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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) 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 (MFC) 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 MFC was determined as the lowest concentration giving no visible growth from culture medium transferred to Sabouraud dextrose agar (Sigma, Poole, Dorset, 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 $4\times$ $SMIC_{50}$ of the two most effective PPs [curcumin (CUR) and pyrogallol (PYG)] every 10 min over 1 h. As a positive control, 70% ethanol (Sigma, Poole, Dorset, UK) 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, Poole, Dorset, UK) and measuring fluorescence at excitation and emission wavelengths of 535 nm and 617 nm, respectively, using a microplate reader (FLUOstar Omega; BMG Labtech, Aylesbury, Buckinghamshire, 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, Poole, Dorset, UK) 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× $SMIC_{50}$ concentrations, 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 Ltd.). The primers and conditions used for real-time quantitative polymerase chain reaction (RT-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^{TM} coverslips (Nunc Inc., Thermo Fisher Scientific, Paisley, UK) were placed in a Corning® Costar® 24-well, flat-bottom plate (Sigma, Poole, Dorset, UK) 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, Shinjuku, 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 post adhesion, cells were treated with a 1× $SMIC_{50}$ concentration 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 inhibition (PMIC) and killing (PMFC). The PMFC generally ranged from 2–8-fold higher than the PMIC for PYG and CUR.

Of all the PPs, PYG and CUR displayed potent activity against sessile *C. albicans* (Table 1), with PYG being more effective ($SMIC_{50}$ = 39.06 μg/mL) than CUR ($SMIC_{50}$ = 50 μg/mL), although neither of these compounds could significantly reduce and disrupt biofilm biomass [88.76% (P = 0.19) and 87.27% (P = 0.29), respectively] compared with the control (100%). The remaining PPs tested displayed relatively poor antifungal activity against sessile growth of the test strains, with all growing at concentrations exceeding the maximum solubility of each PP. Therefore, on this basis, all further testing was performed only with only PYG and CUR. It was also shown that neither PYG or CUR had any discernable effects of membrane integrity

using a PI uptake assay, where neither showed any significant PI uptake over 1-h exposure of compound, whereas significant PI uptake was observed after 10 min for ethanol-treated cells ($P < 0.001$).

3.2. Curcumin and pyrogallol affect Candida albicans biofilm development

We investigated whether transcription of genes involved in adhesion and hyphal formation, critical factors for the development of *C. albicans* biofilms [9], were impacted by CUR and PYG treatment using qRT-PCR. Expression of the adhesin *als3* was shown to be significantly downregulated by CUR at 1 h ($P < 0.05$) and 24 h ($P < 0.001$), whereas PYG showed no significant downregulation of *als3* ($P > 0.05$) following treatment (Fig. 1). These effects were also evaluated using phenotypic assays, where it was demonstrated that pre-coating coverslips with CUR significantly reduced *C. albicans* adhesion by 55.3% ($P < 0.01$), whereas PYG coating only led to a slight reduction in adhesion of 15.63% ($P > 0.05$). Analysis of the filamentation-associated gene *hwp1* showed a significant downregulation for CUR at all time points tested (1 h, $P < 0.01$; 4 h, $P < 0.05$; 24 h, $P < 0.001$). In contrast, PYG did not significantly alter *hwp1* expression, although a 16-fold downregulation was observed at 4 h (Fig. 1). Microscopy analysis confirmed this, where both PYG and CUR can clearly be observed to impede filamentation and biofilm formation (Fig. 2A).

The effect of delayed treatment on *C. albicans* biofilm formation was also explored. Fig. 2B illustrates that compared with the untreated control cells, both CUR and PYG were able to significantly reduce biofilm biomass when added at 0, 1, 2 ($P < 0.001$) and 4 h ($P < 0.01$) post adhesion. These effects were shown to diminish the longer *C. albicans* was permitted to colonise, form hyphae and initiate biofilm formation.

CUR was shown to inhibit biofilm development by 80% following early co-incubation (0 h), although this was significantly reduced to 50% if added 4 h post inoculation ($P < 0.05$). PYG was less effective, inhibiting biofilm development by 70% initially at 0 h, but only by 20% after 4 h. Significant differences between the two compounds were observed at 2 h ($P < 0.01$) and 4 h ($P < 0.05$).

4. Discussion

The past two decades have witnessed a significant increase in the prevalence of multidrug-resistant *C*. *albicans* strains, which is partly due to increased usage of conventional antifungal agents along with a rise in the number of immunocompromised individuals [1]. Here we report the potential for PPs to be used against planktonic and sessile growth of clinically important *C. albicans*. We have shown that CUR and PYG were the most active of the tested PPs, inhibiting growth and biofilm formation of *C. albicans* via suppression of genes responsible for adhesion and hyphal growth. To the best of our knowledge, this is the first study to explore the antifungal potential of PP compounds (flavonoids and non-flavonoids) both against planktonic and biofilm growth of *C. albicans* laboratory and clinical isolates.

Of all the PPs tested, CUR and PYG possess the greatest antifungal activity against planktonic *C. albicans* cells including both clinical and laboratory isolates, as evidenced by their lower MIC values. This was largely in agreement with other studies [10], although published data on PYG is limited. In fact, data in the current study showed lower MIC values compared with previous studies (>180 μg/mL) [11]. This may have been due to intra-assay variation, including methodology, purity and

source of PPs, and fungal strains used. The most notable feature of this study was the activity against biofilms. Both CUR and PYG compounds effectively killed sessile *C. albicans*, although they showed no direct physical effects per se when evaluated through membrane disruption and microscopically at high concentrations. Others have suggested that prolonged exposure at high concentrations (185 μg/mL) permeabilises membranes and disrupts cell wall integrity {Kumar, 2013 #10}, although we did not test these concentrations in this study.

Although PYG numerically has lower MICs, these are very minor compared with CUR, which may be a limitation of broth microdilution testing. The superior inhibitory activity of CUR in the subsequent biofilm assays may be attributed to its chemical structure. Compared with PYG (containing only one phenol group in its structure), CUR has two phenol groups connected by two α,β-unsaturated carbonyl groups. In addition, the presence of two unsaturated double bonds in the central connecting chain may further potentiate its activity [13], although further studies are required to elucidate the precise mechanism. Notably, downregulation of key biofilm genes, such as the adhesin *als3* and the hyphal protein *hwp1*, explains the impeded biofilm phenotype. Delay in exposure to these molecules has a negative impact on the effectiveness of the PPs. Despite this, it has been shown that CUR possess synergistic potential with other antifungal compounds [11], suggesting that these could be used to augment the treatment of oral candidosis for example.

PPs are ubiquitous in plant foods, with CUR readily available in common foods such as dried turmeric and curry powder. However, intake, bioavailability and safety assessments are important considerations. PYG is available in low amounts in cocoa

products, coffee and beer, and whilst the brief exposure of the oral mucosa to PPs during food intake may not be sufficient for potential therapeutic purposes, their high affinity for surface adsorption, including oral tissues, may be important [14]. Moreover, although bioavailability of PPs may be low, some PP metabolites may also appear in saliva, adding to their overall localised bioactivity in the oral cavity. CUR has been shown to possess adsorption potential and has been extensively used for controlled delivery of protein molecules [15]. Adsorption properties can be employed in increasing the local (oral) availability of CUR at the site of action and preventing initiation of biofilm formation on tissue surfaces and prosthetic devices.

Pharmaceutically and dietary, there are clear opportunities for the development of oral care products containing PPs. Whilst CUR and similar PPs are tantalising targets for development owing to their natural background, further cellular studies are required to progress these molecules for the treatment and prevention of fungal, and possibly bacterial, diseases in humans.

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Table 1| Activity of polyphenols against planktonic and sessile *Candida albicans*

Test polyphenol	Solvent	Stock (μg/mL)	Test range (μg/mL)	Minimum inhibitory concentration (μg/mL)					
				PMIC		PMFC		SMIC	
				$PMIC_{50}$ [a]	Range	$PMFC_{50}$ [a]	Range	$SMIC_{50}$ [a]	Range
Epigallocatechin	ddH_2O	2000	0.976–500	>500	˃500	˃500	˃500	˃500	˃500
Epigallocatechin gallate	ddH_2O	2000	1.95–1000	>1000	˃1000	˃1000	˃1000	˃1000	˃1000
Curcumin	DMSO	2000	0.19–100	100	12.5 to >100	>100	100 to ˃100	50	12.5 to >100
Apigenin	DMSO	1000	7.81–125	˃125	˃125	˃125	˃125	˃125	˃125
Hesperetin	DMSO	5000	0.976–500	˃500	˃500	˃500	˃500	˃500	˃500
Naringenin	DMSO	5000	0.976–500	˃500	500 to ˃500	˃500	˃500	˃500	˃500
Pyrocatechol	ddH_2O	10000	9.76–5000	625	312.5–5000	2500	625–5000	2500	312.5 to ˃5000
Pyrogallol	ddH_2O	10000	9.76–5000	78.12	19.53–625	635	156.2 to ˃5000	39.06	9.76–156.25
Gallic acid	ddH_2O	10000	9.76–5000	˃5000	2500 to ˃5000	˃5000	2500 to ˃5000	˃5000	˃5000
3,4-dihydroxyphenylacetic acid	ddH_2O	10000	9.76–5000	˃5000	˃5000	˃5000	˃5000	˃5000	˃5000
Caffeic acid	DMSO	2000	1.95–1000	>1000	1000 to >1000	>1000	1000 to >1000	>1000	>1000
Quercetin	DMSO	2000	0.39–200	>200	50 to >200	>200	100 to >200	>200	>200
Malvidin	ddH_2O	1000	0.976–500	˃500	˃500	˃500	˃500	˃500	˃500
Pelargonidin	DMSO	5000	4.88–2500	˃2500	˃2500	˃2500	˃2500	˃2500	˃2500

ddH_2O, double-distilled water; DMSO, dimethyl sulphoxide; PMIC, planktonic minimum inhibitory concentration; PMFC, planktonic minimum fungicidal concentration; SMIC, sessile minimum inhibitory concentration. [a] The $PMIC_{50}$, $PMFC_{50}$ and $SMIC_{50}$ represent the concentration at which the median number of strains from a total of 20 were either inhibited (planktonic), killed (fungicidal) or their metabolic activity was reduced by 50% (sessile).

Fig. 1. Effect of curcumin (CUR) and pyrogallol (PYG) on expression of *Candida albicans* adhesin (*als3*) and hyphal (*hwp1*) genes. Standardised *C. albicans* SC5314 (1×10^8 cells/ml) was grown for 1, 4 and 24 h in ±1× $SMIC_{50}$ of CUR or PYG in RPMI 1640 medium. RNA was extracted and purified for quantitative RT-PCR analysis and expression of *als3* and *hwp1* using *act-1* as a housekeeping gene. Error bars represent the standard error of mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 1.

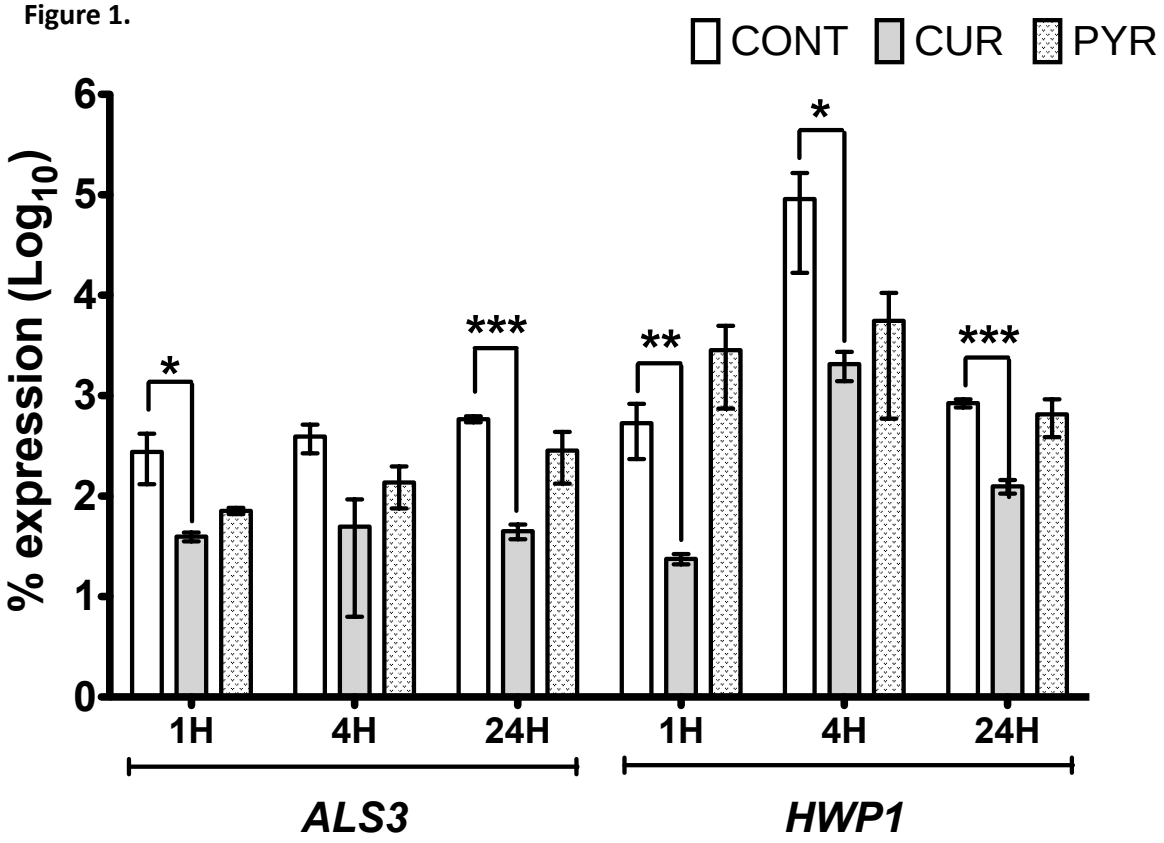

Fig. 2. Effect of pre-exposure to curcumin (CUR) and pyrogallol (PYG) on *Candida albicans* biofilm formation. Standardised *C. albicans* (1×10^6 cells/mL) were added to either Thermanox™ coverslips pre-coated with 1× SMIC of CUR and PYG or to a 96-well, flat-bottom plate treated with ±1× $SMIC_{50}$ of CUR and PYG at 0, 1, 2, and 4 h post adhesion and allowed to grow for a further 24 h. The resultant biofilm was assessed (A) visually by scanning electron microscopy or (B) the biomass was quantified using five isolates on two independent occasions (*n* = 6). Error bars represent the standard error of mean. (** *P* < 0.01;* *P* < 0.05).

Figure 2