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Short Communication

Utilising polyphenols for the clinical management of *Candida albicans* biofilms

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ABSTRACT

Polyphenols (PPs) are secondary metabolites abundant in plant-derived foods. They are reported to exhibit antimicrobial activity that may offer an alternative to existing antimicrobials. The aim of this study was to evaluate the antifungal potential of PPs against *Candida albicans* biofilms that are commonly recalcitrant to antifungal therapy. The antifungal activity of 14 PPs was assessed in terms of planktonic and sessile minimum inhibitory concentrations (PMICs and SMICs, respectively) against various *C. albicans* clinical isolates. The most active PPs were further tested for their effect on *C. albicans* adhesion and biofilm growth using standard biomass assays, microscopy and quantitative gene expression. Of the 14 PPs tested, 7 were effective inhibitors of planktonic growth, of which pyrogallol (PYG) was the most effective ($PMIC_{50}$ = 78 μg/mL), followed by curcumin (CUR) ($PMIC_{50}$ = 100 μg/mL) and pyrocatechol ($PMIC_{50}$ = 625 μg/mL). Both PYG and CUR displayed activity against *C. albicans* biofilms ($SMIC_{50}$ = 40 μg/mL and 50 μg/mL, respectively), although they did not disrupt the biofilm or directly affect the cellular structure. Overall, CUR displayed superior biofilm activity, significantly inhibiting initial cell adhesion following pre-coating ($P < 0.01$), biofilm growth ($P < 0.05$) and gene expression ($P < 0.05$). This inhibitory effect diminished with prolonged CUR exposure, although it still inhibited by 50% after 4 h adhesion. Overall, CUR exhibited positive antibiofilm properties that could be used at the basis for development of similar molecules, although further cellular and in vivo studies are required to explore its precise mechanism of action.

1. Introduction

Candida albicans is an opportunistic fungal pathogen most commonly associated with superficial infections of the oral cavity, although in immunocompromised individuals it can cause life-threatening forms of invasive candidiasis [1]. An important contributing factor in *C. albicans* pathologies is its flexibility in adapting to different environmental conditions by virtue of its morphological plasticity that enables growth preferentially as biofilms, which provides protection both from host defences and antimicrobial therapies [2]. Therefore, alternative approaches to manage and impede these infections more effectively are highly desirable.

Recently, natural compounds have been reported to demonstrate antibiofilm activity [3], which is important given that the likelihood of developing resistance to these molecules is low. Therefore, harnessing and exploiting the chemical diversity provided by Mother Nature's larder as a means of combating oral candidal infections is an attractive option [4]. Polyphenols (PPs) are an abundant source of macromolecular structures containing phenolic hydroxyl rings that can be found in various diets naturally, but are also found as synthetic and semisynthetic compounds. The aim of this study was to explore the possibility that PPs prevent growth and biofilm formation by *C. albicans*. We report for the first time the antifungal activity of specific PPs with biofilm inhibitory potential, acting through modulation of proteins responsible for adhesion and biofilm formation.

2. Materials and methods

2.1. Strains and culture conditions

Candida albicans reference strains MYA-2876 (SC5314) and MYA-4788 (3153A) as well as a range of oral (n = 6), denture (n = 6)

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and bloodstream ($n = 6$) clinical isolates from the culture collection of Glasgow Dental School (University of Glasgow, Glasgow, UK) were used in this study, which were confirmed using an API® 32C biochemical testing panel (bioMérieux UK Ltd., Basingstoke, UK). Isolates were propagated in yeast–peptone–dextrose medium (Oxoid Ltd., Cambridge, UK), washed in phosphate-buffered saline (PBS) (Sigma–Aldrich, Poole, UK) by centrifugation and then re-suspended in RPMI 1640 medium (Sigma–Aldrich).

2.2. Planktonic and sessile antifungal sensitivities

Fourteen high-performance liquid chromatography (HPLC)-grade PP compounds purchased from Sigma–Aldrich were used in this study (Table 1). Depending on their solubility, fresh stock concentrations of each PP compound were prepared either in double-distilled water or dimethyl sulphoxide (DMSO). Dilutions of the stock were made in RPMI 1640 medium, with the final concentration of DMSO adjusted to <5% (v/v). The antifungal activity of these PPs against 20 *C. albicans* was evaluated in terms of their planktonic minimum inhibitory concentration (PMIC) and planktonic minimum fungicidal concentration (PMFC) by the broth microdilution method following Clinical and Laboratory Standards Institute (CLSI) guidelines [5]. The PMIC was defined as the lowest concentration of PP that inhibited visible growth, and the PMFC was determined as the lowest concentration giving no visible growth from culture medium transferred to Sabouraud dextrose agar (Sigma, Poole, UK). Sessile minimum inhibitory concentrations (SMICs) were defined as a ≥50% reduction in metabolic activity compared with the untreated control [3]. The $PMIC_{50}$, $PMFC_{50}$ and $SMIC_{50}$ were defined as the concentrations at which the median number of isolates tested was inhibited, killed or metabolically reduced, respectively.

2.3. Evaluating the physical effects of polyphenols

To assess the effect on membrane integrity, a propidium iodide (PI) uptake assay was performed. *Candida albicans* SC5314 was standardised in RPMI 1640 medium to 1×10^7 cells/mL in a haemocytometer as described in our previous study [3]. Cells were then treated with 4× $SMIC_{50}$ of the two most effective PPs [curcumin (CUR) and pyrogallol (PYG)] every 10 min over 1 h. As a positive control, 70% ethanol (Sigma) was used. Following individual treatments, cells were centrifuged, washed in PBS and stained with PI (20 μM) for 15 min in darkness. PI uptake was then quantified by transferring 100 μL of the solution into a Corning® Costar® flat-bottom, 96-well, black microtitre plate (Sigma) and measuring fluorescence at excitation and emission wavelengths of 535 nm and 617 nm, respectively, using a microplate reader (FLUOstar Omega; BMG Labtech, Aylesbury, UK). To assess biofilm disruption, an established crystal violet (CV) biomass assay was performed [3]. Briefly, *C. albicans* SC5314 biofilms were grown for 24 h and were treated with CUR and PYG for 24 h, after which these were washed with PBS, air-dried and stained with 0.05% (w/v) CV (Sigma) for 20 min, and then quantified by destaining with 100% ethanol and measuring the absorbance at 570 nm (Tecan Sunrise™; Jencons, East Grinstead, UK). Scanning electron microscopy was also performed on treated *C. albicans* SC5314 and untreated controls as previously described [3].

2.4. Gene expression analysis of biofilm-associated genes

The effect of PPs on *C. albicans* SC5314 adhesion (*ALS3*) and filamentation (*HWP1*) was analysed by quantitative transcriptional analysis. Standardised cells (1×10^8 cells/mL) were placed in a 50 mL tube on an orbital shaker (200 rpm) at 37 °C in the presence of CUR or PYG at 1× $SMIC_{50}$, or in RPMI alone, for durations of 1, 4 and 24 h, in triplicate. At each time point, cells were removed, washed by centrifugation with PBS, and RNA was extracted using the TRIzol® method (Invitrogen, Paisley, UK) [6]. After DNase treatment (QIAGEN, Crawley, UK) and purification (RNeasy MinElute Cleanup Kit; QIAGEN), cDNA was synthesised using a High-Capacity RNA-to-cDNA™ Kit (Life Technologies, Paisley, UK), and quantitative PCR (qPCR) was performed using a SYBR® GreenER™ assay (Life Technologies). The primers and conditions used for real-time qPCR throughout this study are described elsewhere [7]. Each parameter was analysed in duplicate using an MxProP Quantitative PCR machine and MxProP 3000 software (Stratagene, Amsterdam, The Netherlands). Gene expression was normalised to the housekeeping gene *act-1* according to the $2^{-\Delta\Delta CT}$ method, and the percentage of gene expression was expressed as the $\log_{10}$ mean ± standard deviation [8].

2.5. Evaluating the effect of polyphenols on Candida albicans biofilm development

Thermanox™ coverslips (Nunc Inc., Thermo Fisher Scientific, Paisley, UK) were placed in a Corning® Costar® 24-well, flat-bottom plate (Sigma) containing SMIC concentrations of CUR or PYG and were incubated overnight at 4 °C. Unbound compound was removed prior to adding 500 μL of standardised suspensions of *C. albicans* SC5314 (1×10^6 cells/mL) and allowing them to adhere for 30 min at 37 °C. Following incubation, coverslips were gently washed in PBS and were transferred to a glass slide for quantification of cell numbers under a light microscope (Model BX40F4; Olympus, Tokyo, Japan) at 40× magnification. Cells were counted manually from three separate fields, averaged, and the cell count for the whole surface area of the coverslip was calculated. The effect of CUR and PYG on hyphal growth and on the initial stages of biofilm formation were also evaluated by plating a standardised (1×10^6 cells/mL) *C. albicans* suspension in 96-well, flat-bottom microtitre plates. At 0, 1, 2 and 4 h postadhesion, cells were treated with 1× $SMIC_{50}$ of CUR or PYG and were incubated for a further 24 h at 37 °C. The resultant biofilm biomass was quantified using the CV assay and was compared with untreated controls. This experiment was performed on two independent occasions using six replicates of each strain ($n = 5$).

2.6. Statistical analysis

Data distribution and statistical analysis were performed using GraphPad Prism v.5 (GraphPad Software Inc., La Jolla, CA). Unpaired *t*-test was used to assess the difference between two independent samples. Student's *t*-test was employed to measure statistical differences between the time points assessed in gene expression studies. One-way analysis of variance (ANOVA) with Tukey's post hoc test was used on data obtained from the PI uptake assay and biofilm inhibition assay.

3. Results

3.1. Curcumin and pyrogallol possess antifungal activity against Candida albicans

In total, 14 PPs were tested for their antifungal potential against *C. albicans* (Table 1). Seven PPs were shown to be effective at inhibiting planktonic growth of *C. albicans* (CUR, PYG, pyrocatechol, quercetin, gallic acid, caffeic acid and naringenin). PYG ($PMIC_{50}$ = 78 μg/mL) and CUR ($PMIC_{50}$ = 100 μg/mL) were the most effective, showing dose-dependent inhibition of growth at 1×, 2× and 4× MIC (data not shown). The remaining compounds showed no discernible effect on planktonic growth, both with respect to