.62	2.01	Indifferent/64×	0.125	15.62	2.01	Indifferent/64×
Ca#63	1.0	1.95	0.18	Synergistic/8×	0.125	15.62	0.51	Additive/64×
Ca#105	0.5	0.48	0.07	Synergistic/16×	0.25	31.25	1.03	Indifferent/32×
Ca#124	0.5	0.12	0.12	Synergistic/8×	0.25	15.62	0.28	Synergistic/32×

MIC values are expressed in micrograms per milliliter.
C3 = propyl caffeate; FCZ = fluconazole; NYST = nystatin.

Table 5
IC_{50} value and SIof propyl caffeate, fluconazole, and nystatinshowing 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

3.2. 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 2 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 2 of the 10 strains when compared with the MIC values of the compounds tested alone, with potentiation of nystatin by 8- to 32-fold. Additive activity was observed against 1 clinical isolate and *C. albicans* ATCC 90028 strain, with FICI values ranging from 0.51 to 0.53. The additive activity of the combination showed potentiation of nystatin between 32- and 64-fold.

3.3. 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).

Fig. 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 ($P < 0.05$).

4. Discussion

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

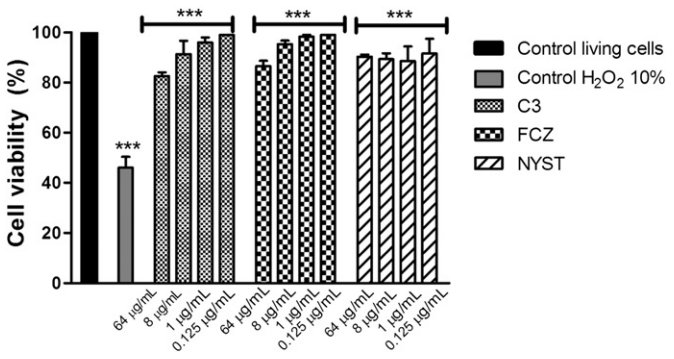


Fig. 1. Representation of the viability of oral mucosa keratinocytes (NOK cells) after treatment with the ester derivative of caffeic acid (C3), fluconazole and nystatin. Statistical analysis was performed by GraphPad Prism software 5.0v, One way ANOVA with Bonferroni post-test's multiple comparison test. *** P <0.05.

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 2–8 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 and 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 et al., 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 Han and Lee (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, fluconazole, and micafungin (Aprotosoaie et al., 2008; Rodrigues et al., 2012; Rosato et al., 2009; 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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