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Susceptibility of Clinical Isolates of *Candida* to Photodynamic Effects of Curcumin

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Background and Objective: The resistance of *Candida* species to antifungals represents a major challenge for therapeutic and prophylactic strategies. This study evaluated photodynamic therapy (PDT) mediated by Curcumin (CUR) against clinical isolates of *C. albicans*, *C. tropicalis*, and *C. glabrata*, both in planktonic and biofilm forms.

Study Design/Materials and Methods: Suspensions of *Candida* were treated with three CUR concentrations and exposed to four LED fluences. The protocol that showed the best outcomes for inactivation of the planktonic phase was selected to be evaluated against *Candida* biofilms. In addition, two higher CUR concentrations were tested. The metabolic activity of biofilms was evaluated by means of XTT reduction assay and the biofilm biomass was evaluated using crystal violet (CV) staining assay. Data were analyzed in a mixed model nested ANOVA, Wilcoxon's nonparametric tests, and the Kruskal–Wallis test ($\alpha = 5\%$).

Results: The use of CUR in association with light was able to promote a significant antifungal effect against the planktonic form of the yeasts. When using 40 μM of CUR, the metabolic activity of *C. albicans*, *C. glabrata*, and *C. tropicalis* biofilms was reduced by 85%, 85%, and 73%, respectively, at 18 J/cm^2 . CUR-mediated PDT also decreased the biofilm biomass of all species evaluated. In addition, CV staining showed that *C. albicans* isolates were strong biofilm-forming strains, when compared with *C. glabrata* and *C. tropicalis* isolates.

Conclusion: The results from the present investigation showed that low CUR concentrations can be highly effective for inactivating *Candida* isolates when associated with light excitation. Lasers Surg. Med. 43:927–934, 2011. © 2011 Wiley Periodicals, Inc.

Key words: *Candida albicans*; *Candida glabrata*; *Candida tropicalis*; photodynamic therapy; turmeric

INTRODUCTION

In healthy individuals, *Candida* species are considered commensal yeasts of the oral cavity. *Candida albicans* represents the predominant species, while *Candida tropicalis* and *Candida glabrata* are considered the second

most frequent [1,2]. These microorganisms can also act as opportunist pathogens, being the so-called non-*albicans* *Candida* species (NAC) increasingly recognized as important agents of human infection. Some predisposing factors can markedly increase the host's susceptibility to oral candidiasis, such as the use of ill-fitting dentures, AIDS and long-term antibiotic therapy [3–5]. For immunocompromised individuals, the disseminated forms of the disease can be a serious problem, often resulting in high mortality rates [6]. A number of surveys have documented increased rates of *C. glabrata* and *C. tropicalis* in systemic fungal infections [6–8]. *C. tropicalis* may be of particular concern in these cases because of its high pathogenicity in causing fungaemia [8].

Treatments available for *Candida* infection are typically drug based and can involve topical and systemic antifungal agents [4]. However, the use of standard antifungal therapies can be limited because of toxicity, low efficacy rates, and drug resistance [9]. In fact, the resistance of NAC to antifungals represents a major challenge for therapeutic and prophylactic strategies [6]. Some of these species, such as *C. glabrata*, are inherently less sensitive to fluconazole and other antimycotic drugs frequently used to combat fungal infections. In addition to the intrinsic resistance of some species, different clinical isolates belonging to a single species can vary widely in their susceptibility to antifungal agents [10]. This diversity could predispose the selection of more resistant strains and the persistence of the disease [10]. Moreover, most manifestations of candidiasis are associated with the formation of *Candida* biofilms on surfaces such as oral tissues, prostheses, and implanted devices [9]. Biofilms

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represent a protective reservoir for oral microorganisms that provides survival advantage to the yeast and elevated resistance to antifungal drugs [11]. Therefore, the increasing worldwide occurrence of antifungal resistance has driven research to the development of alternative strategies to inactivate *Candida* species and to treat oral candidiasis.

Photodynamic inactivation of microorganisms is based on the combination of a drug, known as photosensitizer (PS), and the delivery of a visible light of the appropriate wavelength to excite the PS molecule [12]. The microbial killing is a result of chemical and phototoxic reactions, in which PS absorbs photons and induces the formation of free radicals and reactive oxygen species (ROS). These ROS can rapidly react with nonspecific targets, such as cell membranes and proteins which lead to microbial destruction [12]. When compared with other therapies, photodynamic therapy (PDT) has several advantages, such as high target specificity (PS can be delivered to cells and light can be focused on the site of the lesion), few undesired side effects, and little likelihood of leading to the development of resistance by microorganisms [13]. For this reason, there has been an increased interest in developing PDT for local infections treatment. Different types of PS have been proposed in laboratory investigations, including porphyrins [14,15], phenothiazine dyes [16,17], chlorins [18,19], and phthalocyanines [20]. However, complete killing of the microorganisms is not frequently achieved and some of the available PS showed limited effectiveness against *Candida* biofilms and animal models of candidiasis [15,21]. Such facts have driven research to find adequate parameters of PDT prior to clinical investigations and the search for new PSs remains an important goal.

Curcumin (CUR) is an intensely yellow pigment, isolated from rhizomes of *Curcuma longa*, which is in worldwide used as a cooking spice, flavoring agent, and colorant [22]. An increasing number of investigations have suggested that CUR exhibits potential therapeutic applications that may be enhanced by combination with light, as it displays a high light absorption in the visible spectral region, around 400–500 nm [23–26]. Recently, this natural compound has been shown to possess phototoxic potential against yeast cells [23]. The authors found that CUR was an effective PS for the inactivation of one reference strain of *C. albicans*, in planktonic form. It was also showed that the therapy was more effective in inactivating the yeast cell than a macrophage cell line, suggesting certain specificity of CUR-mediated PDT. However, some aspects need to be further investigated before a clinical recommendation of the therapy, such as the adequate protocol for the effective photosensitization of *Candida* biofilms. As PDT mediated by CUR already showed preliminary results for yeast inactivation, the present study evaluated different PDT protocols against *C. albicans* and non-*albicans* species, both in planktonic and biofilms form. Considering the important clinical relevance of investigating more than one isolate belonging to a single species, we evaluated a total of 15 clinical isolates belonging to the species *C. albicans*, *C. tropicalis*, and *C. glabrata*.

MATERIALS AND METHODS

Photosensitizer and Light Source for PDT

Natural CUR (Fluka Co.) was obtained from Sigma-Aldrich (St. Louis, MO). A stock solution of CUR (600 μ M) was prepared in DMSO and then diluted in saline solution to obtain the concentrations to be tested. A light emitting diode (LED) based device, composed of eight royal blue LEDs (LXHL-PR09, Luxeon[®] III Emitter, Lumileds Lighting, San Jose, CA), was used to excite CUR [23]. The LED device provided a uniform emission from 440 to 460 nm, with maximum emission at 455 nm. The mean irradiance delivered was of 22 mW/cm², which was measured at the same location in which the microorganisms were exposed to PDT.

Candida Strains and Grown Conditions

A total of 15 clinical isolates of *Candida* comprising *C. albicans*: Ca1, Ca2, Ca3, Ca4, and Ca5; *C. glabrata*: Cg1, Cg2, Cg3, Cg4, and Cg5; and *C. tropicalis*: Ct1, Ct2, Ct3, Ct4, and Ct5, were used in this study. The strains were previously isolated from patients with oral candidiasis and identified by CHROMagar technique and the carbohydrate assimilation tests (ID 32C-BioMérieux, Paris, France). Isolates were maintained in Yeast–Peptone–Glucose medium and frozen at -70°C . Prior to each experiment, yeasts were aerobically cultured at 37°C for 24 hours on Sabouraud Dextrose Agar containing 5 mg/l gentamicin (SDA).

Planktonic Culture of *Candida* and PDT Treatments

To obtain standardized suspensions of *Candida*, each isolate was individually inoculated in Tryptic Soy Broth, incubated at 37°C , harvested after centrifugation, washed twice and resuspended in sterile saline to a turbidity of 10^7 cells/ml. The yeast suspension was standardized by adjusting the optical density to a turbidity corresponding to spectrophotometric absorbance 0.38 at 520 nm. Aliquots of 100 μ l of the standardized suspensions of *Candida* were individually transferred to separate wells of a 96-well microtitre plate. Curcumin solutions were added to the wells to give the final concentrations of 5, 10, and 20 μ M, which correspond to 1.8, 3.7, and 7.4 mg/l [23]. After dark incubation for 20 minutes (pre-irradiation time), the plate was placed on the LED device and illumination was performed at 5.28, 18, 25.5, and 37.5 J/cm² (PDT groups). To determine whether CUR alone, at the tested concentrations, induced any effect on cell viability (dark toxicity), additional wells containing the yeast suspensions were exposed to CUR under identical conditions to those described above, but not to LED light (P+L-). The effect of LED light alone was determined by exposing cells to light without being previously exposed to CUR (P-L+). The overall control consisted of *Candida* suspensions not exposed to CUR or LED light (P-L-). Ten-fold serial dilutions of aliquots of the contents of each well were obtained and plated on SDA. After incubation (37°C for 48 hours), colony counts were quantified using a digital colony counter (CP 600 Plus, Phoenix Ind Com Equipamentos

Científicos Ltda, Araraquara, SP, Brazil). The number of colony forming units per milliliter (CFU/ml) was determined and transformed into logarithm (\log_{10}).

C. albicans Biofilm and PDT Treatments

Based on the results of the experiments reported above, the concentration of CUR (20 μ M) and light fluences (5.28 and 18 J/cm²) that achieved the most promising results against planktonic cultures were selected to be tested toward *Candida* biofilms. In addition, two higher concentrations of CUR were evaluated (30 and 40 μ M). For *in vitro* biofilm formation, the isolates were individually inoculated in 5 ml of RPMI-1640 medium and incubated overnight in an orbital shaker (AP 56, Phoenix Ind Com Equipamentos Científicos Ltda, Araraquara, SP, Brazil) at 120 rpm, 37°C, for 16 hours. After incubation, the yeast were washed twice with 5 ml of phosphate-buffered saline (PBS), and suspended to 10⁷ cells/ml by adjusting the optical density of the suspension to 0.38 at 520 nm [27,28]. Aliquots of 100 μ l of the standard cell suspension were transferred into each well of pre-sterilized polystyrene, flat-bottomed 96-well microtiter plates. The plate was incubated at 37°C for 1.5 hours in an orbital shaker at 75 rpm (adhesion phase). After that, the suspensions were carefully aspirated, and each well was washed twice with 150 μ l of PBS to remove nonadherent cells. In order to allow biofilm growth, 150 μ l of fresh RPMI-1640 was transferred into each well, and the plates were incubated at 37°C for 48 hours at 75 rpm.

After biofilm formation, the wells were carefully washed twice with 200 μ l of PBS to remove remaining nonadherent cells. Then, 100 μ l of CUR was added to each well. The CUR concentrations tested were 20, 30, and 40 μ M, which corresponded to 7.4, 11.05, and 14.7 mg/l. The plates were incubated in the dark for 20 minutes, followed by illumination of 5.28 and 18 J/cm². Control wells received PBS instead of CUR solution and samples were not illuminated (P–L–).

The antifungal effects against the biofilms were evaluated by a metabolic assay based on the reduction of XTT, a tetrazolium salt (Sigma–Aldrich). To prepare the XTT solution, the salt was dissolved in Milli-Q water (1 mg/ml) and stored at –70°C. For each experiment, 158 μ l of PBS prepared with glucose at 200 mM, 40 μ l of XTT plus 2 μ l of menadione at 0.4 mM were mixed and transferred to each well. The plates were incubated in the dark at 37°C for 3 hours. After this, 100 μ l of the reacted XTT salt solution was transferred to a new 96-well microtiter plate and the cell viability was analyzed by proportional colorimetric changes. Light absorbance measured by a microtiter plate reader (Thermo Plate–TP Reader) at 492 nm.

In order to evaluate whether PDT could cause the detachment of *Candida* biofilms from the plastic surface, the quantification of the cells adhered to the wells was performed by means of Crystal Violet (CV) assay [27]. For this purpose, additional samples from P–L– and PDT groups (40 μ M of CUR with 18 J/cm² of illumination) were included in this evaluation. The biofilm coated wells of microtiter plates were washed twice with 200 μ l of PBS

and then air dried for 45 minutes at room temperature. Then, each of the washed wells was stained with 110 μ l of 0.4% aqueous CV solution for 45 minutes. Afterwards, each well was washed three times with 200 μ l of sterile distilled water and immediately destained with 200 μ l of 95% ethanol. After 45 minutes at room temperature, 100 μ l of the destaining solution was transferred to a new well and the amount of the CV stain was measured with a microtiter plate reader (BIO-RAD, model 3550-UV, microplate reader, Hercules, CA) at 595 nm.

Statistical Analysis

For each *Candida* isolate, the experimental treatments (PDT, P+L– and P–L–) were tested in five independent samples. Descriptive analysis was performed to summarize the results of each *Candida* isolate. Then, the results from the five isolates of the same species were joined to obtain a general response of the species. Data from colony counts and XTT assay were analyzed in a mixed model nested ANOVA: experimental (PDT) and control (P+L–, P–L+, and P–L–) groups were treated as fixed factors, whereas the five *Candida* isolates were random factors nested within groups. The test was based on the assumption of a linear model with errors normally distributed around zero with corrected variance between groups to fit heteroscedasticity for fixed effects and random effects normally distributed with constant variance. The ANOVA was followed by Tukey's post-hoc tests for multiple comparisons among experimental and control groups (fixed effects). Data obtained after the evaluation of CV staining assay were evaluated using Wilcoxon's nonparametric tests for pairwise comparisons between control (P–L–) and PDT groups of each species and the Kruskal–Wallis test was used to test the null hypothesis among P–L– groups of the three species. The significance level adopted was 5% ($P < 0.05$).

RESULTS

Photodynamic Inactivation of Planktonic Cultures of *Candida*

Table 1 shows the minimal CUR concentration required to achieve at least 4 log decrease in log(CFU/ml) values after PDT. It can be seen from this table that the efficiency of CUR as a fungicidal PS was not homogeneous among the different isolates belonging to the same species. Considering the mean response obtained from the five isolates of each species, the 4 log reduction in *C. albicans* and *C. tropicalis* viability occurred with the use of 10 μ M of CUR at all light fluences tested, while *C. glabrata* required 20 μ M of CUR associated with light fluences starting at 18 J/cm².

The mixed model nested ANOVA showed that PDT groups presented a significant level of variation within the same species, which suggested a different response of the distinct isolates to photoinactivation. Confidence intervals of 95% showed that the random effect represented by the five *Candida* isolates of each species was the major factor influencing the variability in colony

TABLE 1. Minimal CUR Concentration (μM) Required to Achieve 4 log Decrease in $\log(\text{CFU/ml})$ Values After PDT at Different Light Fluences

<i>Candida</i> isolate	5.28 J/cm ²	18 J/cm ²	25.5 J/cm ²	37.5 J/cm ²
Ca1	10	10	5	5
Ca2	10	10	10	10
Ca3	20	20	10	10
Ca4	20	10	10	10
Ca5	5	5	5	5
Ca mean ^a	10	10	10	10
Cg1	20	20	20	20
Cg2	NA	NA	NA	NA
Cg3	20	20	20	20
Cg4	20	20	20	20
Cg5	NA	20	20	20
Cg mean ^a	NA	20	20	20
Ct1	10	5	5	5
Ct2	NA	10	10	20
Ct3	10	10	10	10
Ct4	10	5	5	5
Ct5	20	10	10	10
Ct mean ^a	10	10	10	10

NA, 4 log drop was not achieved at the light fluence.

^aResults obtained from the mean of the five clinical isolates.

counts within each PDT group. The use of different strains accounted for 98.0; 99.3% and 98.8% of the total variability observed in the PDT groups, for *C. albicans*, *C. glabrata*, and *C. tropicalis*, respectively. ANOVA also showed a significant variation of the mean colony counts among the P-L-, P+L-, P-L+, and PDT groups. The results of multiple comparisons are shown in Table 2. All the associations of CUR and LED light were able to promote a significant reduction in *C. albicans* and *C. tropicalis* viability, when compared with the P-L- condition. A significant reduction in *C. glabrata* viability was observed with the use of 10 and 20 μM of CUR, at the four LED fluences. Complete inactivation of *C. glabrata* was not observed. On the other hand, the five *C. albicans* isolates were completely killed after PDT mediated by

20 μM of CUR, even at the lowest LED fluence. Similarly, *C. tropicalis* isolates were inactivated with 20 μM of CUR, but at fluences starting at 18 J/cm². Control groups, comprising the P-L-, P+L-, and P-L+ conditions, showed similar values of colony counts.

Photodynamic Inactivation of *Candida* Biofilms

Figure 1 shows the effect of PDT mediated by different concentrations of CUR on the metabolic activity of biofilms (absorbance after XTT reduction assay). When compared with the control, PDT induced a significant decrease in absorbance values of all *Candida* species. For *C. albicans* and *C. tropicalis* isolates, the use of CUR concentrations of 30 and 40 μM associated with 5.28 J/cm², as well as the use of 20, 30, and 40 μM at 18 J/cm², promoted a similar reduction in the metabolic activity of biofilms ($P < 0.05$). However, the lowest values of metabolic activity of *C. albicans* biofilms were observed with the use of 40 μM of CUR, associated with both 5.28 and 18 J/cm². Under these experimental conditions, the percentage of reduction in metabolic activity was over 80% in comparison with the P-L- group (Table 3). Similar results were observed for *C. glabrata* biofilms. For *C. tropicalis*, the association of 40 μM with 18 J/cm² showed the lowest values of metabolic activity of biofilms, reaching 73% of reduction compared to control group (Table 3).

It can be seen from Table 3 that variations in response to PDT were detected in the individual strains of each species. Similar to the results found for the planktonic phase, the mixed ANOVA was able to verify that PDT groups presented a significant level of variation within the same species. Considering the microorganisms in the biofilms phase, the use of different isolates accounted for 85.3; 42.0% and 80.1% of the total variability of XTT values observed in the PDT groups, for *C. albicans*, *C. glabrata*, and *C. tropicalis* respectively.

The quantification of biofilm biomass using the CV assay was performed in P-L- and PDT (40 μM at 18 J/cm²) samples. Biofilm biomass was significantly lower in the PDT samples ($P < 0.05$), suggesting that PDT promoted the partial detachment of biofilms from the wells. The reduction in biomass observed in PDT samples

TABLE 2. Mean Values and Standard Deviation of $\log(\text{CFU/ml})$ Calculated From the Results of the Five Clinical Isolates of Each *Candida* Species

Species (mean)	P-L+		P+L-			PDT 5.28 J/cm ²			PDT 18 J/cm ²			PDT 25.5 J/cm ²			PDT 37.5 J/cm ²		
	P-L-	37.5 J/cm ²	5 μM	10 μM	20 μM	5 μM	10 μM	20 μM	5 μM	10 μM	20 μM	5 μM	10 μM	20 μM	5 μM	10 μM	20 μM
<i>C. albicans</i>	6.43 ^a 0.17	6.44 ^a 0.17	6.48 ^a 0.09	6.43 ^a 0.15	6.42 ^a 0.12	3.21 ^b 1.14	1.55 ^{bc} 1.37	0.00 0.00	2.98 ^{bc} 1.23	1.45 ^{bc} 1.34	0.00 0.00	2.56 ^{bc} 1.52	0.86 ^c 0.86	0.00 0.00	2.52 ^{bc} 1.50	1.05 ^{bc} 0.93	0.00 0.00
<i>C. glabrata</i>	6.55 ^a 0.07	6.53 ^a 0.08	6.58 ^a 0.05	6.55 ^a 0.06	6.56 ^a 0.04	5.19 ^{ab} 0.46	3.96 ^{bc} 0.86	2.84 ^{cde} 0.78	4.86 ^{ab} 0.59	3.62 ^{bcd} 0.94	1.85 ^{de} 1.26	4.90 ^{ab} 0.58	3.59 ^{bcd} 0.74	1.83 ^{de} 1.43	4.97 ^{ab} 0.56	3.64 ^{bed} 0.85	1.79 ^e 1.45
<i>C. tropicalis</i>	5.59 ^a 0.33	5.71 ^a 0.36	5.65 ^a 0.29	5.62 ^a 0.32	5.64 ^a 0.31	2.87 ^b 0.30	0.92 ^c 0.92	0.30 ^c 0.62	1.59 ^{bc} 0.90	0.27 ^c 0.54	0.00 0.00	1.61 ^{bc} 0.91	0.28 ^c 0.56	0.00 0.00	1.62 ^{bc} 0.92	0.29 ^c 0.58	0.00 0.00

Different lower case letters denote significant differences among columns (groups) according to Tukey's Test ($P < 0.05$).

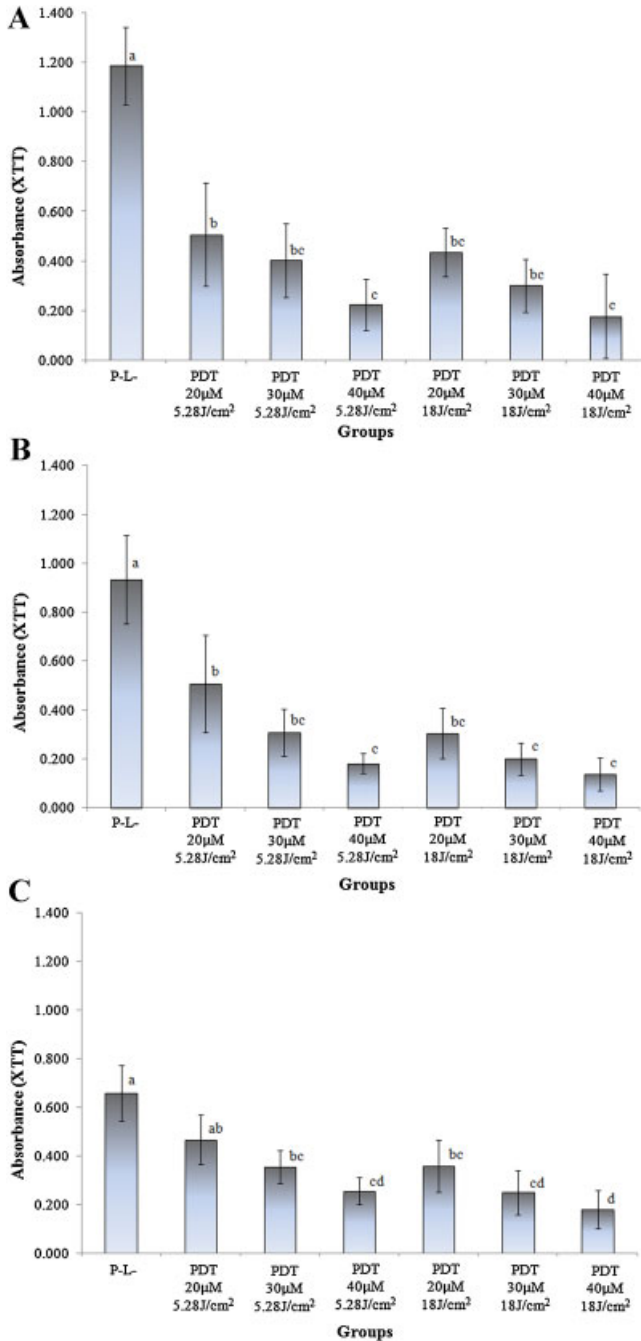


Fig. 1. Graphic representation of mean values and standard deviation of absorbance values (XTT assay) observed after experiments with the *C. albicans* (A), *C. glabrata* (B), and *C. tropicalis* (C) isolates. Different lower case letters denote significant differences among columns (groups) according to Tukey's Test ($P < 0.05$).

corresponded to 52.3%, 69.1%, and 64.1% in comparison with the P–L– groups for *C. albicans*, *C. glabrata*, and *C. tropicalis*, respectively. In addition, the use of CV staining revealed that the ability to produce biofilms

in vitro was higher in *C. albicans* isolates when compared with *C. glabrata* and *C. tropicalis* ($P < 0.05$).

DISCUSSION

Some previous investigations have already suggested that CUR *per se* may have antifungal properties [29,30] and its combination with light has been proposed in order to improve its antifungal action [23]. The results showed that the use of CUR in association with light was able to promote a significant antifungal effect against the planktonic form of the yeasts. According to Jori et al.[31], the efficiency of a PS can be expressed as the minimal concentration which induces a 4 log decrease in survival of microorganisms. For *C. albicans* and *C. tropicalis* isolates, the 4 log drop in viable counts was observed with 10 μM (3.7 mg/L) of CUR at all light fluences tested, while *C. glabrata* isolates required 20 μM (7.4 mg/L) of CUR with illumination of 18 J/cm^2 or higher. In addition, no evidence of colony growth was observed for *C. albicans* and *C. tropicalis* isolates after photosensitization with 20 μM of CUR and illumination at 5.28 and 18 J/cm^2 of LED light, respectively. These results are in agreement with those recently published in which a reference strain of *C. albicans* (ATCC 90028) was efficiently killed after CUR-mediated PDT [23]. The photodynamic effect of CUR against *Candida* species has not been widely documented in the medical literature, but its antibacterial effect has been described with the use of comparable CUR concentrations (1–25 μM) and light fluences (0.5–30 J/cm^2) [26]. In the present study, the reduction in viability of the clinical isolates was observed only when CUR was used in conjunction with LED light. In contrast, Garcia-Gomes et al. [32] found that 50 μM -CUR, without light excitation, was able to inhibit the growth of one strain of *C. albicans*. A recent publication found that the minimum inhibitory concentration (MIC) of CUR against 14 *Candida* strains ranged from 250 to 2,000 mg/L while fluconazole MICs were between 4 and 64 mg/L [33]. In comparison with these investigations, the present study used lower CUR concentrations to sensitize the *Candida* isolates in the planktonic form ($\leq 20 \mu\text{M}$ or 7.4 mg/L). Thus, although promising results were described with the use of CUR without light excitation, higher concentrations of the compound were needed to induce the antifungal effect. Likewise, the studies of Dahl et al. [34] and Tønnesen et al. [35] reported that the antibacterial activity of some CUR concentrations was greatly enhanced by light. Another finding in the present study was that PDT effectiveness was not always fluence-dependent. The use of fluences higher than 18 J/cm^2 did not improve the effectiveness of PDT for inactivating the *Candida* species. This could be as a result of the CUR photobleaching which leads to photodegradation of the PS solution and decreases in reactive oxygen species production [23,25].

Since the light fluences of 5.28 and 18 J/cm^2 showed the best outcomes for *Candida* photoinactivation (planktonic phase), these parameters were selected to be tested against biofilms. When using 40 μM of CUR, the

TABLE 3. Percentage of Reduction (%) in Metabolic Activity (XTT Reduction Assay) Obtained After Treatment of *Candida* Biofilms With PDT

<i>Candida</i> isolate	PDT 5.28 J/cm ²			PDT 18 J/cm ²		
	20 μ M	30 μ M	40 μ M	20 μ M	30 μ M	40 μ M
Ca1	67	71	88	65	74	90
Ca2	30	48	70	56	76	87
Ca3	59	72	86	62	75	73
Ca4	69	71	82	73	76	87
Ca5	62	70	81	61	72	84
Ca mean ^a	57	66	81	63	75	85
Cg1	43	61	80	59	79	81
Cg2	71	71	80	76	86	84
Cg3	43	69	79	64	67	86
Cg4	53	70	83	74	84	90
Cg5	22	67	81	66	76	87
Cg mean ^a	46	67	81	67	79	85
Ct1	28	55	67	60	73	76
Ct2	33	54	69	47	80	89
Ct3	31	45	47	39	48	57
Ct4	22	39	66	33	53	71
Ct5	36	36	54	53	57	72
Ct mean ^a	29	46	61	46	62	73

The percentage was calculated versus the XTT value obtained with P–L– group.

^aResults obtained from the mean of the five clinical isolates.

metabolic activity of *C. albicans*, *C. glabrata*, and *C. tropicalis* biofilms was reduced by 85%, 85%, and 73%, respectively, at 18 J/cm². The ability of CUR to act as an antifungal agent against *C. albicans* biofilms has been previously reported [23,36]. Accordingly, a similar protocol (40 μ M of CUR and 18 J/cm²) was used against one ATCC strain of *C. albicans* and the authors observed a decrease of 87% in biofilm viability [23]. On the other hand, the photosensitization of *C. glabrata* and *C. tropicalis* biofilms by CUR is a completely new result. It was interesting to note that *C. glabrata* isolates in the planktonic phase seemed to be less susceptible to PDT in comparison with *C. albicans* and *C. tropicalis*, but this observation was not true when biofilms were considered. *C. glabrata* required a higher CUR concentration to achieve 4 log reduction in CFU/ml counts and it was not completely inactivated in any of the protocols tested. Other investigations also found the reduced susceptibility of *C. glabrata* suspensions to photoinactivation [14,15]. However, this reduced susceptibility was not observed in *C. glabrata* biofilms, which showed similar levels of photoinactivation in comparison with *C. albicans* biofilms. According to Seneviratne et al. [11], *C. albicans* isolates usually form thicker biofilms with the presence of hyphal elements that support the exopolymeric substances and blastospores, promoting a three-dimensional multilayered community. In contrast, *C. glabrata* isolates are more prone to producing thinner and compact biofilms [11]. In the present investigation, a liquid medium was used to grow the biofilms (RPMI-1640) which induces hyphal formation in *C. albicans* cultures [37]. Thus, the presence of

a robust architecture in *C. albicans* biofilms was probably an important factor influencing the penetration of CUR and light during PDT, differently from the thinner *C. glabrata* biofilms mainly composed of blastospores. For this reason, it is believed that even with the *C. glabrata* cells being more resistant to photoinactivation, the effect against biofilms was equivalent between the two species. In addition, the comparison made among the mean absorbance values (CV staining) found in control biofilms showed that *C. albicans* isolates were strong biofilm-forming strains, when compared with *C. glabrata* and *C. tropicalis* isolates ($P < 0.05$). Conversely, other investigations have found that *C. tropicalis* may have a similar ability to form biofilms when compared with *C. albicans* [38]. These different results probably reflect the different growth conditions, that is, culture medium and substrate, and physiological variations among the strains used in different investigations. The results also showed significant variation in response to PDT among the individual strains of each species. The level of variation within the same species was more pronounced in the planktonic cultures exposed to PDT, but biofilms also showed significant discrepancies. Significant differences observed in the present study among *Candida* species and strains with regard PDT susceptibility also concur with previous studies [15,32,39]. Therefore this investigation once again confirms the importance of testing more than one isolate of *Candida* belonging to a given species to characterize the species-specific behavior. In general, significant variation in the response of individual strains was observed, but all of the isolates were susceptible to CUR-mediated PDT.

The CUR-phototoxicity to microbial systems seems to be mediated through the excited states of CUR, their subsequent reactions with oxygen and formation of reactive species [40]. The production of nonspecific reactive components has important advantages over traditional antifungal treatments. It is unlikely that yeasts could develop resistance to them and microorganisms with innate or acquired resistance to antifungal drugs should be susceptible to photodynamic damage. *C. glabrata* is recognized as having a stable resistance to fluconazole, while *C. tropicalis* has shown rapid development of resistance to fluconazole and amphotericin B, especially in cancer patients [6]. Our results showed that although *Candida* isolates differed in the response to PDT, the five clinical strains belonging to each species were susceptible to CUR-mediated PDT indicating its broad spectrum of action against *Candida*. According to CV staining assay, CUR-mediated PDT not only reduced the metabolic activity of *Candida* biofilms, but also decreased the biofilm biomass of all species evaluated. It has previously been suggested that PDT can target bacterial biofilms by killing the cells and disrupting biofilm architecture [41]. Light micrographs performed by Goulart et al. [42] showed that PDT was able to detach bacterial cells from the well in which biofilms were formed. Thus, it is possible to suggest that CUR-mediated PDT can act against *Candida* biofilms in a similar way, simultaneously altering yeast metabolism and causing biofilm disarticulation. It has been suggested that lethal photosensitization is likely to occur in the outermost layers of *C. albicans* biofilms probably due to the superficial penetration of PS into the biofilm layers [43]. Since PDT can superficially disrupt the biofilm structure after a single application, it is possible to suppose that repeated cycles of the therapy may be capable of reducing the biofilm biomass, allowing deeper penetration of the PS and promoting higher levels of biofilm inactivation. This mode of action can have important relevance for the design of future clinical investigations on CUR-mediated PDT.

In conclusion, the results from the present investigation showed that low CUR concentrations could be highly effective for inactivating *Candida* isolates when associated with light excitation. The three main *Candida* species that are frequently associated with fungal infections were efficiently photosensitized with the protocols tested. Thus, the parameters established in this *in vitro* investigation will shortly be used in an animal model of *Candida* infection in order to verify the effectiveness of the therapy for the treatment of oral candidiasis.

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