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ORIGINAL ARTICLE

Twice-daily red and blue light treatment for *Candida albicans* biofilm matrix development control

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Abstract

Phototherapy has been proposed as a direct means of affecting local bacterial infections. However, the use of phototherapy to prevent fungal biofilm development has received comparatively less attention. This study aimed to determine the effects of red light treatment and blue light treatment, without a photosensitizer, on the development of *Candida albicans* biofilm. During the development of 48-h biofilms of *C. albicans* SN 425 ($n = 10$), the biofilms were exposed twice-daily to noncoherent blue and red light (LumaCare; 420 nm and 635 nm). The energy density applied was 72 J cm^{-2} for blue light and 43.8 J cm^2, 87.6 J cm^2, and 175.5 J cm^2 for red light. Positive control (PC) and negative control (NC) groups were treated twice-daily for 1 min with 0.12% chlorhexidine (CHX) and 0.89% NaCl respectively. Biofilms were analyzed for colony forming units (CFU), dry-weight, and exopolysaccharides (EPS-soluble and EPS-insoluble). Data was analyzed by one-way ANOVA and Tukey post hoc test ($\alpha = 0.05$). Dry-weight was lower than NC ($p < 0.001$) and approached PC levels with both red and blue light treatments. CFU were also lower in groups exposed to blue light and higher durations of red light ($p < 0.05$). EPS-soluble and EPS-insoluble measures were variably reduced by these light exposures. In conclusion, twice-daily exposure to both blue and red lights affect the biofilm development and physiology of polysaccharide production and are potential mechanisms for the control of *C. albicans* biofilm matrix development.

Keywords *Candida albicans* · Biofilm · Red light · Blue light · Phototherapy

Introduction

Candida albicans is the primary species associated with oral candidiasis and has been increasingly observed in both immunocompromised and non-immunocompromised individuals [1]. This increased incidence of oral candidiasis is related to an increased incidence of several factors, including diabetes, decreased life expectancy, HIV infection and AIDS epidemic, immunosuppressive therapy utilization, practice of invasive clinical procedures (e.g., solid organ or bone marrow transplantation) and broad spectrum antibiotic use in recent years [1, 2]. *C. albicans* is a frequent fungal biofilm-forming pathogen that can cause life-threatening infections by colonizing medical and dental devices (i.e., prostheses, implants, and catheters) [2]. Dimorphism is an important characteristic experienced by *C. albicans* in response to adverse environmental conditions, which increases its virulence. *C. albicans* can present as either yeast cells or long filamentous cells known as hyphae [3]. The yeast form facilitates the colonization of different sites while the hyphal form has an important role in causing disease by invading epithelial cells and causing tissue damage [3].

C. albicans attachment to mucosal tissues and abiotic surfaces and the formation of biofilms are crucial steps for its

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survival and proliferation in the oral cavity [4]. It is estimated that most microorganisms in nature occur in biofilms [5]. The beginning of biofilm growth is characterized by the initial adherence of planktonic cells to a surface, proliferation of the yeast cells across the substrate surface, and hyphal development. The final step of biofilm development is the maturation stage, in which yeast-like growth is repressed, hyphal growth is augmented, and extracellular matrix encases the biofilm [6]. Biofilm formation is one of the most important virulence factors in *C. albicans* species and contributes to increased resistance to current antifungal agents, environmental stress, and host immune mechanisms [7]. It has been shown that *C. albicans* exopolysaccharides (EPS) in the matrix are composed by β-glucans (EPS-insoluble) and α-mannans (EPS-soluble) interacting to form a mannan-glucan complex (MGCx) [8, 9]. This exopolysaccharide interaction is essential for the protection of the biofilms from drug treatment [10, 11]. Thus, targeting EPS from *C. albicans* extracellular matrix may lend to the designing of novel antibiofilm therapies for topical oral candidiasis treatment.

Photodynamic antimicrobial chemotherapy (PACT) has been indicated as an alternative to conventional antimicrobial therapy in killing oral microorganisms [12]. It is based on the use of photosensitizers that begin a photochemical response when exposed to light of a specific wavelength [12]. A previous study investigated the antimicrobial effects of blue (wavelength range, 400–440 nm) and red (wavelength range, 570–690 nm) light-emitting diode (LED) for different exposure times to active different concentrations of curcumin and toluidine blue on planktonic suspensions of *Streptococcus mutans* [12]. It was observed that the LED device in combination with curcumin and toluidine blue promoted photoinactivation of *S. mutans* suspensions at ultrashort light illumination times. However, the greatest PACT limitation is the challenge for the photosensitizer to penetrate the depth of the biofilm [12]. A mucosal adhesive film system for local release of photosensitizer (toluidine blue) has been developed and it was observed that *C. albicans* biofilms were more resistant to PACT than planktonic cultures, since higher concentration and longer incubation time with the photosensitizer were necessary to achieve complete fungal inactivation in biofilms when compared to planktonic cultures [13].

Studies that evaluate the efficacy of PACT suggest that the therapy is effective and promotes complete inactivation of the microorganisms in suspension [12, 13]. However, the complete inactivation of the microorganisms organized in biofilms has not yet been observed. This is probably due to the protective effect of the extracellular matrix of the biofilm, which hinders the action of the photosensitizing agent. The phototherapy appears to be a hopeful alternative to PACT, as it has been shown to overcome this issue [14]. The antimicrobial effect of blue light alone has been shown to inhibit *S. mutans* matrix-rich biofilm development [14]. However, the effect of blue and red light alone in fungal biofilms, such as *C. albicans*, has been less investigated. Therefore, the aim of this study was to determine how phototherapy using blue and red light affects the development and composition of *C. albicans* biofilm.

Methods

Light sources

A noncoherent light with spot size of 113.1 mm^2 was used (LumaCare LC-122 A, LumaCare Medical Group, Newport Beach, CA, USA) [12]. Two lights of different spectrums (blue and red) were applied to the biofilms in continuous wave. The blue light (wavelength range, 400–440 nm) has a central wavelength peak at 420 ± 20 nm and a fixed output power of 95.5 mW cm^{-2} [12]. The radiant exposures tested for this specific wavelength were 72 J cm^{-2}. Thus, biofilms were irradiated for 12.56 min, with the samples receiving a total dose of 36 J. The red light (wavelength range, 570–690 nm) has a central wavelength of 635 ± 10 nm with a fixed output power of 1460 mW cm^{-2} [12]. As the red wavelength presents a higher potency compared to blue light, ultrashort irradiation times were necessary. Thus, three different radiant exposures were tested: 43.8 J cm^2, corresponding to 30 s of irradiation; 87.6 J cm^2, corresponding to 1 min of irradiation; and 175.5 J cm^2, corresponding to 2 min of irradiation. Samples received a total dose of 21.9, 43.8, and 87.75 J, respectively. The irradiation time was calculated using the following formula: Light dose (J/cm^2) = Power density (W/cm^2) × time (seconds). A working distance of 5 mm between the light source and the biofilm surface was applied, a safe distance to avoid heating the sample [12]. The energy density for red light applied in the current study was based on a pilot study performed in our laboratory (data not published).

Inoculum

The biofilms were obtained from a wild-type *C. albicans* strain (SN 425). The microorganism kept at − 80 °C was seeded onto Petri dishes with Sabouraud dextrose agar and incubated at 37 °C for 48 h. Then, the pre-inoculum was prepared by taking approximately five colonies of the microorganism with a loop and adding in YNB medium (Yeast Nitrogen Base - DIFCO, Detroit, MI, USA) supplemented with 100 mM. The pre-inoculum was incubated at 37 °C for 16 h. After 16 h of incubation, the pre-inoculum was diluted with fresh YNB medium supplemented with 100 mM glucose (1:20 dilution) to form the inoculum. The inoculum was incubated at 37 °C until the strain reached the mid-log growth phase [15]. Then, it was adjusted to reach 10^7 cells/mL at the optical density of 540 nm [15].

Biofilm formation and phototherapy

One milliliter of the inoculum was added to the wells of a 24-well polystyrene plate. The culture plate was incubated at 37 °C for 90 min (adhesion phase). After this period, the wells were washed twice with sterile 0.89% NaCl solution to remove non-adhered cells. Afterwards, 1 mL of RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, St. Louis, MO, USA) at pH 7 was added to each well. After 18 h of initial biofilm formation, the biofilms were exposed to red light and blue light without a photosensitizer twice-daily (9 a.m. and 3 p.m.). To do that, the RPMI+MOPS medium was removed, biofilms were washed twice with sterile 0.89% NaCl, and then the exposure to the lights was performed. After exposition, fresh RPMI+MOPS medium was added to the wells (1 mL) and the culture plate was incubated at 37 °C. This was done until 48 h of biofilm development was achieved. Positive and negative control groups were treated twice-daily with 0.12% chlorhexidine-CHX (1 min) and 0.89% NaCl (1 min), respectively.

Biofilm analysis

At the end of the experimental period, biofilms were washed twice with 1 mL of sterile 0.89% NaCl solution. For biofilm removal, 2 mL of sterile 0.89% NaCl solution was added to wells and the bottom of the wells were scraped with a sterile spatula. The removed biofilms were transferred to sterile tubes. These tubes were vigorously vortexed for 1 min and an aliquot of 100 mL was separated for the dry-weight [16] and another aliquot of 100 μL was separated for colony forming units (CFU/mL). The remaining content was centrifuged (10,000 rpm, 10 min). Using a micropipette, 1 mL of the resulting supernatant was collected for EPS-soluble analysis by phenol:chloroform method [17] and the biofilm pellet was resuspended and washed with Milli-Q water; this procedure was repeated three times. Following wash, the biofilm pellet was resuspended in 1 mL of Milli-Q water, and this aliquot was used for EPS-insoluble analysis by phenol:chloroform method [17].

Statistical analyses

All the experiments were repeated on five separate occasions with two replicates in each. The polysaccharide content was normalized by the dry-weight (μg/mg). Colony forming units were Log_{10} transformed. Group differences were analyzed by one-way ANOVA and Tukey's post hoc test ($\alpha = 0.05$), using IBM SPSS (v 23, IBM Corp., Armonk, NY, USA). Statistical significance indicates $p < 0.05$.

Results

Figure 1 shows the results of Log_{10} CFU/mL of *C. albicans* after twice-daily treatments and Table 1 shows the CFU/mL results. Log_{10} CFU/mL and CFU were lower in the positive control (PC) samples than any other group ($p < 0.001$), and each light group, except red light 30 s, was lower than the negative control (NC) ($p < 0.05$). Figure 2 shows the results of the dry-weight (mg) of *C. albicans* biofilms after periodic light treatments. Dry-weight was lower in all light-treated samples than the negative control ($p < 0.001$) and the levels observed in the light-treated groups approached those seen in the PC. Figures 3 and 4 show lower levels of *C. albicans* EPS-soluble and EPS-insoluble content in PC than NC groups ($p < 0.001$). Although not statistically significant, it was observed that twice-daily light exposure to red light for 1 and 2 min as well as to blue light for 12.56 min numerically reduced the EPS-soluble content in comparison to the negative control (primarily the blue light treatment). In contrast, the EPS-insoluble content was numerically reduced by twice-daily exposure to red light for 1 min in comparison to the negative control, blue light treatment, as well as the remaining periods of exposure for red light treatment. Thus, there is a pattern of EPS-soluble reduction by twice-daily treatment with the blue

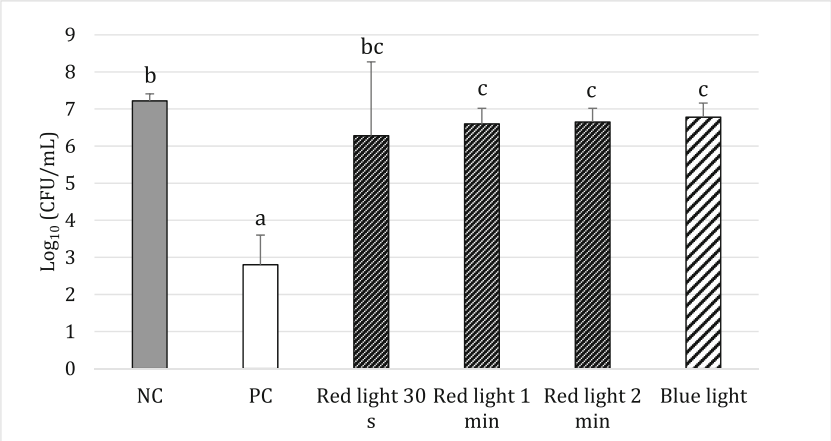

Fig. 1 Mean and 95% confidence limits of Log_{10} CFU/mL of *C. albicans*. Comparisons were made between the twice-daily light treatments with red and blue lights and the controls—0.12% CHX (PC) and 0.89% NaCl (NC). Equal letters denote statistical similarity between groups ($p > 0.05$)

Table 1 Mean and standard deviations of colony forming units (CFU)

Groups	CFU	
NC	2×10^7 (9×10^6)	b
PC	2×10^5 (2×10^5)	a
Red light 30 s	8×10^6 (4×10^6)	bc
Red light 1 min	5×10^6 (3×10^6)	c
Red light 2 min	6×10^6 (3×10^6)	c
Blue light	9×10^6 (9×10^6)	c

Comparisons were made between the twice-daily light treatments with red and blue lights and the controls—0.12% CHX (PC) and 0.89% NaCl (NC). Equal letters represent statistical similarity between groups ($p > 0.05$)

light and of EPS-insoluble by twice-daily treatment with red light for 1 min.

Discussion

C. albicans is the most frequent species isolated from superficial and systemic fungal infections and is associated with high rates of mortality [1]. There is an increasing number of strains of this microorganism that are resistant to antifungal agents [1]. Treatments of oral infections caused by *Candida* use topical antifungal medication, such as nystatin [18], and systemic antifungal medication, such as fluconazole [19]. Due to the antifungal resistance and difficulties associated with the use of conventional medications, PACT has been indicated for inactivating *Candida* and for the treatment of superficial fungal infections [20–22].

Studies have demonstrated that *Candida* species present susceptibility to PACT [20–22], especially within planktonic cultures [12, 13]. However, this method has limitations, such as nonselective antimicrobial characteristics and difficulty to penetrate to the depth of biofilm, resulting in reduced effectiveness on biofilms [23]. As an alternative to PACT, a previous study described the use of blue light without a photosensitizer to prevent the biofilm development of *S. mutans* in vitro [14]. The study found that twice-daily treatment with blue light prevented in vitro *S. mutans* biofilm matrix development, being more effective in reducing the production of EPS-insoluble than the gold-standard, antiplaque 0.12% chlorhexidine [14]. Exopolysaccharides (EPS) are abundant polymers in the biofilm matrix of *C. albicans* [8, 24]. *Candida* resistance to drugs is multifactorial, being related to the physiological state of the cells, activation of drug efflux pumps and protective effect of the EPS from the matrix [25]. β-Glucans from the extracellular matrix of *C. albicans* biofilms (EPS-insoluble) bind to fluconazole and amphotericin B [26], evading the diffusion of these drugs into the biofilm [27].

LumaCare™ was previously proposed as an effective device to be applied to *S. mutans* planktonic suspensions [12] and biofilms [14], demonstrating photoinactivation of *S. mutans* suspensions with red and blue lights [12] and inhibition of matrix-rich *S. mutans* biofilm development by the blue light [14]. Considering that the greatest advantage of this device is the associated decrease in treatment time (making the device more viable for clinical applications [12]) and there are no studies regarding the effectiveness of this device in *C. albicans* (a frequent species associated with oral infections), this study's aim was to determine how twice-daily treatment with blue or red light affects the development and composition of *C. albicans* biofilms. Because *C. albicans*' extracellular matrix is composed through a complex interaction between

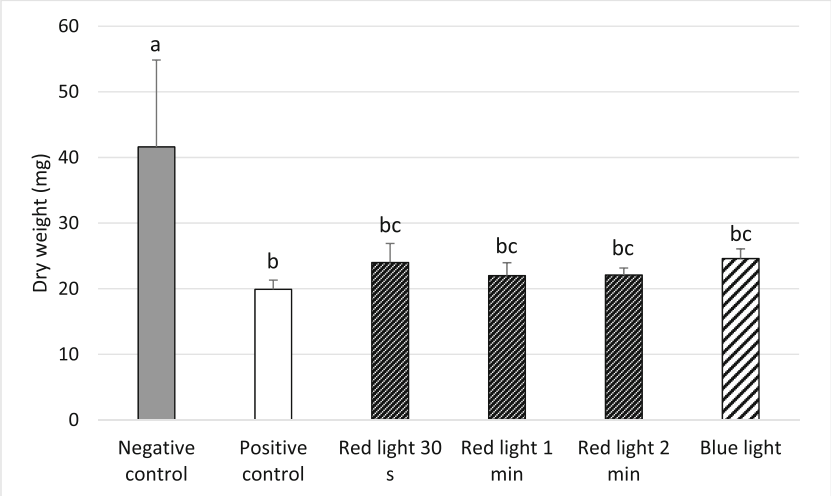

Fig. 2 Mean and 95% confidence limits of dry-weight (mg) of *C. albicans*. Comparisons were made between the twice-daily light treatments with red and blue lights and the controls—0.12% CHX (PC) and 0.89% NaCl (NC). Equal letters denote statistical similarity between groups ($p > 0.05$)

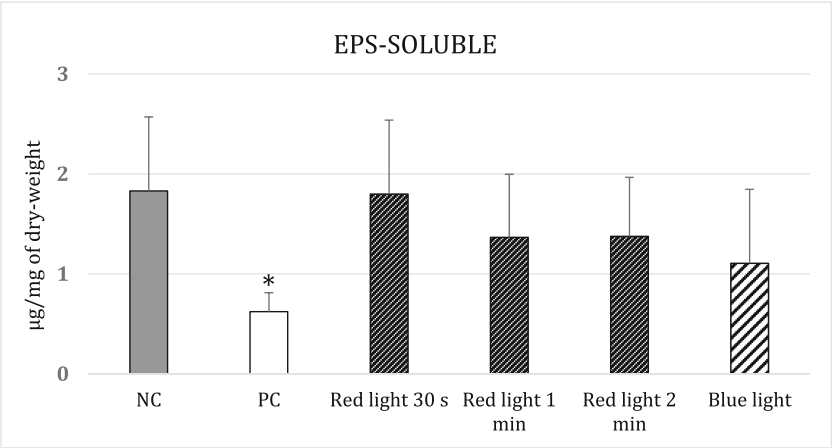

Fig. 3 Mean values and 95% confidence limits of EPS-soluble content in *C. albicans* biofilm (µg/mg of dry-weight). Comparisons were made between the twice-daily light treatments with red and blue lights and the controls—0.12% CHX (PC) and 0.89% NaCl (NC). The * indicates significant difference ($p < 0.001$) in comparison to the other groups

soluble (α-mannan) and insoluble (β-glucan) EPS [8], the present study focused on these components.

The present study showed a significant reduction in the viable colony count (CFU) by the light treatments in comparison to the NC (except for red light 30 s) (Fig. 1 and Table 1), but completely killing of *C. albicans* was not reached and the reductions observed might not be biologically relevant. A previous study using red light (wavelength 660 nm) and Photodithazine (PDZ) as a photosensitizer have shown that the use of PDZ to mediate PACT was effective to eliminate planktonic clinical isolates cultures of *C. albicans* [28]. When PACT mediated by PDZ was applied against *C. albicans* biofilms, substantial reductions in the viability were observed; however, totally killing was not achieved [28, 29]. Similarly, the use of blue LED (wavelength peak at 455 nm) and curcumin as a photosensitizer showed that PACT eradicated planktonic cultures of *C. albicans* but was not able to eradicate *C. albicans* biofilms [30]. Such studies focused on *C. albicans* viability after PACT; however, the effect of red and blue lights with or without photosensitizers on the extracellular matrix of *C. albicans* biofilms is still scarce.

In the present study, it was observed a significant reduction in total biomasses (dry-weight) of the biofilms after treatments with red and blue lights (Fig. 2). The total biomass comprises the total weight of both the cells and extracellular matrix of the biofilm after treatments and washes. By reducing the biomasses and viable colony count, the lights prevented biofilm development. A numerical reduction of EPS-soluble was observed for those treatments using red light for 1 and 2 min (Fig. 3) and for those using the blue light, although the reduction was not statistically significant. However, EPS-insoluble was numerically reduced by red light for 1 min (Fig. 4). These results suggest that EPS-soluble are more reactive to the treatments with the lights than EPS-insoluble. Moreover, together with the reduction of viable colony count, these outcomes may also have contributed to the significant reduction of the biomasses.

Besides EPS-soluble and EPS-insoluble, the extracellular matrix of *C. albicans* biofilms is composed of proteins and extracellular DNA (eDNA) [8]. These were not quantified in the present study. Thus, further studies might consider evaluating eDNA and proteins for the matrix of *C. albicans* biofilms after phototherapy, as biomass reduction observed in the present study could also be related to the reduction of these components from the matrix. However, the reduction of

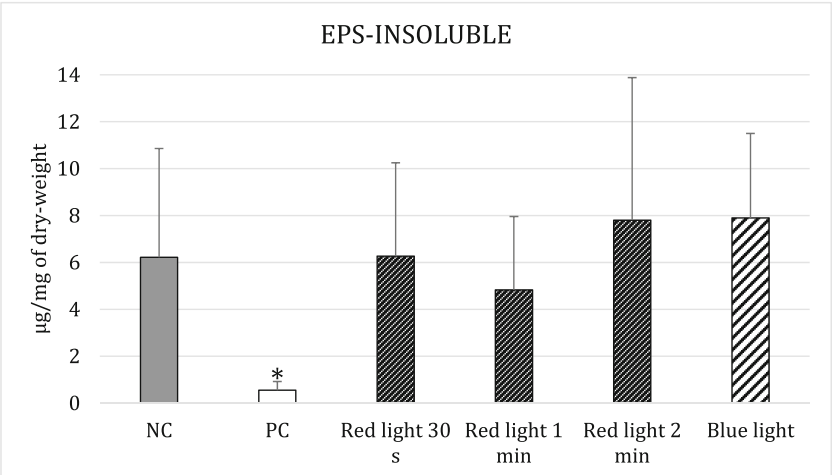

Fig. 4 Mean values and 95% confidence limits of EPS-insoluble content in *C. albicans* biofilm (µg/mg of dry-weight). Comparisons were made between the twice-daily light treatments with red and blue lights and the controls—0.12% CHX (PC) and 0.89% NaCl (NC). The * indicates significant difference ($p < 0.001$) in comparison to the other groups

polysaccharides from *C. albicans* biofilms matrix is important, as these components are involved in protecting the biofilm from antifungals [8–10]. Phototherapy without the use of a photosensitizer has been recognized as a powerful instrument for several areas in medical sciences. The effects of blue light without a photosensitizer on oral bacteria have been published, generally related to its antimicrobial effects [31–34]. Previous studies [14, 35] showed that blue light may be considered an effective and safe therapy against the matrix-rich biofilm development. In many microorganisms, exopolysaccharides are indispensable for biofilm formation, and mutants that cannot synthesize exopolysaccharides are strictly compromised and incapable to form mature biofilms [6]. Thus, by reducing EPS formation, the lights can be used to improve the mechanical removal of oral biofilms, since less EPS in the biofilm's matrix will facilitate its removal.

Proper oral hygiene is indispensable for preventing oral diseases. Thus, the use of mouth rinses that have bactericidal, bacteriostatic, fungicidal, and fungistatic effects is recommended. The golden standard for this purpose is CHX [36]. For this reason, CHX was chosen as a positive control for the present study. Phototherapy showed a numerical reduction of EPS-soluble and EPS-insoluble compared to the NC; however, it failed to show EPS-soluble and EPS-insoluble reduction compared to CHX. Nevertheless, CHX has side effects including suppression of the oral microbiota [37] and restricted timeframe of use of 14 days because of its collateral effects (such as teeth staining) [38]. Therefore, utilizing light instead of CHX is useful, in that it avoids these effects.

Twice-daily treatment of oral candidiasis with blue light for 12.56 min and twice-daily treatment of oral candidiasis with red light for at least 1 min might serve as an adjuvant to topic antifungal application such as Nystatin. Further studies should focus on a combination of treatments, beginning with phototherapy and later commencing topical antifungal application. This approach would help to disorganize the extracellular matrix protecting *C. albicans* biofilm, allowing improved drug penetration throughout the biofilm to reach and ultimately kill *C. albicans* cells. Moreover, phototherapy presents a unique advantage compared to the thoroughly studied PACT; it is noticeably faster, considering phototherapy does not require a period of incubation with a photosensitizer.

Our results demonstrated the effects of blue and red light without a photosensitizer on *C. albicans* biofilm. This exposure prevented *C. albicans* biofilm development by significantly reducing viable colony count and biomasses. Moreover, even though no statistical differences for EPS reduction were noted, there was a slight tendency of reduction of EPS-soluble by blue light for 12.56 min and red light for at least 1 min. Additionally, there was a pattern of EPS-insoluble reduction by exposure to red light for 1 min. Hence, future studies with red and blue light therapy should focus on other *C. albicans* extracellular matrix components in addition to EPS-soluble and EPS-insoluble (such as eDNA and proteins). Therefore, we conclude that *C. albicans* viable colony count and biofilms biomasses were significantly reduced by the treatment with blue light and higher durations of red light. In conclusion, we believe that light is a promising therapeutic approach for biofilm-related *C. albicans* diseases such as oral candidiasis, indicating that twice-daily treatment of *C. albicans* biofilms with either blue light or red light can function as an adjuvant to topic antifungal application.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent The authors have attested that for this type of study, formal consent is not required.

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