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Daily phototherapy with red light to regulate Candida albicans biofilm growth

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TITLE:

Daily Phototherapy with Red Light to Regulate *Candida albicans* Biofilm Growth

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KEYWORDS:

biofilm, *Candida albicans*, phototherapy, red light, extracellular matrix, polyssacharides.

SUMMARY:

Here, we present a protocol to assess the outcome of red light application on the growth of *Candida albicans* biofilm. A non-coherent red light device with the wavelength of 635 nm and energy density of 87.6 J·cm^{-2} was applied throughout the growth of *Candida albicans* biofilms for 48 h.

ABSTRACT:

Here, we present a protocol to assess the outcomes of per diem red light treatment on the growth of *Candida albicans* biofilm. To increase the planktonic growth of *C. albicans* SN425, the inoculums grew on Yeast Nitrogen Base media. For biofilm formation, RPMI 1640 media, which have high concentrations of amino acids, were applied to help biofilm growth. Biofilms of 48 h were treated twice a day for a period of 1 min with a non-coherent light device (red light; wavelength = 635 nm; energy density = 87.6 J·cm^{-2}). As a positive control (PC), 0.12% chlorhexidine (CHX) was applied, and as a negative control (NC), 0.89% NaCl was applied to the biofilms. Colony forming units (CFU), dry-weight, soluble and insoluble exopolysaccharides were quantified after treatments. Briefly, the protocol presented here is simple, reproducible and provides answers regarding viability, dry-weight and extracellular polysaccharide amounts after red light treatment.

INTRODUCTION:

The increased incidence of diabetes, immunosuppressive therapy applications, HIV infection, AIDS epidemic, invasive clinical procedures and broad-spectrum antibiotic consumption in the

past years have increased the incidence of *Candida albicans* related diseases[1-2]. *C. albicans* infections are commonly related to biofilm development and may cause clinical manifestations, such as candidiasis, or systemic manifestations, such as candidemia[1-2]. One of the most noteworthy virulence factors of biofilm growth is the extracellular polysaccharide matrix establishment. Biofilm formation cooperates to increase the resistance to existing antifungal drugs, environmental stress, and host immune mechanisms[3].

The biofilm growth of *C. albicans* begins with the early adherence of planktonic cells to a substrate, followed by the propagation of yeast cells through the substrate surface, and hyphal growth. The last phase of biofilm growth is the maturation phase, wherein yeast-like development is suppressed, the hyphal development expands, and the extracellular matrix encloses the biofilm[4]. *C. albicans* exopolysaccharides (EPS) in the matrix interact to form the mannan-glucan complex[5-6]. The interaction of exopolysaccharides is critical for the defense of the biofilms against drugs[7]. Hence, the reduction of EPS from the *C. albicans* extracellular matrix could support the development of new antibiofilm protocols for oral candidiasis control.

Light regulates the growth, development, and behavior of several organisms[8] and it has been applied as an antimicrobial in photodynamic antimicrobial chemotherapy (PACT). PACT applies a visible light of a specific wavelength and a light-absorbing photosensitizer[9]. However, the photosensitizers have difficulties in penetrating the biofilm, causing lower efficacy[10]. The failure of therapeutic agents to fully infiltrate biofilms is a reason that biofilms occasionally resist traditional antimicrobial therapy[3,5]. To deactivate the enclosed microbial cells, antimicrobials need to permeate through the extracellular matrix; nevertheless, the EPS characterizes a diffusional obstacle for such molecules by prompting their level of carriage into the biofilm or by influencing the response of the antimicrobial with the matrix itself[11].

Considering the disadvantages of PACT, the use of light by itself emerges as a valuable improvement. Preliminary data revealed that the treatment with blue light twice a day significantly inhibited the production of EPS-insoluble in *Streptococcus mutans* biofilm. By the decrease of EPS-insoluble, blue light diminished biofilm growth. Nevertheless, the outcomes of phototherapy using red light in *C. albicans* biofilms are scarce. Hence, the objective of this investigation was to evaluate in what manner phototherapy using red light influences the growth and arrangement of *C. albicans* biofilm. For the twice-daily treatment, we adapted our laboratory's previous protocols[9,12] to provide an easy and reproducible biofilm model that delivers answers regarding viability, dry-weight and extracellular polysaccharides amounts after red light treatment**.** The same protocol can be used for testing other therapies.

PROTOCOL:

1. Preparation of culture media

1.1 Prepare sabouraud dextrose agar (SDA). Suspend 65 g of SDA supplemented with chloramphenicol (50 mg/L) in 1,000 mL of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 PSI (121°C) for 30 min. Cool to 45-50 °C. Mix well and pour 20 mL of

SDA into sterile Petri plates (size: 100 mm x 15 mm).

1.2 Prepare yeast nitrogen base (YNB) medium supplemented with 100 mM glucose by mixing 6.7 g of YNB and 18 g of dextrose to 1,000 mL of ultrapure water. Mix using a stirrer and sterilize the medium using a 0.22 µm vacuum filter system.

1.3 Prepare RPMI by mixing 10.4 g of RPMI 1640 and 34.32 g of 3-(N-morpholino)propanesulfonic acid (MOPS) to 1,000 mL of ultrapure water. Mix in a stirrer without heat and adjust the pH to 7 by adding 1 N NaOH (Add 2 g of NaOH to 50 mL of ultrapure water). Sterilize the medium using a 0.22 µm vacuum filter system.

2. Pre-inoculum and inoculum

2.1 Remove the microorganism *C. albicans* SN425 from the −80 °C freezer and let it thaw at ambient temperature. Seed 10 µL of the stock culture on Petri dishes containing SDA supplemented with chloramphenicol (50 mg/L). Incubate the Petri dishes aerobically at 37 °C for 48 h.

2.2 After 48 h of incubation, add 10 colonies of *C. albicans* to 10 mL of yeast nitrogen base (YNB) medium supplemented with 100 mM glucose, and incubate aerobically at 37 °C for 16 h for the pre-inoculum.

2.3 After incubating for 16 h, prepare the inoculums by diluting the starter cultures into fresh YNB medium supplemented with 100 mM glucose in a 1:10 proportion (1 mL of the starter culture diluted in 9 mL of YNB). Measure the initial optical density (OD) at 540 nm.

2.4 Incubate the inoculums aerobically at 37 °C for 8 h until the strains reach the mid-log growth phase and measure the final OD at 540 nm. Subtract the final OD from the initial OD to check if the OD of the inoculums are on the mid-log growth phase (8 h, based on the growth curves in **Figure 1**).

2.5 To reach an inoculum with 10^7 cells mL^{-1}, centrifuge the inoculum for 5 min at 5,500 x *g*, discard the supernatant and concentrate the inoculum to half of the volume of the tubes.

3. Biofilm formation and phototherapy

3.1 Add 1 mL of the inoculum to the wells of a 24-well polystyrene plate. Incubate aerobically at 37 °C for 90 min to allow cell adhesion to the bottom of the wells.

3.2 Subsequently, wash the wells two times with sterile 0.89% NaCl to remove non-adherent cells and then expose the biofilms to red light (1 min).

3.3 Use a non-coherent red light (see **Table of Materials**) as the light source with wavelength of 635 ± 10 nm and a fixed output power of 1,460 mW cm^{-2}. The radiant exposure is 87.6 J cm^{-2},

equivalent to 1 min of irradiation. Apply a distance of 5 mm between the light source and the biofilm to avoid warming the sample.

3.4 Add 1 mL of RPMI 1640 buffered with 3-(N-morpholino)propanesulfonic acid (MOPS) at pH 7 to each well.

3.5 To prepare RPMI 1640, add 10.4 g of RPMI 1640, 34.32 g of MOPS and 1000 mL of ultrapure water in a beaker. Mix without heat and adjust the pH to 7 by adding 1 N NaOH. Sterilize the medium using a filter.

3.6 After adding RPMI 1640 to the wells, incubate the plates at 37 °C overnight. In the morning, apply the same treatments as steps 3.2 and 3.3, wash the biofilms twice with 0.89% NaCl, add new RPMI buffered with MOPS (1 mL, pH 7) to the wells and incubate aerobically at 37 °C.

3.7 The same day, after 6 h, wash the biofilms twice with sterile 0.89% NaCl and expose them to red light (1 min, 87.6 J/cm^2). Treat the positive control biofilms with 0.12% chlorhexidine for 1 min and negative control biofilms with 0.89% NaCl for 1 min.

3.8 Repeat the steps 3.6-3.7 until achieving 48 h of biofilm development.

NOTE: Basically, one treatment will happen in the morning and the other one will happen in the afternoon on the same day (6 h apart).

4. Processing

4.1 Add 1 mL of sterile 0.89% NaCl to the well to remove the biofilm by scratching it from the well using a pipette tip. Add the removed biofilm to a sterile tube.

4.2 Add another 1 mL of sterile 0.89% NaCl to the well. Scratch it again and add the suspension to the same tube for a total volume of 2 mL.

4.3 Colony-forming units (CFU)

4.3.1 Vigorously vortex the biofilm suspension for 1 min. From the biofilm suspension, use an aliquot of 0.1 mL for the serial dilution.

4.3.2 Dilute the biofilm suspension from 10^{-1} to 10^{-4} in 0.89% NaCl solution.

4.3.3 Seed 50 µL of each dilution to SDA plates and incubate the plates at 37 °C for 48 h.

4.3.4 Count the colonies and apply the formula CFU/mL = number of colonies x 10^n / q (n= dilution; q= seeded volume).

4.4 Dry weight

4.4.1 Weigh and label microcentrifuge tubes.

4.4.2 From the biofilm suspension, use 0.1 mL for dry weight (biomass) determination.

4.4.3 Add 1 mL of absolute ethanol to an aliquot of 0.1 mL of the biofilms and store it at -20 °C for 18 h. After 18 h, centrifuge at 10,000 x *g* for 10 min.

4.4.4 Discard the supernatant, place the tubes on a desiccator to dry the samples for 2 h and weigh the microcentrifuge tubes again.

4.5 Water-soluble polysaccharides (WSPs) and alkali-soluble polysaccharides (ASPs)

4.5.1 From the biofilm suspension, vigorously vortex the remainder of the volume (1.8 mL) for 1 min and centrifuge at 5,500 × *g* for 10 min at 4 °C. Store the supernatant in a new tube and wash the precipitate twice with the cells, which contain the insoluble constituents of the extracellular matrix with sterile ultrapure water (5,500 × *g*, 10 min at 4 °C).

4.5.2 Re-suspend the insoluble content at 1.8 mL of sterile ultrapure water.

4.5.3 Water-soluble polysaccharides (WSPs)

NOTE: Perform this experiment at a fume hood.

4.5.3.1 From the stored supernatant, reserve 1 mL for the quantification of water-soluble polysaccharides (WSPs).

4.5.3.2 Homogenize the stored supernatant for 1 min. Then, transfer to sterile tubes and add 2.5 volumes of 95% ethanol. Store the tubes for 18 h at -20 °C for WSPs precipitation.

4.5.3.3 After 18 h, centrifuge the tubes at 9,500 x *g* for 20 min at 4 °C. After centrifugation, discard the supernatants.

4.5.3.4 Wash the samples three times with 75% ethanol and air-dry the pellets. Re-suspend the pellet with 1 mL of sterile ultrapure water, and quantify the WSP using the phenol-sulfuric acid method.

4.5.3.5 Use 0.1% glucose (0.01 g of glucose to 10 mL of ultrapure water) for the standard curve (0, 2.5, 5, 10, 15, 20, and 25 µL of glucose per tube).

4.5.3.6 Add 200 µL of 5% phenol (2.7 mL of 90% phenol to 47.3 mL of ultrapure water) to a glass tube comprising 200 µL of the sample or standard curve point (in triplicate per sample) and mix cautiously.

4.5.3.7 Add 1 mL of sulfuric acid to the tube and mix. Wait 20 min for the reaction and measure the samples using a spectrophotometer (490 nm).

4.5.4 Alkali-soluble polysaccharides (ASPs)

NOTE: Perform this experiment in a fume hood.

4.5.4.1 From the insoluble re-suspended amount, separate 1 mL for the determination of ASPs. Centrifuge the aliquots of each biofilm suspension (13,000 x *g*, for 10 min at 4 °C).

4.5.4.2 Cautiously remove the supernatant of each tube. Then place the samples on a vacuum concentrator to dry the pellets.

4.5.4.3 Weigh the pellets and add 300 µL of 1 N NaOH (2 g of NaOH to 50 mL of ultrapure water) per 1 mg of the dry weight. Incubate them for 2 h at 37 °C and then centrifuge at 13,000 × *g* for 10 min.

4.5.4.4 Cautiously collect the supernatants with a pipette and transfer to microcentrifuge tubes, maintaining the pellet.

4.5.4.5 Once again, add an equal volume of 1 N NaOH to the tubes comprising the pellets, and repeat the same steps as above for the extraction of ASP.

4.5.4.6 After incubation for 2 h, centrifuge the samples (13,000 x *g* for 10 min), carefully collect the supernatants and add to the previously collected supernatants.

4.5.4.7 For the third extraction, repeat the same steps as above, but this time, do not incubate the samples for 2 h before centrifugation.

4.5.4.8 Afterwards, add three volumes of 95% ethanol to each sample. Then, stock the samples at −20 °C for 18 h for precipitation of ASP.

4.5.4.9 Centrifuge the tubes (9,500 × *g* for 20 min at 4 °C) and discard the supernatants.

4.5.4.10 Wash each pellet three times with 75% ethanol and air-dry (same procedures did for WSP). Re-suspend the pellets in equal total volume of the first extraction with 1 N NaOH.

4.5.4.11 Read the samples for quantification of ASP using the phenol–sulfuric (same as for WSP analysis).

REPRESENTATIVE RESULTS:

Figure 2 displays the outcomes of Log_{10} CFU/mL of *C. albicans* after per diem treatments with red light for 1 min. Red light significantly reduced the Log_{10} CFU/mL compared to the NC ($p = 0.004$).

Figure 3 presents the outcomes of the biomass (mg) of *C. albicans* biofilms after daily treatments. All treated groups showed reduction of the biomass compared to the NC ($p = 0.000$) and the red light treated groups presented similar reduction of biomass to that observed in the PC. **Figure 4** and **Figure 5** display inferior amounts of *C. albicans* EPS-soluble and EPS-insoluble in PC compared to NC ($p = 0.000$). Even though not statistically significant, per diem application of red light for 1 min to *C. albicans* biofilms numerically diminished the amounts of EPS-soluble and EPS- insoluble.

FIGURE AND TABLE LEGENDS:

Figure 1. Growth curve of *C. albicans* strain SN 425. Planktonic culture was made in YNB medium supplemented with 100 mM of glucose and incubated at 37 °C. The optical density (OD at 540 nm) and Log10 CFU/mL were determined over time. Standard deviation is shown.

Figure 2. Mean and standard deviations of Log_{10} CFU/mL of *C. albicans*. Assessments were made between the treatment with red light twice a day and the controls—0.12% CHX (PC) and 0.89% NaCl (NC). Equal letters represent statistical similarity between groups ($p > 0.05$).

Figure 3. Mean and standard deviations of dry-weight (mg) of *C. albicans*. Assessments were made between the treatment with red light twice a day and the controls—0.12% CHX (PC) and 0.89% NaCl (NC). Equal letters represent statistical similarity between groups ($p > 0.05$).

Figure 4. Mean and standard deviations of EPS-soluble amount in *C. albicans* biofilm (µg/mg of dry-weight). Comparisons were made between the treatment with red light twice a day and the controls—0.12% CHX (PC) and 0.89% NaCl (NC). Equal letters represent statistical similarity between groups ($p < 0.001$).

Figure 5. Mean and standard deviations of EPS-insoluble content in *C. albicans* biofilm (µg/mg of dry-weight). Assessments were made between the treatment with red light twice a day and the controls—0.12% CHX (PC) and 0.89% NaCl (NC). Equal letters represent statistical similarity between groups ($p < 0.001$).

DISCUSSION:

The most critical steps for successful culturing of *C. albicans* biofilm are: 1) to do the pre-inoculum and the inoculum in YNB medium complemented with 100 mM glucose; 2) to wait 90 min for the adhesion phase and carefully wash twice the wells with 0.89% NaCl to remove non-adhered cells; and 3) to add RPMI medium to the adhered cells to start biofilm formation, since RPMI will stimulate hyphae growth.

A study limitation is that it was performed in vitro. Whilst in vitro studies have greatly increased the understanding of the biology of biofilm, they do not precisely represent in vivo conditions[13]. However, in vitro tests provide high-throughput screening in addition to being a cheap and simple methodology[13] to answer questions regarding new antibiofilm therapies. Choosing the correct culture media for each phase of biofilm development is an important step for the success of the method. RPMI 1640 is a nutrient-rich medium that has amino acids and simulates the

composition of human fluids[14]. RPMI 1640 contains L-glutamine, L-arginine, L- asparagine in addition to vitamins and inorganic salts. Nevertheless, YNB media has an elevated amount of glucose (18 g/L) compared to RPMI 1640 medium (2 g/L glucose). The high glucose content has been described to increase the planktonic growth of *Candida* species[15]. In contrast, the existence of higher concentrations of amino acids will help biofilm growth in RPMI medium compared to YNB medium[15]. Qualitative data applying SEM micrographs showed that the structural design of *C. albicans* biofilms in RPMI presents a complex organization with a solid growth of yeasts with ramifying hyphae, budding elements and bud scars with an abundant extracellular matrix[15]. Such outcomes are in accordance with a previous study that reported that RPMI 1640 augmented the hyphal formation in *C. albicans* biofilms[14]. These results demonstrate the differences in substrate utilization by *Candida* throughout different biofilm growth phases and show the importance of changing media during planktonic growth and biofilm formation.

The selection of the correct temperature and pH to cultivate *C. albicans* biofilms is also important to accomplish the method with the adequate formation of hyphae. *C. albicans* undertakes the conversion from blastospores to filaments in reaction to conditions that mimic the milieu of mammalian host tissues[16]. These conditions involve growing at body temperature (37 °C) and at neutral pH[16], and this is the reason why the pH of the RPMI medium is adjusted to 7.

The methods applied in this study are significant since biofilms of *C. albicans* SN 425 were characterized before[12], showing to have great amounts of EPS-soluble and –insoluble, which makes it a reliable method to analyze the outcome of the lights on the extracellular polysaccharides. Moreover, the same non-coherent light device applied in the experiments was successfully applied to bacterial planktonic suspensions[17] and biofilms[9], and the utmost benefit of this device is the reduction in treatment time, what makes the device more feasible for clinical applications[17]. The analysis of daily phototherapy applied to *C. albicans* biofilms is significant since the methodology applied in the present study simulates a treatment that is fast and can be easily done by the patient at home.

Red light daily treatment meaningfully reduced *C. albicans* viable colony count and biofilm biomasses. More studies might try a combination of treatments, starting with phototherapy and later applying topical antifungal. This strategy might assist in disorganizing the extracellular matrix shielding *C. albicans* biofilm, permitting better drug infiltration through the biofilm to reach and finally eradicate *C. albicans* cells. Considering the limitations of this in vitro experiment, the use of red light for 1 min may assist as an adjuvant to topic antifungals on the treatment of oral candidiasis.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

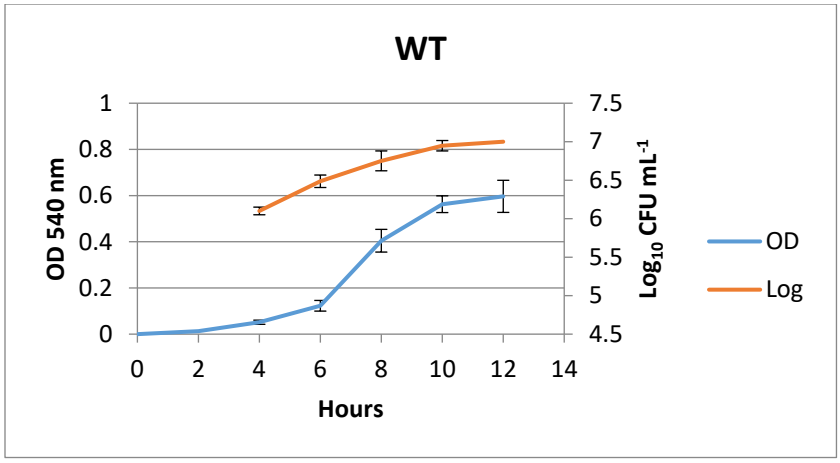


Figure 2

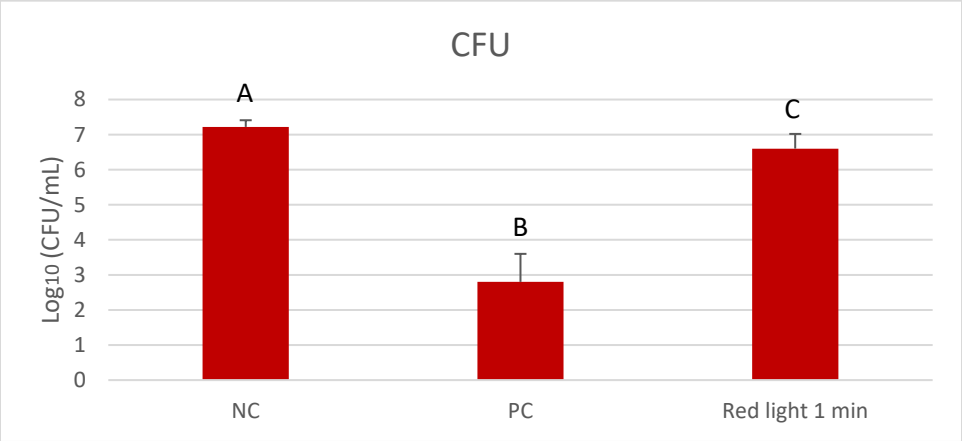
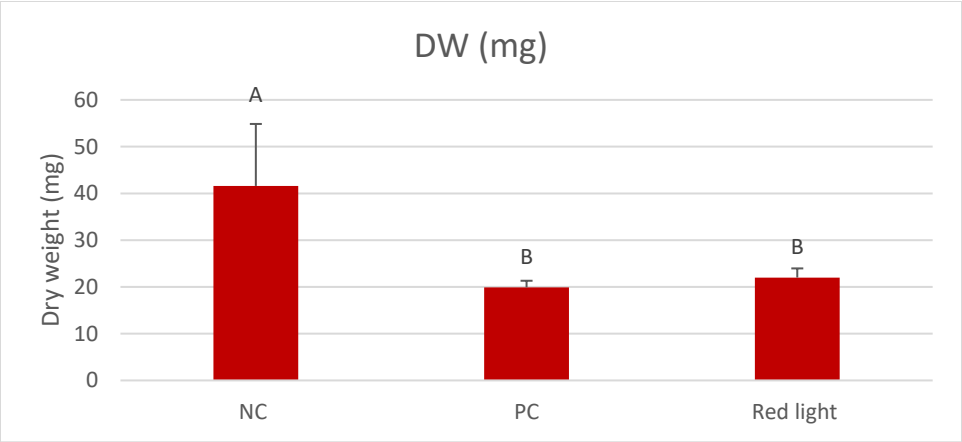


Figure 3



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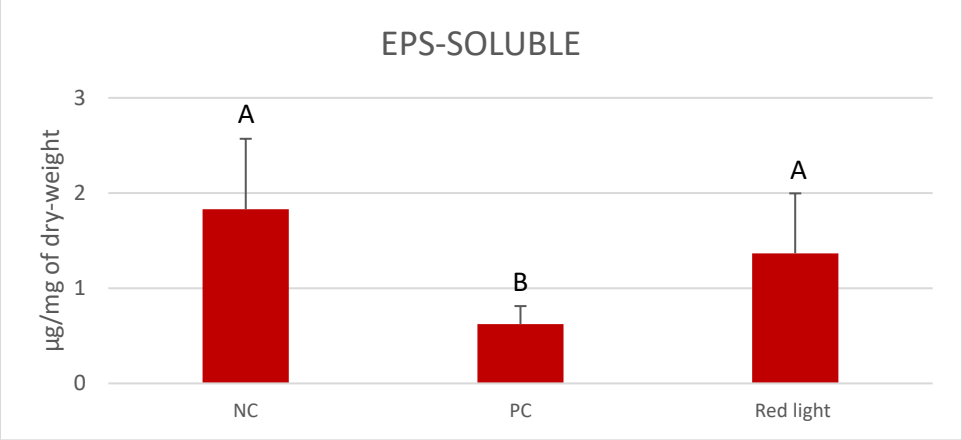

Figure 5

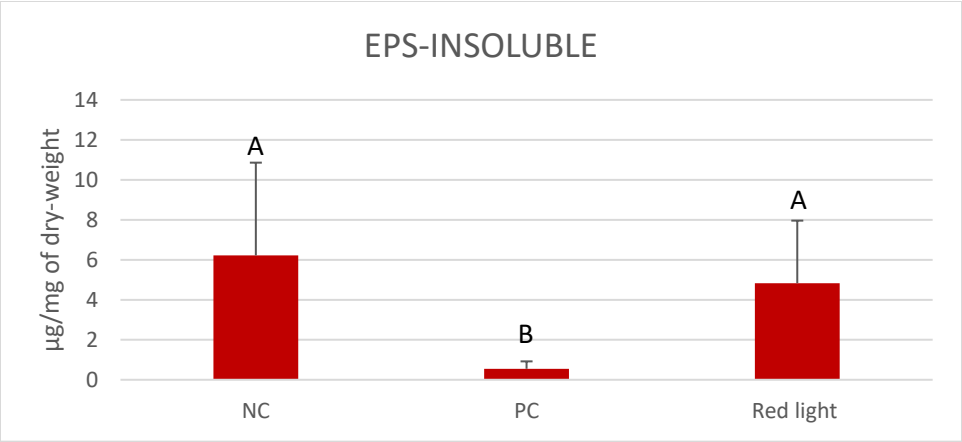

Table of Materials

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Name of Reagent/ Equipment	Company
Clorhexidine 20%	Sigma-Aldrich
Dextrose (D-Glucose) Anhydroous	Fisher Chemical
Ethanol 200 proof	Decon Laboratories
LumaCare LC-122 A	LumaCare Medical Group, Newport Beach, CA, USA
NaCl	Fisher Chemical
NaOH	Fisher Bioreagents
Phenol 5%	Milipore Sigma
RPMI 1640 buffered with 3-(N-morpholino)	Sigma
Sabouraud dextrose agar supplemented with chloramphenicol	Acumedia
Sulfuric acid	Fisher Chemical
Yeast nitrogen base	Difco
3-(N-morpholino)propanesulfonic acid MOPS	Sigma-Aldrich
24-well polystyrene plate	Falcon

Catalog Number	Comments/Description
C9394	
D16-500	
DSP-MD.43	
S641-500	
BP 359-500	
843984	
R7755	
7306A	
SA200-1	
DF0392-15-9	
M1254	
353935	

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Dear Editor,

Ref.: JoVE59326R2- Daily phototherapy with red light to regulate *Candida albicans* biofilm growth

I am re-submitting to Journal of Visualized Experiments - JoVE an updated version of the manuscript entitled "Daily phototherapy with red light to regulate *Candida albicans* biofilm growth". We thank the editorial team for their generous comments on the manuscript and we edited it to address their concerns. Bellow the changes made in the current manuscript are described:

- The item 1 of the protocol was changed for "Prepare culture media". As we added this step, the sequence of the other steps were altered.
- The growth curve of *C. albicans* SN 425 was added to the manuscript as Figure 1. We provided a title and a short description for figure 1 and added these items to the manuscript at the "figure and table legends" session. All the other figures were numbered in the order of their appearance in the manuscript.
- "Step 1.1: Please write this step in the imperative tense". This step was rewritten and it is the step 2.1 in the current manuscript.
- *"3.3.2: What is used to dilute the suspension? Water?".* It was used 0.89% NaCl solution. We altered the sentence and it is step 4.3.2 in the current manuscript.
- *"Please do not highlight notes for filming".* We removed highlights from notes.
- *"Please ensure that the references appear as the following:*
  Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR)". The references were double-checked and are according to the standards.

Sincerely yours,

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On behalf of all authors.