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Effect of different pre-irradiation times on curcumin-mediated photodynamic therapy against planktonic cultures and biofilms of *Candida* spp

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ABSTRACT

Objectives: The aim of this study was to evaluate the effects of pre-irradiation time (PIT) on curcumin (Cur)-mediated photodynamic therapy (PDT) against planktonic and biofilm cultures of reference strains of *Candida albicans*, *Candida glabrata* and *Candida dubliniensis*.
Materials and methods: Suspensions and biofilms of *Candida* species were maintained in contact with different concentrations of Cur for time intervals of 1, 5, 10 and 20 min before irradiation and LED (light emitting diode) activation. Additional samples were treated only with Cur, without illumination, or only with light, without Cur. Control samples received neither light nor Cur. After PDT, suspensions were plated on Sabouraud Dextrose Agar, while biofilm results were obtained using the XTT-salt reduction method. Confocal Laser Scanning Microscopy (CLSM) observations were performed to supply a better understanding of Cur penetration through the biofilms after 5 and 20 min of contact with the cultures.
Results: Different PITs showed no statistical differences in Cur-mediated PDT of *Candida* spp. cell suspensions. There was complete inactivation of the three *Candida* species with the association of 20.0 μM Cur after 5, 10 and 20 min of PIT. Biofilm cultures showed significant reduction in cell viability after PDT. In general, the three *Candida* species evaluated in this study suffered higher reductions in cell viability with the association of 40.0 μM Cur and 20 min of PIT. Additionally, CLSM observations showed different intensities of fluorescence emissions after 5 and 20 min of incubation.
Conclusion: Photoinactivation of planktonic cultures was not PIT-dependent. PIT-dependence of the biofilm cultures differed among the species evaluated. Also, CLSM observations confirmed the need of higher time intervals for the Cur to penetrate biofilm structures.

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1. Introduction

Species of the genus *Candida* are considered commensal yeasts frequently isolated from the oral cavity of healthy patients.[1–3] However, these microorganisms can act as opportunistic pathogens under certain circumstances, such as impairment of salivary glands, long-term use of immunosuppressive drugs and antibiotics, denture wear, and malignancies.[4,5] *Candida albicans* is the most commonly isolated species, being present in around 20–50% of the cases of oral infections.[6] Recently, infections with species other than *C. albicans*, notably *Candida glabrata and Candida dubliniensis* have been increasingly described.[7–9] *C. glabrata* has become the second most frequently isolated commensal yeast from the oral cavity,[2,7,8] and it is responsible for 15% of mucosal lesions.[2] *C. dubliniensis* is a recently described species of the genus *Candida*[10] primarily associated with oral candidiasis[11] in acquired immunodeficiency syndrome (AIDS) patients.

Denture stomatitis is a common superficial infection of the palate oral mucosa that affects more than 65% of denture wearers.[12] This condition develops under the influence of denture plaque, which consists of a structured community of microorganisms surrounded by a self-produced polymeric matrix and adherent to an inert or living surface.[13] Studies[14,15] have demonstrated that different species of yeast and bacteria are associated with denture biofilm, including *Candida* spp., *Staphylococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Pseudomonas* spp., *Enterobacter* spp. and *Actinomyces* spp. Clinically, denture stomatitis is characterised by erythematous points on the denture-bearing tissues and diffuse erythema.[16] The most susceptible hosts are the elderly, who concomitantly wear dentures and use immunosuppressive medications or prophylactic antifungal agents, which can promote substantial switching of the oral ecology,[17] and further facilitate the installation[6] and dissemination of opportunistic infections.[22]

Oral candidiasis may be treated with either topical[19,20] or systemic[21] antifungal therapy, according to the severity of the infection. The therapy of choice for immunocompromised patients is usually a course of systemic antifungal agents such as fluconazole or amphotericin B.[6] However, some conventional antifungal drugs, such as azoles, present fungistatic activity rather than fungicidal, resulting in an inadequate treatment outcome for immunocompromised patients.[9,22,23] Therefore, recurrent candidiasis is common, and retreatments are often needed. In this context, *C. glabrata* and *C. dubliniensis* are of special importance because of the innate resistance to antifungal agents of the former,[2,9] and the ability of the latter to develop rapid *in vitro* stable fluconazole resistance.[23–25]

With the increase of microbial resistance, many researchers have focused on finding non-conventional therapies to treat oral infections. Photodynamic therapy (PDT) is a promising therapeutic method[26–30] originally developed for the treatment of tumours.[28] Recently, PDT has been investigated to treat other pathologies such as viral, fungal and bacterial infections.[28,30] Although PDT does not replace conventional systemic antimicrobial therapy, improvements may be obtained using the photodynamic approach in the clinical treatment of local infection.[30] PDT involves the application of a photoactive drug denominated photosensitiser (PS) and its exposure to a light source with appropriate wavelength to activate the PS. After the absorption of photons, and in the presence of oxygen, an excited state of the PS can be generated.[28] These events result in a cytotoxic photodynamic reaction, involving the production of reactive oxygen species and sequential oxidative reactions, which lead to cell death.[31] It seems that PDT acts primarily against the cell membrane, and after increasing its permeability, the PS moves into the interior of the cell, and damages the intracellular organelles.[32,33] Therefore, differently from conventional antifungal drugs, whose mode of action is limited to a single target,[23] PDT acts against several targets, thus it is unlikely that resistance will emerge.[34] Among other factors, successful PDT depends on the pre-irradiation time (PIT),[35] which is the time required by the PS to remain in contact with the target cells before irradiation. This period will enable the PS to bind to the cytoplasmic membrane and/or penetrate into the cells.[33,34] The following exposure to light will allow the PSs to exert their function in promoting cell death.

Many researchers have focused their attention on effective PSs for the photoinactivation of microorganisms.[26,27,29,32–45] Curcumin (Cur) is a yellow-orange dye extracted from the rhizomes of the plant *Curcuma longa*.[46] It is commonly used as a spice in traditional Asian cookery, and has been shown to exhibit a variety of pharmacological properties such as antitumor, anticancer, anti-inflammatory, antioxidants, and antimicrobial activities,[18,46,47] some of which can be enhanced by light application.[44,48] Cur has been used as a PS in antimicrobial PDT, mainly on photoinactivation of *Candida* species, with positive results.[41] However, some studies have stated that in contrast to that which occurs with several PSs, Cur does not bind to cells, or binds to them weakly, leaving about 90% in an extracellular bulk phase.[37] The removal of the non-associated Cur promotes a substantial reduction in its phototoxic effects.[36,41]

The aim of this study was to evaluate the effects of PIT on curcumin-mediated PDT in the inactivation of planktonic and biofilm cultures of three *Candida* species: *C. albicans*, *C. glabrata*, and *C. dubliniensis*.

2. Materials and methods

2.1. Microorganisms

Two *Candida* strains obtained from American Type Culture Collection (ATCC) and one from the Centraal bureau voon Schimmelcultures (CBS) were evaluated in this study: *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 2001), and *C. dubliniensis* (CBS 7987). All three *Candida* strains were maintained in a freezer at −70 °C until the assay.

2.2. Photosensitiser and light source

Curcumin (Sigma–Aldrich, Saint Louis, Missouri, USA) was prepared with 10% of Dimethyl Sulfoxide (DMSO) to originate a stock solution, from which other solutions were prepared at final concentrations of 5.0, 10.0, 20.0, 30.0 and 40.0 μM. A light emitting diode (LED) was used to activate the PS. The LED

device emitted 22.0 mW/cm^2 of light intensity and 455 nm of predominant wavelength, and was designed at São Carlos Physics Institute, University of São Paulo, Brazil.

2.3. Planktonic cultures and photodynamic therapy

Aliquots of 25 μL of each microorganism were spread in Petri dishes containing Sabouraud Dextrose Agar with chloramphenicol (SDA) and were incubated for 48 h at 37 °C. After this, a loopful of each cultivated yeasts was individually subcultured in 5 mL of Tryptic Soy Broth (TSB) and grown aerobically *overnight* at 37 °C. Each culture tube was centrifuged at 4000 rpm for 7 min and the supernatants were discarded. The cells were then washed twice with sterile saline solution and centrifuged again. *Candida* suspensions were spectrophotometrically standardised to a concentration of 1×10^6 cells/mL. The resulting suspensions were used for all the further procedures. Aliquots of 100 μL of *Candida* standardised suspension were individually transferred to separate wells of a 96-well microtitre plate. After inoculation, an equal volume of diluted Cur solutions (100 μL) was added to the appropriate wells to give final concentrations of 5, 10 and 20 μM. After dark incubation of 1, 5, 10 and 20 min, the samples were irradiated on the LED device for 4 min, which corresponded to 5.28 J/cm^2 (P+L+).[41] To determine whether LED light alone had any effect on cell viability, additional samples were made with no PS (P−L+). The effect of Cur alone was also determined by exposing the yeast suspensions to the PS in an identical manner to those described above, but with no light exposure (P+L−). The suspensions that were not exposed to LED light or Cur acted as overall control (P−L−). All experiments were performed five times on two independent occasions. The microtitre plate containing the no-light samples was kept in the dark for 24 min, corresponding to the pre-irradiation time plus light exposure time. Ten-fold serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) were generated from the fungal suspensions and plated on SDA in duplicate. The plates were then aerobically incubated at 37 °C for 48 h. After incubation, yeast colony counts of each plate were quantified and the colony forming unit per millilitre (CFU mL^{-1}) was determined.

2.4. Biofilm cultures and photodynamic therapy

A loopful of recently cultivated yeast was subcultured in RPMI 1640 *overnight* in an orbital shaker (AP 56, Phoenix Ind Com Equipamentos Científicos Ltda, Araraquara, SP, Brazil) at 120 rpm and 37 °C. The cells grown were harvested by centrifugation at 4000 rpm for 7 min, and the supernatants were discarded. The pellet was washed twice in PBS, and finally resuspended in PBS. *Candida* suspensions were spectrophotometrically standardised to a concentration of 1×10^6 cells/mL. Aliquots of 100 μL of the resulting standardised *Candida* cell suspensions were transferred to appropriate wells of a 96-well microtitre plate and incubated at 37 °C in an orbital shaker (75 rpm). After 90 min of the adhesion phase, the supernatants were removed from the plate wells and gently washed twice with 150 μL of PBS to remove the non-adherent cells. Next, 150 μL of freshly prepared RPMI 1640 were added to each well and the plates were incubated in an orbital shaker for 48 h at 37 °C in order to generate single-species biofilms. After incubation, the wells were carefully washed twice with PBS to remove non-adherent cells. Aliquots of 150 μL of Cur at 20, 30 and 40 μM were added to each appropriate well directly onto the biofilm. The experimental conditions were identical to those of the planktonic cultures: P+L+, P−L+, P+L− and P−L−. All experiments were performed five times on three independent occasions. To estimate the viability of the yeasts and the effects of PIT on Cur-mediated PDT, the biofilm samples were evaluated by XTT reduction assay. XTT (Sigma, MO, USA) was prepared in ultrapure water at a final concentration of 1 mg/mL. The solution was filter sterilised and stored at −70 °C until use. Menadione (Sigma, MO, USA) solution was prepared in acetone at 0.4 mM immediately before each assay. After experimental procedures, 158 mL PBS with 200 mM glucose, 40 mL XTT and 2 mL menadione were inoculated to each well. The plates were incubated for 3 h[49] in the dark at 37 °C. The resulting colorimetric changes were considered to be proportional to the number of living cells and their metabolic activity. Aliquots of 100 μL of the supernatant were transferred to a new 96-well microtitre plate and measures were read by a microtitre plate reader (Thermo Plate—TP Reader) at 492 nm.

2.5. Confocal laser scanning microscopy observations

Biofilm cultures of *C. albicans*, and *C. glabrata* ATCC, and *C. dubliniensis* CBS in were formed on 8-mm round coverslips as described previously, and placed on confocal microscopy.[50] The biofilms were incubated at 37 °C for 48 h, and washed twice with PBS. Following 5 and 20 min of incubation with Cur 40 μM, the coverslips containing the biofilms were flipped and placed on a glass-bottom and observed using a Leica TCS SPE confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Curcumin excitation wavelength is known to be dependent of the solvents used.[51] The selection of the excitation and emission parameters for fluorescence analysis was made in accordance of a previous published paper which also used curcumin in 10%-DMSO saline solution.[41] Curcumin-treated biofilms were observed under fluorescence mode using a 405-nm excitation wavelength and a green fluorescence (emission from 450 to 600 nm). Corresponding Cur fluorescence allows observation of the biofilms cells. Serial sections in the *xy* plane were obtained at 1 μm intervals along the *z* axis.

2.6. Statistics

Statistical comparison among groups was performed within each species. The data obtained from the planktonic cultures were evaluated by Kruskal–Wallis test and complemented by Dunn test for multiple comparisons. For the biofilm groups, analysis of variance followed by Tukey's and the Student's-*t* test (using paired data) were used for evaluating the data obtained. *P* values of less than .05 were considered significant.

3. Results

3.1. Planktonic cultures

Fig. 1 shows the descriptive statistics, median, minimum and maximum, of calculated colony forming units transformed

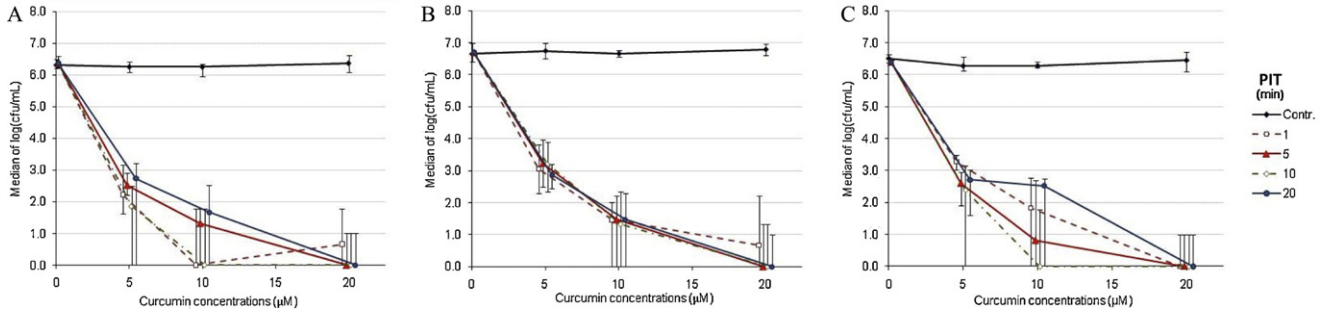

Fig. 1 – Graphic representation of median logarithms of CFU/mL of *C. albicans* (A), *C. glabrata* (B) and *C. dubliniensis* (C) cell suspensions. Vertical bar indicates minimum and maximum.

into their decimal logarithms. All the control groups maintained cell counts in the same order of magnitude (1×10^6 cells/mL) from the initial standardised suspensions ($p > 0.05$). Inactivation of *Candida* species was observed only when Cur and LED light were associated, which indicates the photodynamic effect. For the three species evaluated, no microbiological growth was observed after associating 20 μM Cur-mediated PDT with PITs of 5, 10 and 20 min. In addition, the PIT of 1 min was able to promote complete inactivation of *C. dubliniensis* (20 μM Cur-mediated PDT) and 89.5% reduction in *C. albicans* and *C. glabrata* CFU/mL.

Non-parametric statistics found significant differences between the P+L+ and control groups ($p < 0.001$). PIT presented no statistical differences irrespective of the Cur concentration tested. For *C. albicans*, the use of 1 and 10 min of PIT resulted in similar CFU/mL values among the three Cur concentrations tested ($p > 0.05$). However, when the PIT time intervals of 5 and 20 min were considered, 20 μM Cur promoted the highest reduction in cell counts, while 5 μM Cur presented the lowest reductions ($p < 0.05$). The concentration-dependence was also observed for *C. glabrata* and *C. dubliniensis* since 20 μM Cur always promoted the highest reduction in cell counts, and 5 μM Cur always presented the lowest reductions, irrespective of the pre-irradiation period ($p < 0.05$).

3.2. *Biofilm cultures*

Figs. 2–4 present mean values and 95% confidence intervals of the absorbance values (XTT) obtained for *C. albicans*, *C. glabrata* and *C. dubliniensis* (respectively) after experimental procedures with the biofilm cultures irradiated for 4 and 8 min. All the control groups presented significantly higher mean absorbances than the P+L+ groups, demonstrating that PDT in association of Cur and LED light had a significant effect on diminishing cell metabolism of all species evaluated.

The mean absorbance values for both 4 and 8 min irradiation groups were calculated and compared using the Student's-*t* test ($p < 0.05$). The results are presented in Fig. 5. In general, the use of 8 min of illumination resulted in lower absorbance values in comparison with those of the 4 min samples, but in some cases the difference was not statistically significant.

For *C. albicans* biofilms, the two-way analysis of variance of the P+L+ groups (irradiated for 4 and 8 min) indicated the significant effect of PIT ($p < 0.001$) and Cur concentration ($p < 0.001$), but no significant effect of the interaction of these factors ($p > 0.05$). Therefore, PIT and Cur concentration had independent effect on cell metabolism. Figs. 5 and 6 present details of the multiple comparisons obtained by Tukey's test, separately exhibiting comparisons among each PIT within the same Cur concentration (Fig. 5), and among each Cur concentrations within the same PIT (Fig. 6).

For *C. albicans*, analysis of the data allowed the observation that after either 4 or 8 min of illumination, as the PIT increased, the cell viability diminished proportionally, irrespectively of the concentration. The lowest absorbance values were reached in 20 min of PIT and 40 μM Cur.

For *C. glabrata*, the analysis of variance of the P+L+ group irradiated for 4 and 8 min indicated significant effect of the PIT and Cur-concentration interaction ($p = 0.001$ and $p = 0.015$, respectively). To detect this interaction, Tukey's test was performed, and the results are presented in Figs. 5 and 6. The highest reduction in cell viability was obtained with 40 μM Cur and the lowest with 20 μM Cur, and 30 μM Cur promoted intermediate values. PIT dependency was not clear.

For biofilms of *C. dubliniensis*, the analysis of variance showed significant interaction of PIT and Cur concentration ($p = 0.001$) in the P+L+ groups irradiated for 4 min. On the other hand, the interaction was not significant in the P+L+ groups irradiated for 8 min, with a significant effect of PIT ($p < 0.001$) and Cur concentration ($p < 0.001$). Tukey's test was applied to study the cases, and the results are presented in Figs. 5 and 6. The groups illuminated for 4 min were concentration-dependent for the extreme values (40 and 20 μM). No PIT dependency was clearly observed. Whereas, groups illuminated for 8 min were concentration and PIT-dependent.

3.3. *Confocal laser scanning microscopy observations*

For all the microorganisms, CSLM was used to investigate Cur penetration into the deepness of the biofilms. Images of *Candida* spp. biofilms were captured by fluorescence mode (Figs. 7 and 8) following incubation of the biofilms with Cur 40 μM for 5 min (Fig. 7A, C and E) and 20 min (Figs. 7B, D, F and 8). In spite of the light green fluorescence observed after a 5-min incubation (Fig. 7A, C and E), brighter fluorescence was observed following a 20-min incubation (Fig. 7B, D and F). Fig. 8 presents cross sections and side views of *C. albicans* biofilms after 5 and 20 min of incubation with Cur 40 μM (Fig. 8B and C,

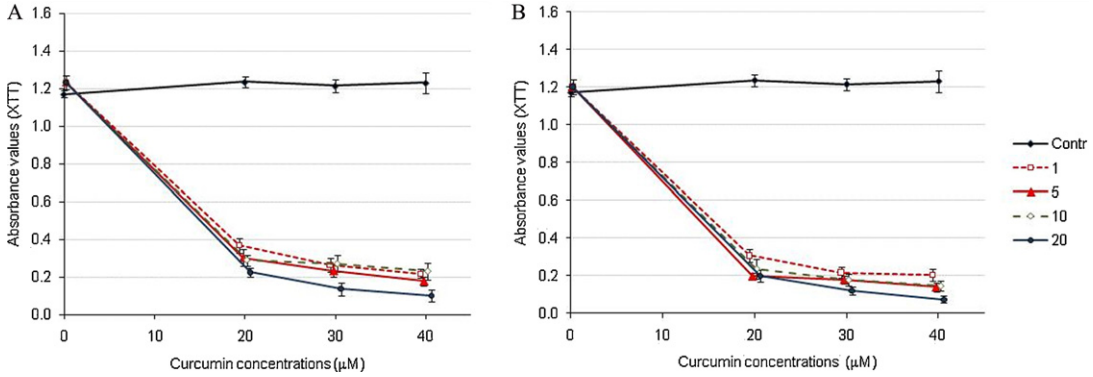

Fig. 2 – Graphic representation of mean values of absorbance of *C. albicans* biofilms, illuminated for 4 min (A) and 8 min (B). Vertical bar indicates a 95% confidence interval for a population mean.

respectively). Fig. 8A presents an image of the transmittance mode applied to *C. albicans* biofilm before the incubation with curcumin, due to the absence of fluorescence signal from *Candida* biofilms without curcumin. On the side views of the same biofilm (Fig. 8B and C), it is possible to determine the biofilm thickness (yellow lines) and curcumin penetration through the biofilm (red lines). Moreover, it is possible to observe the lack of sensitised cells in the deepest portions (yellow arrow) when compared to the outermost layers with a brighter fluorescence (red arrow).

4. Discussion

Among other factors, the effectiveness of antimicrobial PDT depends on the pre-irradiation time (PIT), which is the period required by the PS to remain in contact with the microorganisms before illumination. It seems that the PIT sufficient to promote effective microbial killing depends on the properties of the PS.

For example, the porphyrins, the phenothiazine and the aluminium phthalocyanine (AlPc)[26,34,35] require shorter PITs when compared with tetrasulfonated aluminium phthalocyanine ($AlPcS_4$).[35] In contrast, other studies have stated that PIT had no significant importance on the effectiveness of PDT, and demonstrated that a longer PIT did not increase the reduction in cell viability.[26,33,34] In addition, the species of the microorganism studied is an important factor influencing PDT effectiveness.[39,45,51] Due to the vast diversity of microorganisms, a PS with distinct physicochemical properties may be required. For these reasons, different types of PS have been proposed for antimicrobial PDT. Demidova and Hamblin[38] compared the effectiveness of PDT mediated by several PSs for the inactivation of bacterial suspensions of *Escherichia coli* (gram-negative) and *S. aureus* (gram-positive), and of fungal suspensions of *C. albicans*. In their study, polylysine conjugate was highly effective against fungal and bacterial suspensions, although lower concentrations were required for bacterial (0.75 μM) than for fungal inactivation (5 μM). Toluidine blue needed concentration and light doses of 10 μM and 32 J/cm^2 to promote *S. aureus* inactivation, 35 μM and 32 J/cm^2 to promote *E. coli* inactivation, and 50 μM and 40 J/cm^2 to promote *C. albicans* inactivation. Moreover, to cause cell inactivation with Rose Bengal as a PS, it was necessary to use 0.25 μM and 4 J/cm^2 for *S. aureus*, 35 μM and 8 J/cm^2 for *E. coli*, and 200 μM and from 40 to 80 J/cm^2 for *C. albicans*. Thus, *C. albicans* was shown to be more resistant to PDT, when compared with bacteria, which may be attributed to differences in cell size. *Candida* species are approximately 25–50 times larger than bacterial cells.[27,39,51] Furthermore, as an eukaryotic microorganism, the

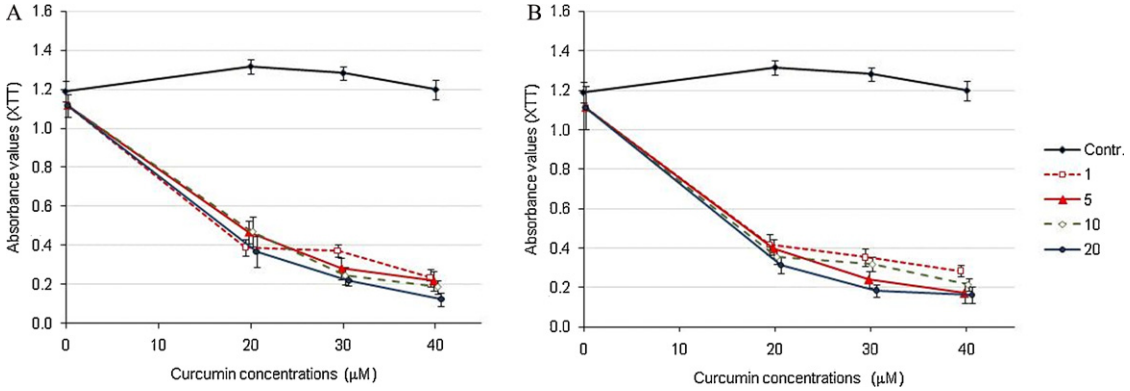

Fig. 3 – Graphic representation of mean values of absorbance of *C. glabrata* biofilms, illuminated for 4 min (A) and 8 min (B). Vertical bar indicates a 95% confidence interval for a population mean.

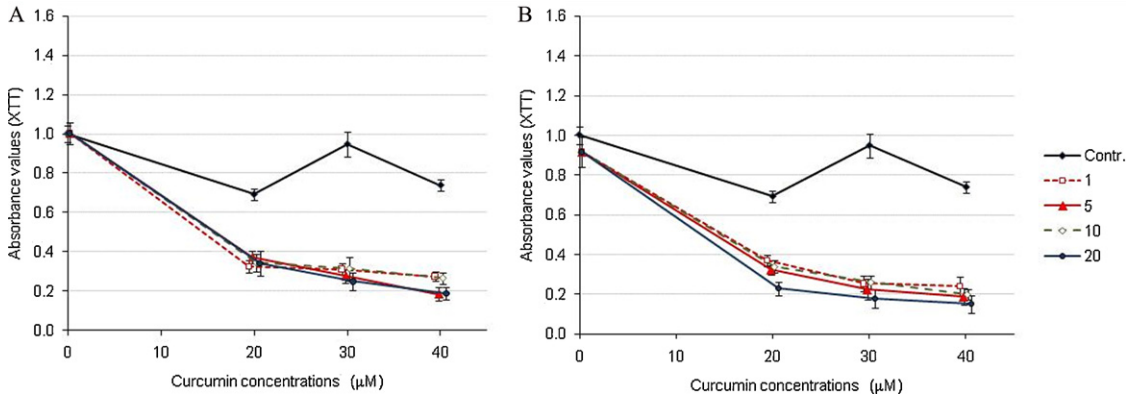

Fig. 4 – Graphic representation of mean values of absorbance of *C. dubliniensis* biofilms, illuminated for 4 min (A) and 8 min (B). Vertical bar indicates a 95% confidence interval for a population mean.

presence of a nuclear membrane could act as an additional barrier to the PS.[39,51]

This study showed the effectiveness of Cur-mediated PDT on the photoinactivation of the three evaluated *Candida* species. For the planktonic cultures of *Candida* spp. the results demonstrated that different PITs presented no statistical differences in photoinactivation of any of the evaluated species. In addition, the association of 20 μM Cur and LED light, after 5, 10 and 20 min of PIT promoted complete inactivation of the *C. albicans*, *C. glabrata* and *C. dubliniensis* cells. These results are in agreement with Dahl et al.[36] whose study demonstrated that a long PIT is not required for Cur phototoxicity. In their study, the authors obtained photoinactivation of both gram-positive and gram-negative bacteria with Cur at 1 and 10 μM, respectively, which is less than the concentration required in the present study for the photoinactivation of *Candida* species. Furthermore, they observed that the Cur which remained in contact with bacterial cells for different times before irradiation did not significantly modify its phototoxic effects. Also, the removal of Cur before illumination promoted a significant reduction in its phototoxicity, suggesting that Cur in the extracellular bulk phase or loosely bound to the cells is responsible for most of the phototoxic effects. As a lipophillic molecule, Cur first interacts with the cell membrane and membrane bound proteins and is

PIT (min)	Cur (μM)	*C. albicans*			*C. glabrata*			*C. dubliniensis*		
		4min*	8min**	t+	4min*	8min**	t+	4min*	8min**	t+
1	20	c	C	+	a	B		a	C	+
5		b	B	+	a	AB		a	B	+
10		b	B		a	AB	+	a	BC	
20		a	A		a	A		a	A	+
1	30	c	C	+	b	C		a	C	+
5		b	B	+	ab	AB		a	B	
10		b	B	+	a	BC	+	a	BC	
20		a	A		a	A		a	A	+
1	40	c	C		b	B		b	C	
5		b	B	+	ab	A		a	B	
10		b	B	+	ab	AB		b	BC	+
20		a	A		a	A		a	A	

Fig. 5 – Results of the statistical comparison among P+L+ groups within each Cur concentration, level of significance set at 5%. * Multiple comparisons (Tukey's test) among different PITs within each Cur concentration at 4 min of illumination. Different lower case letters denote significant differences only among lines within the same Cur concentration and species. ** Multiple comparisons (Tukey's test) among different PITs within each Cur concentration at 8 min of illumination. Different capital letters denote significant differences only among lines within the same Cur concentration and species. + Comparison of mean values of 4 and 8 min P+L+ groups (with the same Cur concentration and same PIT) that resulted in significant differences by Student's-t test.

PIT (min)	Cur (μM)	*C. albicans*		*C. glabrata*		*C. dubliniensis*	
		4min*	8min**	4min*	8min**	4min*	8min**
1	20	c	C	b	B	a	C
	30	b	B	b	AB	a	B
	40	a	A	a	A	a	A
5	20	c	C	b	B	c	C
	30	b	B	a	A	b	B
	40	a	A	a	A	a	A
10	20	c	C	b	B	b	C
	30	b	B	a	B	ab	B
	40	a	A	a	A	a	A
20	20	c	C	b	B	b	C
	30	b	B	a	A	a	B
	40	a	A	a	A	a	A

Fig. 6 – Results of the statistical comparison among P+L+ groups within each PIT, level of significance set at 5%. * Multiple comparisons (Tukey's test) among different Cur concentrations within each PIT at 4 min of illumination. Different lower case letters denote significant differences only among lines within the same PIT and species. ** Multiple comparisons (Tukey's test) among different Cur concentrations within each PIT at 8 min of illumination. Different capital letters denote significant differences only among lines within the same PIT and species.

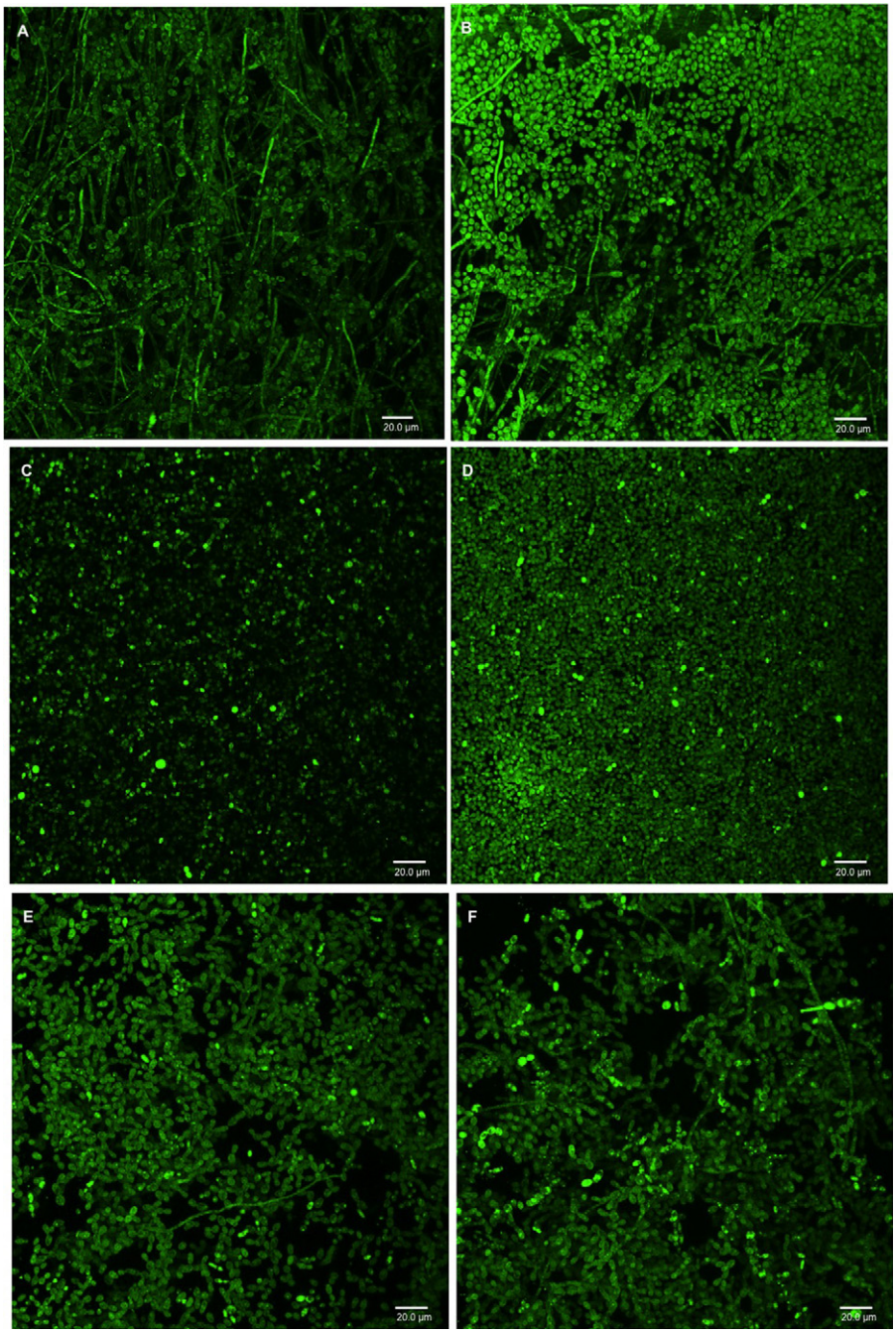

Fig. 7 – Overview of a 46.0 μm thick biofilm of *C. albicans* after 5 min of contact with Cur 40 μM (A), and after 20 min of incubation with Cur 40 μM (B). In spite of the low fluorescence emission after 5 min of incubation (A), brighter green fluorescence can be observed after 20 min of incubation (B). C and D image *C. glabrata* 21.0 μm thick biofilms after 5 and 20 min of incubation with Cur 40 μM: Light green fluorescence is observed on the biofilm and, even managing to observe the presence of cells, they are not brightly stained (C), while bright fluorescence can be seen on the outermost layer of the biofilm after 20 min of incubation with the PS (D); E and F are captured images of *C. dubliniensis* 30.0 μm thick biofilms after incubation with Cur 40 μM. A light green fluorescence can be observed after 5 min of incubation (E), while bright fluorescence can be seen after 20 min of incubation (F). All images used a 40× objective. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

then distributed to different parts of the cell.[45] The nature of these interactions may justify the results obtained for the planktonic cultures of this study, in which an increase in PIT did not promote substantial alterations in photoinactivation of the three evaluated species.

Furthermore, *C. albicans* and *C. glabrata* suspensions pre-incubated with 20 μM Cur for only 1 min resulted in 89.5% photoinactivation. These findings are in agreement with Dahl et al.[37]. In an effort to identify the photodynamically relevant activity that penetration/uptake by cells exerts on Cur

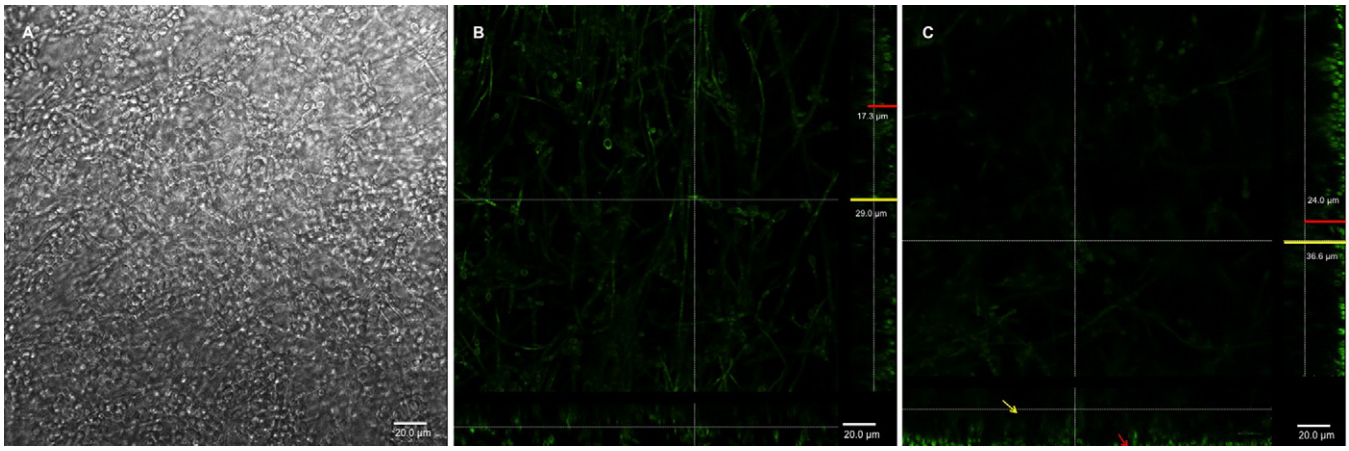

Fig. 8 – (A) Image of the transmittance mode applied to *C. albicans* biofilm before the incubation with curcumin. *Candida* biofilms without curcumin revealed no fluorescence signal; (B) cross sections and side views of a 29.0 μm (yellow line) thick biofilm of *C. albicans* after 5 min of incubation with Cur 40 μM. Curcumin fluorescence was observed at a depth of 17.3 μm (red line). (C) Cross sections and side views of a 36.6 μm (yellow line) thick biofilm of *C. albicans* after 20 min of incubation with Cur 40 μM. Curcumin fluorescence was observed at a depth of 24.0 μm (red line). On the side views, it is possible to observe a lack of sensitised cells in the deepest portion of the biofilm (yellow arrows), when compared to the outer layer (red arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

phototoxic activity, the authors observed that the Cur rapidly penetrated the cells, with maximum penetration reached in 2–4 min. However, the penetration represented only 10% of the Cur added to the solution, leaving about 90% in an extracellular bulk phase.[37] However, 1 min of PIT, the shortest time evaluated in this study, might not have been sufficient to allow cell penetration of the 10% Cur in solution, resulting in approximately 90% reduction, which accounts for the extracellular bulk phase phototoxicity of the Cur.

When organised in biofilm cultures, Cur at 20, 30 and 40 μM promoted significant reduction in cell metabolism after PDT. Nevertheless, these cultures demonstrated lower susceptibility to PDT when compared with their planktonic counterparts. The results are in agreement with Dovigo et al.[40] who analysed biofilm and planktonic cultures of *C. albicans* and *C. glabrata* strains exposed to Photogem® at 25 mg/mL and illuminated by LED light (37.5 J/cm^2). Significant decreases in biofilm viability were observed for *C. albicans* and *C. glabrata*. The results demonstrated that although PDT was effective against *Candida* species, single-species biofilms were less susceptible to PDT than their planktonic counterparts. This might be explained by the sessile organisation of the biofilms that may confer ecological advantages and may be responsible for the increased resistance of microbial biofilms, since it restricts the penetration of antimicrobials.[52] Moreover, it has been demonstrated that efflux pump genes are up regulated during biofilm formation and development of some *Candida* species.[23] In addition, it has long been supposed that the matrix of extracellular polymeric material might exclude or limit the access of drugs to organisms deep within a biofilm.[12] Pereira et al.[42] evaluated the susceptibility of *C. albicans*, *S. aureus* and *S. mutans* biofilms to photodynamic inactivation, and after Scanning Electron Microscopy analyses, they observed that the effects of the therapy occurred predominantly in the outermost layers of the biofilms. Furthermore, Wood et al.[44] evaluated bacterial biofilms by confocal laser scanning microscopy, or processed by transmission electron microscopy, and they verified that after PDT the biofilms were reduced to approximately half the thickness of the control biofilms, were less dense and seemed to be made up of columns of bacterial aggregates. In our study, CLSM imaging led to the observations that the cells located on the superficial layers of the biofilm presented a bright intense fluorescence, while in the basal layer the fluorescence was less intense or not present (Fig. 8B and C). The fluorescence observed was a result of curcumin penetration since the biofilms formed with the three *Candida* species revealed no fluorescence signal, leading to the conclusion that the cells under this experimental conditions and excitation/emission interval have no auto-fluorescence (Fig. 8A). When the biofilms were maintained in contact with the Cur for 5 and 20 min of incubation, brighter fluorescence was observed after 20 min of incubation (Fig. 7B, D and F), suggesting that Cur penetration into the cells of the biofilm after 20 min might have achieved greater amounts than after 5 min. The drugs need to effectively penetrate the extracellular matrix to ensure the occurrence of intimate contact with the microorganisms. For these reasons, in all the P+L+ groups, 20 min of PIT promoted the highest reductions in cell viability.

C. albicans seemed to be the only species whose cell viabilities were clearly dependent on PIT after 4 and 8 min of irradiation. The *C. albicans* biofilms submitted to PDT showed higher reduction in cell viability after 20 min of PIT ($p < 0.01$). When PIT was reduced, cell viability was also reduced proportionally. Cell viability of *C. dubliniensis* biofilms after 8 min of irradiation was PIT-dependent. However, *C. dubliniensis* biofilms after 4 min of irradiation, and *C. glabrata* biofilms (after 4 and 8 min of irradiation) showed no clear tendency to be PIT-dependent, although 1 and 20 min of PIT,

respectively, resulted in the worst and best results. The morphology of the microorganisms seems to have great importance in PDT. A survey by Jackson et al.[26] evaluated whether the hyphae and yeasts forms of *C. albicans* could be killed by PDT. The results demonstrated that both forms are susceptible to photosensitisation. However, hyphal forms presented higher susceptibility to PDT than the yeasts. In the present study, the biofilms were grown in RPMI 1640, which induces hyphae formation.[19] *C. albicans* and *C dubliniensis* are dimorphic fungi (ovoid yeasts and/or filaments).[12,18,52] On the other hand, *C. glabrata* presents itself as a single morphological species and does not transform itself into hyphae.[53] Therefore, considering the possibility that within each PIT, Cur is able to reach the same depth in the biofilms of the three species, fungi that were transformed into hyphae and were sensitised with Cur might have been more susceptible to the phototoxic effects of PDT. This might justify the fact that *C. glabrata* was the only species that did not present a clear tendency to be PIT-dependent under any of the evaluated conditions.

Due to structural and biological differences, different behaviours are expected from distinct *Candida* strains. *C. glabrata* produces adhesins capable of promoting adhesion to buccal epithelial cells.[18] It also has high hydrophobicity values and efficient co-adhesion mechanisms, which allows cells to bind to other cells.[54] In addition, the *C. glabrata* biofilm matrix has higher amounts of both proteins and carbohydrates.[53] Thus, it is possible that drug penetration through the *C. glabrata* biofilm structure is more difficult than it is through the other analysed biofilms, a fact that might be able to explain why *C. glabrata* biofilms were not PIT-dependent and showed higher absorbance values commonly found under most of the experimental conditions presented.

Regarding *C. dubliniensis* biofilms results, after 4 min of irradiation, there was no clear tendency to be PIT-dependent, showing a different behaviour from *C. albicans*. A recent study also found that *C. dubliniensis* tended to be more resistant to PDT effects when compared to *C. albicans*.[55] The authors showed that higher concentrations of erythrosine were necessary to achieve the same microbial reduction observed for *C. albicans* and only a 0.21 $\log_{10}$ reduction on CFU/mL of *C. dubliniensis* biofilms where obtained when exposed to PDT mediated by 400 μM erythrosine and a green LED.[55] Therefore, more studies are necessary to identify biological reasons of different response to PDT among different species of *Candida*.

Cur-mediated PDT was shown to be effective against *Candida* biofilms. Reductions of 94%, 89% and 85% in cell viabilities were observed for *C. albicans*, *C. glabrata* and *C. dubliniensis*, respectively. Photosensitisers may need a longer time to penetrate into the depth of the biofilms[12] to achieve intimate contact with the specimens in order to obtain more effective action. The 20 min PIT associated with 40 μM Cur resulted in the highest reductions in cell viability.

Whilst it is not suggested that PDT will replace conventional therapy, improvements may be obtained using the photodynamic approach in the clinical treatment of local infection,[30] and Cur-mediated PDT may exhibit benefits in the treatment of oral candidiasis of immunocompromised patients and/or in cases of long-term use of medications, in which the emergence of resistant strains is likely to occur.

Based on the experimental conditions of this study and in accordance with the methodology used, it was possible to conclude that PDT with the association of Cur and blue LED light was effective in decreasing cell viabilities of the three *Candida* species evaluated. For the planktonic cultures, photoinactivation was concentration-dependent, but not PIT-dependent. The further combination of 20 μM Cur and LED light at 5.28 J/cm^2 output promoted complete inactivation of the suspensions after 5, 10 and 20 min time intervals of PIT. On the other hand, Cur-mediated PDT was shown to be effective against *Candida* biofilms, with reductions of 94%, 89% and 85% in the cell viabilities of *C. albicans*, *C. glabrata* and *C. dubliniensis*, respectively. As observed in CLSM images, Cur needed a longer time to show a more intense brightness deeper in the biofilm, and, in this way, achieve intimate contact with the organisms and obtain more effective action. Thus, the highest reductions in cell viability for the biofilm cultures were achieved after associating 40 μM Cur with 20 min of PIT.

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Conflict of interest

None to declare.

Ethical approval

This project showed no need for approval by the Ethics Committee.

Author contribution

All authors have made substantial contribution to this work, discussed the results and implications, and commented on the manuscript at all stages:

MCA performed the experiments for data collection and also wrote the article; ACP was responsible for the conception and design of the study, and answer for the overall responsibility; APDR performed the experiments for data collection and made the critical revision of the article; LND was responsible for the conception and design of the study and the statistical analysis and interpretation of the data; ETG was responsible for the conception and design of the study, and made the critical revision of the article; ILB and VSB were responsible for the conception and design of the study, and also supervised the project and helped with the analysis and interpretation of the data.

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