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ORIGINAL ARTICLE

Curcumin-mediated anti-microbial photodynamic therapy against *Candida dubliniensis* biofilms

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Abstract The purpose of this study was to evaluate the effectiveness of anti-microbial photodynamic therapy (aPDT) mediated by curcumin (Cur) associated with LED light against biofilms of *Candida dubliniensis*, and further, investigate cellular uptake and drug penetration through the biofilms under confocal laser scanning microscopy (CLSM). Four *C. dubliniensis* strains were tested: three clinical isolates from HIV-positive patients and one reference strain (CBS 7987). Biofilms were treated with three Cur concentrations (20.0, 30.0, and 40.0 μM). All samples were incubated in the dark for 20 min and exposed to a 5.28 J/cm^2 of LED light fluence. Additional samples of each strain were treated either with Cur or LED light only. Control samples had neither Cur nor light. After aPDT, results were read using the XTT salt reduction method. The data were statistically analyzed by two-way ANOVA followed by Games-Howell post-hoc test ($\alpha = 0.05$). Confocal laser scanning microscopy was used to verify both the uptake of Cur by yeast cells and its penetration through the biofilm. The results showed that aPDT promoted significant reduction on the metabolism of the biofilm-organized cells of *C. dubliniensis.* Further, while Cur was rapidly taken up by *C. dubliniensis* cells, a longer time interval was required to allow Cur penetration into biofilm cells. Based on these results, aPDT associating LED and Cur presents promising potential on fungal control of biofilms of *C. dubliniensis.*

Keywords Photochemotherapy · Biofilm · Curcumin · *Candida dubliniensis* · Light-emitted diode

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Introduction

Although *Candida* species are frequently isolated from human oral cavity as commensal microorganisms, they are important causative agents of healthcare-associated infections, especially in critically-ill immunocompromised patients [1]. In terms of epidemiology, *Candida albicans* remains the predominant cause of local and invasive candidiasis and accounts for more than 50% of all cases [2–5]. However, the literature has shown that the epidemiology of *Candida* infections has changed with emergence of non-*albicans* species, which have been increasingly described in both compromised and non-compromised hosts [2–5]. In vivo studies demonstrated that multiple isolations of *Candida* species, including *C. albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, and *Candida parapsilosis*, are common in patients with oral and systemic infections, both as single cultures and in mixed biofilms [3–8]. Another *Candida* spp. that requires attention is *Candida dubliniensis*. This species was recovered primarily from superficial oral candidiasis in HIV-infected patients in 1995 and its most important feature is the phenotypic similarities to *C. albicans* [9]. Due to this fact, *C. dubliniensis* can be misidentified in routine laboratory methods, and, thus, its incidence of infection may be underreported. According to the literature, *C. dubliniensis* has been isolated from local and systemic infections of diabetes mellitus patients [10], cancer patients [11], cystic fibrosis patients [12],

immunocompromised patients [13], and immunodeficiency virus-infected (HIV+) patients [14–16]. This incidence must be highlighted because *Candida* infections in these patients may be more difficult to eradicate and, consequently, can become life-threatening [13].

One of the strategies used by several *Candida* spp., including *C. dubliniensis*, to cause infections in both compromised and non-compromised hosts is its ability to form biofilms [17]. Biofilms are biological communities with a high degree of organization, in which microorganisms form structured, coordinated, and functional communities surrounded by a self-derived extracellular matrix [18]. In this mode of growth, biofilms act as reservoirs of pathogenic microorganisms [6–8] favoring the dissemination of infections to other body sites [19]. The clinical relevance of the presence of a biofilm in an infection is that its cells are phenotypically distinct from their "free-living" or planktonic counterparts, exhibiting elevated resistance to host defenses and higher tolerance to anti-microbial agents [18]. *C. dubliniensis* as biofilm increases the therapeutic complexity of infections. It is not uncommon the failure of the traditional approaches available to the management of these infections due to the ability of this species to rapidly develop resistance to anti-fungal drugs commonly used in clinical practice [13, 15, 16, 20, 21]. In fact, the recovery of some *C. dubliniensis* isolates with lower susceptibility to echinocandins [21] and resistant to azoles [13, 15, 16, 20] has been reported.

In order to overcome these problems, there is a strong medical motivation for the development of novel anti-fungal biofilm strategies. In this context, anti-microbial photodynamic therapy (aPDT), a treatment modality that has been studied for more than a century against tumoral cells [22], has shown to be effective against several pathogenic microorganisms [23–28]. In addition, aPDT has been considered as an alternative treatment modality for resistant strains [26, 28]. To fill its skills, the employment of a photoactive drug (named as photosensitizer-PS) and a visible light is needed [29]. When exposed to a specific wavelength in the presence of oxygen, the PS generates reactive oxygen species (ROS) that are toxic to cells, promoting cell death [29]. Unlike most anti-fungal drugs that act over a single cell target, the ROS interact with different cell structures [29].

Many authors have focused in searching for a PS that does not cause undesirable side effects to host's healthy cells and, for this purpose, natural products are one of the most searched ones. Curcumin (Cur) is a yellow-orange dye extracted from the root of *Curcuma longa* L. and is commonly used as a spice in traditional Asian cookery [30]. It has already been reported that Cur has several pharmacological effects such as anti-inflammatory, anti-tumoral, anti-fungal, anti-bacterial, and anti-carcinogenic properties [23–27, 30]. Some of which properties can be enhanced after light activating [24]. Also, phototoxicity of Cur has already been demonstrated against bacterial systems [27] and, also, against *C. albicans* [23]. As *C. albicans* showed to be more resistant to aPDT when compared to Gram-positive or Gram-negative bacteria [31], the knowledge of the effects of light-activated Cur against other *Candida* spp. may lead to better understanding of the potential of Cur-mediated aPDT. In this context, previous studies evaluated the anti-microbial effective of Cur-mediated aPDT against planktonic cultures and biofilms of both reference strains and clinical isolates of two non-*albicans* species—*C. glabrata* and *C. tropicalis* [23, 24]. When considering *C. dubliniensis* species, to the best of our knowledge, there is only one investigation that evaluated Cur-mediated aPDT against planktonic and biofilm cultures of a single reference strain [27]. Although promising results were found [27], it is also important to evaluate the effect of this treatment modality against clinical isolates, since the virulence of each strain vary according to the isolate itself, its species, and its source (ATCC × clinical) [23, 24]. Therefore, the purpose of this study was to evaluate the effectiveness of Cur-mediated aPDT on photoinactivation of mature biofilms of three clinical isolates of *C. dubliniensis* obtained from HIV-infected patients. Additionally, this study aimed to evaluate the uptake and biofilm penetration of Cur under fluorescence imaging on confocal laser scanning microscopy (CLSM).

Materials and methods

Clinical isolates of *C. dubliniensis*

In the present study, three clinical isolates of *C. dubliniensis* obtained from the yeast culture collection of the Laboratório Especial de Micologia-UNIFESP, São Paulo, SP, Brazil, were used (*C. dubliniensis* CD6; *C. dubliniensis* CD7; *C. dubliniensis* CD8). These isolates were previously recovered from the oral cavity of HIV-positive patients. Additionally, a reference strain was used as control (*C. dubliniensis* CBS 7987). All isolates were maintained in yeast-peptone-glucose medium (YEPD: 1% yeast extract, 2% Bacto peptone, and 2% D-glucose, 2% agar) with glycerol and frozen at − 70 °C until use.

Photosensitizer and light source

The photosensitizer Cur (Sigma-Aldrich, Saint Louis, MO, USA) was used and prepared with 10% dimethyl sulfoxide (DMSO) to originate a stock solution.

A light-emitting diode (LED, LXHL- PR09, Luxeon1 III Emitter, Lumileds Lighting, San Jose, CA, USA) was used to activate the curcumin. The LED device emits 22.0 mW/cm^2 of light intensity and 455 nm of predominant wavelength.

Planktonic cultures and aPDT

In order to determine the concentrations that would be inhibitory to biofilms, an experiment was conducted with

planktonic cultures of *C. dubliniensis*. Thus, from the stock solution of Cur, solutions were prepared at final concentrations of 5.0, 10.0, and 20.0 μM.

For the planktonic assays, a 25 μL aliquot of each yeast was subcultured onto Sabouraud Dextrose Agar (SDA, Acumedia Manufactures Inc, Lansing, Michigan, USA) with chloramphenicol and incubated for 48 h at 37 °C. To prepare the yeasts' inoculums, colonies from the agar stock cultures recently cultivated were transferred using a sterile loop to a Falcon tube containing 10 mL of Tryptic Soy Broth (TSB, Acumedia Manufactures Inc, Lansing, Michigan, USA) and grown aerobically overnight at 37 °C. After the incubation period, each culture tube was centrifuged at 4000 rpm for 7 min, the supernatants were discarded, and the cell pellets were washed twice by centrifugation in 10 mL of sterile saline solution. Then, all washed suspensions were spectrophotometrically standardized at an optical density of 520 nm (Biospectro, Equipar Ltda, Curitiba, PR, Brasil) to a final concentration of 10^6 cells/mL.

Aliquots of 100 μL of *C. dubliniensis* standardized suspension of 10^6 cells/mL were individually transferred to separate wells of a 96-well microtiter plates. After inoculation on the microtiter plates, an equal volume of the three Cur solutions was added to each well (experimental conditions aPDT 5, aPDT 10, and aPDT 20). After dark incubation for 20 min (pre-irradiation time), the *C. dubliniensis* suspensions were irradiated on the LED device for 4 min, which corresponds to 5.28 J/cm^2 light dose.

To determine whether LED light alone had any effect on cell viability, additional samples were prepared without the PS (experimental condition P-L+). The effect of Cur alone was also determined by exposing the yeast suspensions to the photosensitizer identically to those described above, but without light exposure (experimental conditions P + L- 5, P + L- 10, and P + L- 20). The microtiter plates containing the no-light samples were kept in the dark for 24 min, corresponding to the pre-incubation time plus light exposure time. The suspensions exposed to neither LED light nor Cur acted as overall control (experimental condition P-L-). Five samples were tested for each *C. dubliniensis* yeast, considering each experimental condition, in three independent experiments.

After all assays, tenfold serial dilutions were generated from the fungal suspensions (10^{-1}, 10^{-2}, and 10^{-3}) 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 using a digital colony counter (CP 600 Plus, Phoenix Ind Com Equipamentos Científicos Ltda, Araraquara, SP, Brazil) and colony forming unit per milliliter (CFU/mL) was determined.

Biofilms and aPDT

A loopful of the recently grown yeasts in SDA with chloramphenicol was individually transferred to a Falcon tube containing RPMI 1640 (Sigma-Aldrich, Saint Louis, MO, USA) and incubated overnight in an orbital shaker (AP 56, Phoenix Ind Com Equipamentos Científicos Ltda, Araraquara, SP, Brazil) at 120 rpm and 37 °C. Then, cultures were centrifuged, the supernatants were discarded, and the cell pellets were washed twice by centrifugation in 10 mL of PBS and resuspended in PBS. Standardized cell suspensions at 10^6 cells/mL were obtained as described for the planktonic cultures.

Aliquots of 100 μL of each *C. dubliniensis* standardized suspensions were transferred to a 96-well microtiter plate and incubated for 90 min at 37 °C in the orbital shaker at 75 rpm for the adhesion phase of biofilm formation. After this period, each well was washed twice with PBS and 150 μL of freshly prepared RPMI 1640 was added to each well. The plates were incubated for 48 h at 37 °C in order to generate single-species biofilms. After incubation, the wells were carefully washed twice with 200 μL of PBS to remove non-adherent cells.

To evaluate the anti-fungal efficacy of the treatments, aliquots of 150 μL of Cur at 20, 30, and 40 μM were added to each appropriate well directly over the biofilm (experimental conditions aPDT 20, aPDT 30, and aPDT 40). After 20 min in the dark, biofilms were illuminated (5.28 J/cm^2). To determine the effect of LED light and Cur alone on biofilm viability, the same experimental conditions for planktonic cultures were tested: P-L+, P + L- 20, P + L- 30, P + L- 40. A control condition without exposure to LED light or Cur was also conducted (P-L- group). Five samples were made for each *C. dubliniensis* yeast, considering each experimental condition, in three independent experiments.

The anti-fungal effects of Cur-mediated aPDT against the biofilms were evaluated by a metabolic assay based on the reduction of XTT (Sigma-Aldrich, St Louis, MO), a tetrazolium salt. After all experimental procedures, 158 μL of PBS with 200 mM glucose, 40 μL of XTT, and 2 μL of menadione were inoculated to each well. The plates were covered with aluminum foil and incubated for 3 h in the dark at 37 °C. The resulting colorimetric changes are proportional to the number of living cells and their metabolic activity. Aliquots of 100 μL of the reacted XTT salt solution were transferred to a new 96-well microtiter plate and the cell viability was analyzed by proportional colorimetric changes and light absorbance measured by a microtiter plate reader (Thermo Plate—TP Reader) at 492 nm.

Confocal laser scanning microscopy observations

In order to evaluate the uptake of Cur by yeast cells, *C. dubliniensis* ATCC in planktonic cultures was prepared as described above and placed on 10-mm round coverslips. Confocal microscopy was performed on live organisms. Following 5 and 20 min of incubation with Cur 20 μM, the glass coverslips containing cell suspension of *C. dubliniensis* were flipped and placed on a glass-bottom and observed using a Leica TCS SPE confocal microscope (Leica Microsystems

GmbH, Wetzlar, Germany). Transmission mode using differential interference contrast (DIC) technique was used to image the fungal cells. Fluorescence mode used a 405-nm excitation wavelength and a green fluorescence (emission from 450 to 600 nm). Corresponding fluorescence and transmission images were overlayed to demonstrate Cur localization and uptake by the cells.

Cur penetration through the biofilm was also evaluated under microscope observations. Standardized suspensions were prepared as described before and biofilm formation was performed on polymethylmetacrylate coverslips. After 48 h, the biofilms were washed twice with PBS and incubated with Cur 40 μM for 20 min. Stained biofilms were observed under fluorescence mode. Corresponding Cur fluorescence allows observation of stained cells of the biofilms. Serial sections in the *xy* plane were obtained at 1 μm intervals along the *z* axis.

Statistical analysis

Data from planktonic cultures (CFU/mL values) were analyzed descriptively. The response variable from biofilm testing was the absorbance values from XTT (Abs XTT) while the factors analyzed were the different experimental conditions (P-L+, P + L- 20, P + L- 30, P + L- 40, P-L-, aPDT 20, aPDT 30, and aPDT 40) and the sources of the *C. dubliniensis* isolates (ATCC and three clinical isolates from HIV-infected patients). A two-way ANOVA was used to assess the possible effects of the factors "experimental conditions" and "sources of isolates" on XTT values. Significant differences were explored by Games-Howell post-hoc test. All tests were performed using the SPSS statistical software package (SPSS Inc., Chicago, USA) with a confidence level of 95%.

Results

Anti-fungal effect of aPDT against planktonic cultures of *C. dubliniensis*

The microbial growth observed for all experimental conditions and *C. dubliniensis* strains are presented in Table 1. The mean values of CFU/mL of *C. dubliniensis* exposed only to irradiation (P-L+) or only to Cur (P + L- 5, P + L- 10, and P + L- 20) seemed similar to the values from the control group (P-L-). Only the association of Cur and LED light was capable to cause a reduction in CFU values from 10^6 to 10^3 (CD6 strain) and 10^2 (CD7 and CD8 strains). When the aPDT groups were evaluated, it could be seen that 20.0 μM of Cur was the only concentration that resulted in absence of colony growth of all *C. dubliniensis* isolates. Thus, the Cur concentrations selected to evaluate the effectiveness of Cur-mediated aPDT against *C. dubliniensis* biofilms were 20, 30, and 40 μM.

Table 1 Mean CFU/mL values of the planktonic cultures of all *C. dubliniensis* isolates after assays considering all experimental conditions

Experimental conditions	*C. dubliniensis* isolates			
	ATCC	CD6	CD7	CD8
P-L-	1.83×10^6	8.74×10^5	1.82×10^6	2.94×10^6
P + L- 5	8.25×10^5	6.88×10^5	7.10×10^5	2.47×10^6
P + L- 10	7.50×10^5	4.96×10^5	5.41×10^5	2.80×10^6
P + L- 20	8.44×10^5	5.12×10^5	4.40×10^5	2.83×10^6
P-L+	2.94×10^6	1.82×10^6	1.64×10^6	3.10×10^6
aPDT 5	ND	8.20×10^3	3.16×10^2	4.80×10^2
aPDT 10	ND	1.80×10^3	ND	ND
aPDT 20	ND	ND	ND	ND

ND growth not detected

Anti-fungal effect of aPDT against *C. dubliniensis* in biofilm cultures

Table 2 shows the summary of the two-way ANOVA for the factors analyzed. The interaction between both factors analyzed (sources of isolates and experimental conditions), as well as each factor isolated, showed a significant effect ($p < 0.0001$) on the XTT values of *C. dubliniensis* biofilms.

The XTT mean values of all *C. dubliniensis* biofilms after exposure to the different experimental conditions are presented in Fig. 1. Considering the factor "experimental conditions" (uppercase letters), for all sources of isolates evaluated (control strain and three clinical isolates), aPDT with the three Cur concentrations (aPDT 20, aPDT 30, and aPDT 40) caused a significant reduction of biofilm viability ($p < 0.001$) in comparison to the following groups: control (P-L-), LED light alone (P-L+), and Cur alone (P + L- 20, P + L- 30, and P + L- 40). For these aPDT groups, the mean percentage reduction ranged from 57.70 to 82.05% considering reference and clinical strains (Table 3). There were no significant differences among the three aPDT groups for any source of isolates ($0.266 \leq p \leq 1$). When the other experimental conditions were compared, except for the clinical isolate *C. dubliniensis* CD6, the exposure to Cur alone in the highest concentrations (P + L- 30 and P + L- 40) significantly reduced the biofilm viability ($0.001 \leq p \leq 0.01$) in comparison to the control group. Also, with the exception of the clinical isolate *C. dubliniensis* CD6, exposure of *C. dubliniensis* biofilms to LED light alone (P-L+) and to Cur alone in the lowest concentration (P + L- 20) showed no significant reduction in viability compared to the control group ($0.379 \leq p \leq 1$).

The two-way ANOVA also found a significant effect of the sources of isolates on XTT values of *C. dubliniensis* biofilms. As observed in Fig. 1 (lowercase letters), the four isolates showed different behaviors under the experimental conditions

Table 2 Summary of the two-way ANOVA for both factors analyzed

Source	SQ‡	DF†	MS*	F	p	η^2_p	Power
Sources of isolates	5702	3	1901	133.465	< 0.0001	0.472	1
Experimental conditions	32.949	7	4707	330.508	< 0.0001	0.838	1
Source* conditions	1.88	21	0.09	6286	< 0.0001	0.228	1
Error	6.38	448	0.014				
Total	243.198	480					

‡ *SQ* sum of squares

† *DF* degree of freedom

**MS* mean square

tested. In general, the clinical isolate CD6 tended to be less susceptible to experimental conditions, since it showed significantly higher biofilm viability after exposure to P + L- 20, P + L- 30, P + L- 40, aPDT 20, and aPDT 40 ($0.001 \leq p \leq 0.038$) in comparison to the other clinical isolates. In addition, there were no significant differences in biofilm viabilities among the reference strain and clinical isolates CD7 and CD8 ($0.385 \leq p \leq 0.998$). The exceptions occurred in control group ($p = 0.022$) and aPDT 40 group ($p = 0.006$), where there were significant differences between the reference strain and clinical isolate CD7. The only group where no significant differences ($0.066 \leq p \leq 0.878$) were found among the four isolates tested was the LED light alone group (P-L+).

Uptake of curcumin by *C. dubliniensis*

CSLM was used to investigate the uptake of Cur by the ATCC *C. dubliniensis* cells in planktonic cultures and its penetration through the biofilm. Figures 2 and 3 show the confocal images after incubation of planktonic cultures with Cur 20 μM and biofilms with Cur 40 μM, respectively, for 5 and 20 min.

From Fig. 2, it can be observed that planktonic cultures of *C. dubliniensis* incubated with Cur exhibited bright green fluorescence after 5 and 20 min, with similar uptake patterns in both incubation periods.

When the penetration of Cur through the *C. dubliniensis* biofilm was evaluated (Fig. 3), it was observed that, after 5 min of incubation, a light green fluorescence was seen in specific portions of the biofilm. After 20 min of contact with the biofilms, Cur was able to sensitize a greater amount of cells of the biofilm, and further emit a more intense fluorescence.

Discussion

C. dubliniensis has been reported as the most prevalent non-*albicans* species isolated from oral candidiasis in HIV patients [32]. The recovery of resistant isolates of *C. dubliniensis* to conventional therapy with clinically used anti-fungals, such as fluconazole, has been reported [13, 15, 16, 20, 21]. The resistance of microorganisms to anti-fungal drugs has an important impact on evolution of the diseases because it makes the

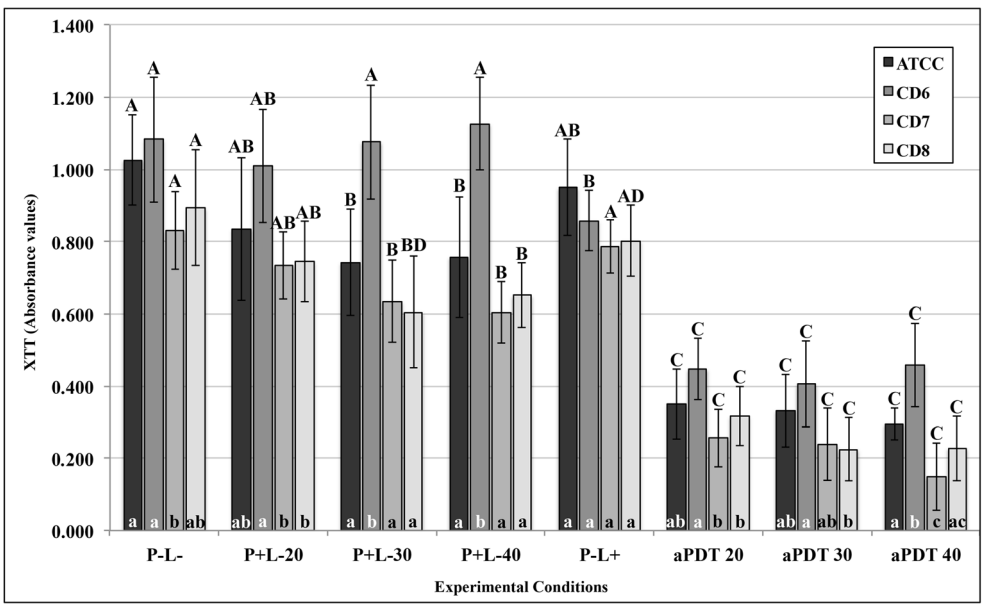

Fig. 1 XTT mean values of all *C. dubliniensis* biofilms after exposure to the different experimental conditions. Error bars represent standard deviation. On the top of the columns, different uppercase letters show significant differences among the experimental conditions; on the bottom of the columns, different lowercase letters show significant differences among the source of isolates (Games-Howell post-hoc; $\alpha = 0.05$)

Table 3 Mean percentage reductions of all experimental groups in relation to control (P-L-)

Isolates	Mean percentage reduction						
	P + L-20	P + L-30	P + L-40	P-L+	aPDT 20	aPDT 30	aPDT 40
ATCC	ND	27.65	26.10	ND	65.81	67.56	71.15
CD6	ND	ND	ND	ND	58.68	62.51	57.70
CD7	ND	23.77	27.29	ND	69.21	71.19	82.05
CD8	ND	32.29	27.11	ND	64.53	74.75	74.42

ND significant reductions were not detected in relation to P-L- ($p > 0.379$ according to Games Howell post hoc test)

infections more difficult to treat, the microorganisms more challenging to eradicate, and it enhances the recurrence rates of the infections. Studies have shown that *Candida* isolates that have demonstrated anti-fungal resistance are susceptible to photodynamic inactivation [26, 28, 33]. Thus, this study evaluated the effectiveness of aPDT mediated by Cur associated with LED light against biofilms of three clinical isolates of *C. dubliniensis* obtained from HIV-infected patients.

In order to establish an aPDT protocol to eradicate *C. dubliniensis*, the planktonic cultures were submitted to different Cur concentrations and LED light, previous to the biofilm assays. The results showed that the complete inactivation of all isolates was verified only with Cur at 20 μM during 20 min followed by 5.28 J/cm^2, which is in agreement with a previous study evaluating a reference strain (ATCC) of *C. dubliniensis* [27]. This result is also in accordance to Dovigo et al. [23], who evaluated the effectiveness of Cur-mediated aPDT against suspensions of *C. albicans* and observed that the same Cur concentration (20 μM) was required to promote photoinactivation of the cells. Therefore, this was the lowest Cur concentration tested against biofilms in the present investigation, followed by 30 and 40 μM. Other studies also observed photoinactivation of suspensions of *C. dubliniensis* using Photogem® [34, 35], erythrosine [36], four dyes [37], and a natural extract of *Althernathera brasiliana* [38].

In the present study, mature biofilms (48 h) of *C. dubliniensis* submitted to aPDT showed significant reduction in their metabolism, regardless of the Cur concentrations (Fig. 1). When the percentage of reduction of the treatments was calculated, the clinical isolate CD6 had the lowest reductions after aPDT with the three Cur concentrations compared with the other strains, suggesting that this isolate was less susceptible to the aPDT (Table 3). In fact, the results concerning the effect of this treatment modality against the four strains evaluated (Fig. 1 and Table 3) show that there was an intra-species difference in the behavior of the isolates against the treatment. This difference may be influenced by the virulence of each strain, which vary according to the isolate itself, as well as to its source (ATCC × clinical). A previous study that evaluated a physical method of *Candida* spp. eradication (microwave irradiation) also detected that, after the treatment, the clinical isolates of several *Candida* species, including *C. dubliniensis*, had significantly higher growth (CFU/mL) than the reference strain [39]. In addition, it has been shown that the expression of some virulence factors, such as adhesion to buccal epithelial cells (BEC) and hydrolytic enzyme production [40], was also more pronounced in clinical isolates of *Candida* spp. from HIV patients. Another recent study also observed that a clinical isolate of *C. albicans* showed a higher adhesion force to an acrylic surface than its reference counterpart [41]. Thus, it is possible that some clinical isolates, such as the *C. dubliniensis* CD6 tested here, may be more pathogenic and, consequently, more difficult to eradicate.

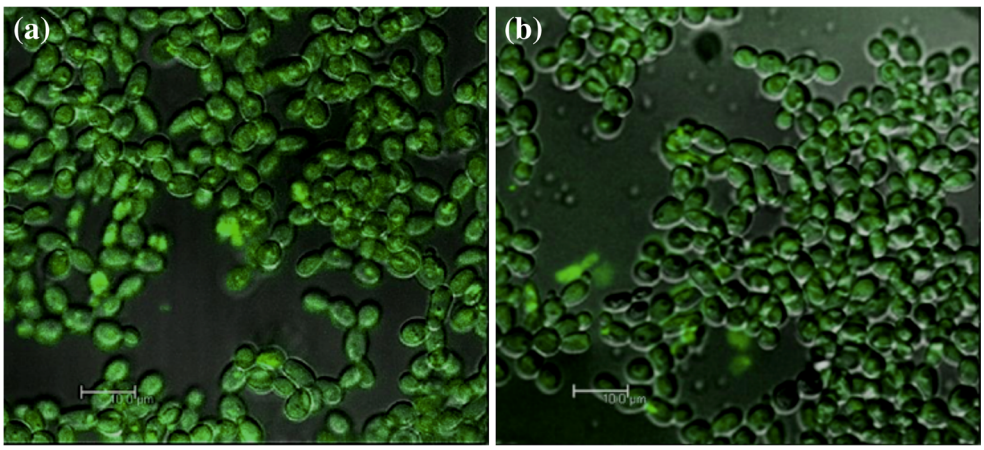

Fig. 2 CLSM imaging of *C. dubliniensis* planktonic suspensions after 5 min (**a**) and 20 min (**b**) of incubation with Cur 20 μM. Bars show magnification of 10.0 μm

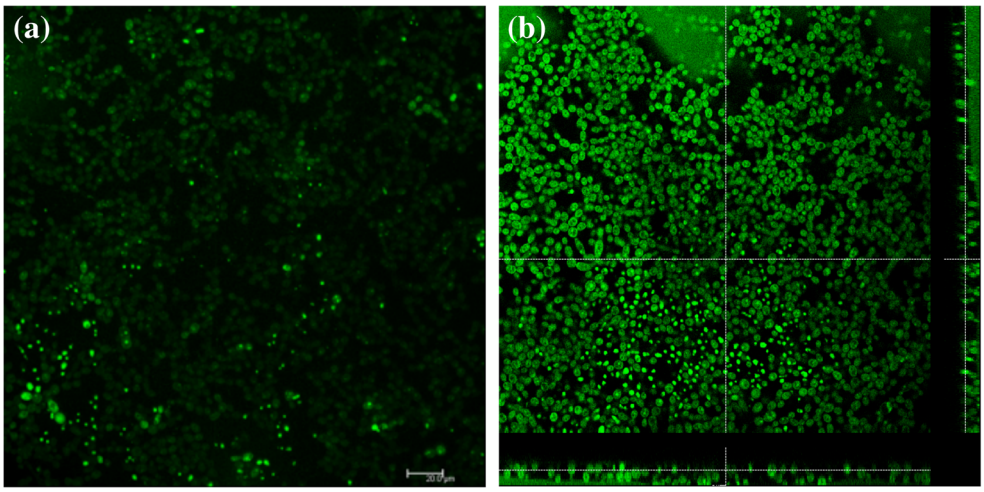

Fig. 3 CLSM imaging of *C. dubliniensis* biofilms after 5 min (**a**) and 20 min (**b**) of incubation with Cur 40 μM. In image B, a brighter fluorescence can be seen of the biofilm than that observed in image A. Bars show magnification of 20.0 μm

Data from the present investigation shows that, although the other *C. dubliniensis* strains (ATCC, CD7, and CD8) were more susceptible to aPDT with Cur at 20, 30, and 40 μM than CD6, the treatment with only Cur at the highest concentrations (30 and 40 μM) was also toxic to them, since small but significant reductions were observed (23.77–32.29% / Table 3). The effect of this PS also influenced some virulence factors of *Candida* spp. Martins et al. [42] observed that Cur alone was able to inhibit the adhesion of *Candida* species to BEC, and *C. dubliniensis* strains isolated from the oral cavity of HIV patients showed the most significant reductions (63 and 74%). Recently, it was also demonstrated that Cur had an anti-adhesive effect on a biofilm of *C. albicans* formed on acrylic surfaces [43]. In this study, the pre-treatment with Cur of both, disks (800 μg/mL for 10 min, corresponding to 50 μg/mL of Cur adsorbed to the surface) and *C. albicans* (50 μg/mL for 3, 30, and 90 min), significantly reduced adhesion of cells to an acrylic surface (70 and 27%, respectively). Moreover, a combined pre-treatment with Cur resulted in the greatest inhibition (93%) [43]. Those results highlight the potential of Cur as an effective anti-fungal and anti-biofilm agent.

Another important finding of the present study was the higher resistance of the biofilms to the aPDT. The results showed that Cur at 20 μM for 20 min followed by 5.28 J/cm^2 inhibited the growth of all *C. dubliniensis* suspensions. However, when the mature biofilms were submitted to aPDT, only a significant reduction in their metabolism was observed (Fig. 1), which is in agreement with other studies evaluating *Candida* spp. [23, 24, 27]. These findings may be related to the sessile organization and ecologic advantages of the biofilms, which warranty protection from the environment, nutrient availability, and metabolic cooperation [44]. In fact, biofilms are more difficult to eliminate, and drug penetration into its organized structure is equally difficult, since extracellular polymeric matrix might limit or exclude the access of drugs to the microorganisms in the deep layers of the biofilm [18].

It is important to highlight that, to aPDT promotes cell inactivation, the PS has to be bonded to or uptaken by the cells. As expected, while *C. dubliniensis* suspensions showed similar Cur uptake in both pre-irradiation times, the biofilms required a longer incubation time with Cur to be sensitized. For biofilms, the CLSM images revealed that, in contrast to the light green fluorescence emitted after 5 min of incubation with Cur (Fig. 3a), a bright green fluorescence could be seen after 20 min (Fig. 3b), suggesting that a longer pre-irradiation time might be required to allow penetration of Cur into the biofilm matrix and sensitization of a greater amount of cells. A similar pattern of sensitization was observed by Andrade et al. [27], who evaluated Cur uptake by biofilms of *C. albicans*, *C. glabrata*, and *C. dubliniensis*.

Cur is a natural pigment of low-molecular weight found in the rhizomes of turmeric and widely used as a food flavoring. Therefore, besides its therapeutic properties (anti-inflammatory, anti-microbial, anti-oxidant, and anti-cancer), its cytotoxicity may be low or improbable. Previous studies evaluated the toxicity of Cur at 5, 10, and 20 μM against mammalian cells, such as macrophages [23] and fibroblasts [26], and verified that these concentrations had no toxic effect on cells metabolism. Another study evaluated the cytotoxicity of Cur at 40 μM on epithelial cells (keratinocytes) co-cultured with *C. albicans* and verified that this concentration had no influence on the metabolism of the cells [45]. Other studies compared the susceptibility of cancer cells and mammalian cells to Cur, and they observed that breast cancer cells were 3.5-fold more susceptible than epithelial cells to Cur up to 100 μM [46], and osteosarcoma cells showed higher reduction in their viability compared with osteoblast cells after incubation with Cur up to 25 μM [47]. In the present study, the concentrations of Cur solution used during experiments with planktonic cells were 5, 10, and 20 μM, while the concentrations used in biofilms were 20, 30, and 40 μM. Despite these favorable results, it is important to mention that Cur at high dosages may be used with caution, since it can cause cell damage. Woo et al. verified that the treatment of

human renal cells with Cur at 50 μM for 24 h resulted in DNA fragmentation and apoptosis [48]. In addition, the treatment of human retinal pigment epithelial cells with Cur at concentrations among 50 and 100 mM for 24 h strongly decreased the viability of the cells and increased the DNA fragmentation rate. When the higher concentration was tested (100 mM), the treatment of the cells for 6 and 24 h resulted in cell apoptosis and almost no viable cells, respectively [49]. However, the susceptibility of cell culture in vitro may differ from in vivo tissue. An in vivo study demonstrated that Cur at 80 μM (associated or not with light) topically applied on tongue of mice with oral candidosis showed no adverse effect on the tissue, since inflammatory response was verified in all animals with candidosis regardless the treatment received [50].

The present study showed that aPDT mediated by Cur promoted significant reductions in the metabolism of *C. dubliniensis* biofilms, which required a longer pre-irradiation time for cell photosensitization compared with the planktonic cultures. However, the viability of cells by conventional plating (CFU) was not assessed. This assessment could be important, since it has been demonstrated that biofilms submitted to aPDT may show different results when evaluated by these two viability assays (XTT and CFU) [51]. In addition, a positive control using a gold standard method of eradication of biofilm, such as chlorhexidine or an anti-fungal agent (fluconazole, nystatin), was not included in order to compare with the aPDT effect. Thus, within the limitations of this investigation, the protocol evaluated in the present study could be an alternative method for *C. dubliniensis* biofilm control and should be further tested in in vivo conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the Ethics Committee of Araraquara Dental School (process number 16/11).

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