Diagnostic Microbiology and Infectious Disease 86 (2016) 387–391

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

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

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ARTICLE INFO

Article history:
Received 7 March 2016
Received in revised form 11 July 2016
Accepted 5 August 2016
Available online 8 August 2016

Keywords:
Synergism
Candida albicans
Caffeic acid
Antifungal

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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http://dx.doi.org/10.1016/j.diagmicrobio.2016.08.002
0732-8893/

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.

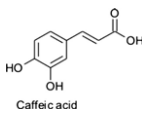
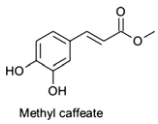
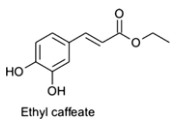
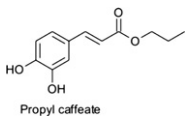
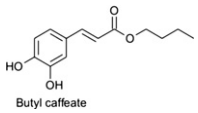
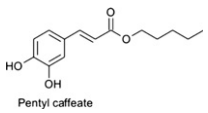
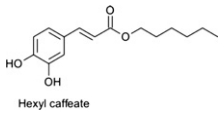
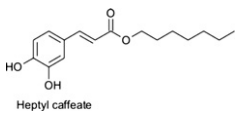
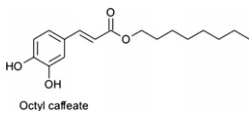
Code	Nomenclature	Molecular formula	Chemical structure
C0	Caffeic acid	$C_9H_8O_4$	 Caffeic acid
C1	Methyl caffeate	$C_{10}H_8O_4$	 Methyl caffeate
C2	Ethyl caffeate	$C_{11}H_{12}O_4$	 Ethyl caffeate
C3	Propyl caffeate	$C_{12}H_{14}O_4$	 Propyl caffeate
C4	Butyl caffeate	$C_{13}H_{16}O_4$	 Butyl caffeate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