

Protocol for evaluating the in vitro effect of violet light-emitting diodes (LEDs) 410 nm ± 10 nm on yeast cultures

Protocolo para avaliação do efeito in vitro de diodos emissores de luz violeta (LEDs) 410 nm ± 10 nm em culturas de levedura

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ABSTRACT | BACKGROUND: *Candida spp* and *Malassezia spp* cause superficial infections that may be resistant to conventional treatments. Violet light-emitting diodes (LEDs) therapy is a therapeutic alternative. **PURPOSE:** To describe the protocol for evaluating the antifungal effect of violet LEDs 410 nm ± 10 nm on *Candida spp* and *Malassezia spp* in vitro. **PROTOCOL:** LEDs 410 nm ± 10 nm are applied to a fungal suspension at fluences of 61.13 J/cm², 91.70 J/cm², and 183.39 J/cm². The isolates are cultured for 48 to 72 hours. Colony forming units (CFUs) are quantified by visual counting and percent culture plate occupancy by digital analysis. Morphology is assessed by light microscopy and Gram staining, and yeast metabolism/function by transmission electron microscopy, assessment of reactive oxygen species, and DNA fragmentation. **DATA ANALYSIS:** the percentage of LEDs inhibition is calculated considering the growth of the negative control condition and the percentage of plate occupancy by yeasts by dividing the number of pixels classified as colonies by the total number of pixels on the plate. The morphological and functional aspects are described for the intervention and negative control. The ANOVA test is used to compare the mean percentages of growth inhibition and plate occupancy between the three fluences of LEDs 410 nm ± 10 nm and the negative control. **ESTIMATED RESULTS:** We intend to determine the antifungal effect of the different fluences of LEDs 410 nm ± 10 nm on *Candida spp* and *Malassezia spp*. The evaluation of other fungal species by this protocol should be investigated.

KEYWORDS: Phototherapy. Antifungal agents. *Candida*. *Malassezia*.

RESUMO | INTRODUÇÃO: *Candida spp* e *Malassezia spp* causam infecções superficiais que podem ser resistentes aos tratamentos convencionais. A terapia com diodos emissores de luz violeta (LEDs) é uma alternativa terapêutica. **OBJETIVO:** Descrever o protocolo de avaliação do efeito antifúngico in vitro dos LEDs violeta 410 nm ± 10 nm sobre a *Candida spp* e *Malassezia spp*. **PROTOCOLO:** LEDs de 410 nm ± 10 nm são aplicados a uma suspensão fúngica em fluências de 61,13 J/cm², 91,70 J/cm² e 183,39 J/cm². Os isolados são cultivados por 48 a 72 horas. As unidades formadoras de colônias (UFCs) são quantificadas por contagem visual e a porcentagem de ocupação da placa de cultura por análise digital. A morfologia é avaliada por microscopia de luz e coloração de Gram, e o metabolismo/função da levedura por microscopia eletrônica de transmissão, avaliação de espécies reativas de oxigênio e fragmentação de DNA. **ANÁLISE DOS DADOS:** O percentual de inibição dos LEDs é calculado considerando o crescimento da condição de controle negativo e o percentual de ocupação da placa por leveduras, dividindo-se o número de pixels classificados como colônias pelo número total de pixels na placa. Os aspectos morfológicos e funcionais são descritos para a intervenção e controle negativo. O teste ANOVA é utilizado para comparar as porcentagens médias de inibição de crescimento e ocupação de placa entre as três fluências dos LEDs 410 nm ± 10 nm e o controle negativo. **RESULTADOS ESTIMADOS:** Pretendemos determinar o efeito antifúngico das diferentes fluências de LEDs 410 nm ± 10 nm sobre *Candida spp* e *Malassezia spp*. A avaliação de outras espécies de fungos por este protocolo deve ser investigada.

PALAVRAS-CHAVE: Fototerapia. Agentes antifúngicos. *Candida*. *Malassezia*.

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Introduction

Fungal infections affect more than one billion people each year¹ and are the fourth leading cause of human disease. *Candida* and *Malassezia* species are among the frequent causes of superficial fungal infections.² Vulvovaginitis is one of the most common genital infections in women and is manifested by vaginal discharge, itching, burning and/or stinging, dysuria, and dyspareunia.³ *Candida albicans*, a saprophyte found in the vaginal mucosa, is the main species associated with vulvovaginitis.⁴ However, nonalbicans *Candida* species, including *C. glabrata*, also cause vulvovaginitis in HIV-infected women.⁵ Although vulvovaginitis is usually sporadic and associated with mild to moderate symptoms, the infection may recur in some women and responds poorly to conventional treatment.⁶

The genus *Malassezia* includes lipophilic and lipodependent fungi.⁷ To date, 18 species of *Malassezia*⁸ are known. *Malassezia spp* is considered one of the most common skin inhabitants in warm-blooded animals.⁹ *M. globosa* and *M. restricta* are involved in the development of seborrheic dermatitis/dandruff (SD/D) and *M. sympodialis* is associated with atopic dermatitis.¹⁰ Seborrheic dermatitis occurs in 2% to 5% of the general population and affects 20% to 83% of immunocompromised individuals with HIV and HTLV.¹¹⁻¹³

Azole antifungals are the first-line agents for the treatment of superficial infections with *Candida spp* and *Malassezia spp*. However, the long duration of treatment and the need for special long-term skin/mucosal care limit the efficacy of the proposed methods.^{14,15} The inability to control the recurrence of fungal infection leads to anxiety and irritation, which aggravates the symptoms and prevents a better prognosis of the pathology.¹⁶⁻¹⁸

Violet/blue LED has shown antifungal activity *in vitro* and beneficial biological effects on skin inflammation *in vivo*.^{19,20} The LED causes excitation of endogenous intracellular porphyrin present in the fungus, which acts as a photosensitizer. The absorption of photons leads to the production of reactive oxygen species, which eventually leads to cell death.^{19,21} The wavelength of violet/blue LED ranges from 380 to 495 nm, the therapeutic window with lower penetration into the skin, as it

mainly reaches the epidermis²², the layer where superficial mycoses occur.² This study describes the experimental protocol to evaluate the *in vitro* antifungal effect of violet light-emitting diodes (LEDs) (410 nm \pm 10 nm) on *Candida spp.* and *Malassezia spp.*

Methods

Study protocol

The protocol was designed to study *in vitro* the antifungal effect of violet light-emitting diodes (LEDs) 410 nm \pm 10 nm on yeast. The study is conducted at the research laboratory Núcleo de Pesquisa e Inovação - NUPI (Research and Innovation Center) at the Bahiana School of Medicine and Public Health (EBMSP) in Salvador-Bahia-Brazil.

Ethical considerations

Submission to the Institutional Research Board is waived because no human subjects are involved. The study is registered in the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado - SisGen (National System for the Management of Genetic Heritage and Associated Traditional Knowledge) (SisGen AE39DE1 and AED7762).

LEDs equipment 410 nm \pm 10 nm

The LED device used is the cluster e-light V of the brand DMC Equipamentos Ltda. (São Carlos, Brazil), which has an array of 8 violet LEDs with a light emission wavelength of 410 nm \pm 10 nm and a power of 480 mW per emitter. The device is registered with the Agência Nacional de Vigilância Sanitária - ANVISA (National Health Surveillance Agency) (registration 80030810174).

Isolates of *Candida* and *Malassezia* species

The isolates of *Candida spp* used were obtained from the Clinical Analysis Laboratory Jaime Cerqueira, Salvador, Brazil, and are seeded in Sabouraud Dextrose Agar (SDA) culture. The isolates of *Malassezia spp* used were obtained from the School of Veterinary Medicine and Animal Sciences, Universidade Federal da Bahia (UFBA), and are seeded in SDA culture medium plus 1% olive oil.

Culture and expansion of yeast isolates

First, yeast are seeded in a class 2 biological safety cabinet with a 10 µL microbiology loop in 90x15mm sterile Petri dishes containing 20 mL SDA with chloramphenicol (Neogen, Lansing Michigan, United States). For *Malassezia spp.* cultures, 1% extra virgin olive oil with 0.50% acidity (Gallo, Abrantes, Portugal) is added to the culture medium. The plates are then incubated in a Quimis® oven at $36 \pm 2^\circ\text{C}$ for 48 hours and at $33 \pm 2^\circ\text{C}$ for 72 hours for *Candida spp.*²⁰ and *Malassezia spp.*²³, respectively. To prepare the suspension, remove well-defined, ovoid yeast colonies from the Petri dishes using a 10 µL microbiology loop and place them in an 8-mm glass tube containing 4 mL of 0.85% saline. Then mix the colonies in a vortex and adjust the turbidity to 0.5 McFarland (McF), which corresponds to 1.5×10^8 CFU/mL in the DEN -1 turbidimeter (McFarland Densitometer, Biosan).

LEDs application

Use LEDs on 60 x 15 mm Petri dishes (CRAL®, Cotia, Brazil) containing 5 mL yeast suspension (10-2 and 10-3) in a biological safety cabinet. Irradiate the plates with LEDs 410 nm \pm 10 nm for 10 (T1), 15 (T2), and 30 (T3) minutes, corresponding to fluence of 61.13 J/cm², 91.70 J/cm², and 183.39 J/cm², respectively. These fluences were adjusted after initial experiments based on the study described elsewhere.²⁴

According to the manufacturer's instructions, a 5-minute break is taken every 10 minutes to allow the instrument to cool. The positive control consists of a yeast suspension in the presence of an antifungal agent with a 50% growth inhibitory concentration (IC50). The negative control (C-) consists of the yeast suspension without any type of intervention. After the intervention, 50 µL of the suspension of each condition is added in triplicate to 90 x 15 mm Petri dishes containing ASD medium with chloramphenicol (*Candida spp.*) and enriched with 1% olive oil for *Malassezia spp.* cultures. The solutions are spread with a sterile disposable L-shaped spreader bar. The culture plates containing *Candida spp.* are incubated at $36 \pm 2^\circ\text{C}$ for 48 hours, whereas those containing *Malassezia spp.* are incubated at $33 \pm 2^\circ\text{C}$ for 72 hours. Colony forming units (CFUs) are then quantified by visual counting and the percentage of occupancy of the culture plate is determined by digital analysis.

Morphology is assessed by light microscopy and Gram stain, and metabolism/function by transmission electron microscopy and DNA fragmentation. The experiments are repeated 3 times.

Morphological evaluation

CFUs are examined macroscopically for shape (circular, ovoid, or fusiform), color (white, yellowish-creamy), brightness (light, opaque), surface (smooth or rough), and elevation (flat, convex, roof-shaped).²⁵ Homogeneity of growth is assessed by the distribution of colonies on the surface of the medium. For microscopic evaluation of yeast, a CFU in a delineated quadrant of plate 1 of each condition is randomly selected for preparation of a new slide and Gram staining. Slides are prepared by placing the selected CFU in 10 µL of 0.85% saline on a glass slide. For fresh slides, the solution is immediately covered with a coverslip. In addition, after the suspension dries, the slides are subjected to Gram staining to exclude bacterial contamination of the plates.^{4,26} The slides are viewed under a light microscope (Olympus® CX22LED, Tokyo, Japan) with a 100x magnification objective, photographed, and examined for cell morphology, staining, and the presence of pseudomycelium.

Quantification of colony forming units (CFU)

CFU is counted in the quadrant previously drawn on the bottom of the Petri dish with a permanent marker. The CP 608 colony counter (PHOENIX® Equipamentos Científicos, Araraquara, Brazil) is used to identify budding and/or converging CFUs.

Evaluation of the colony occupation rate of the culture plate

Triplicate plates of each condition are identified and placed without cover in front of a dark background in the biological safety cabinet with cold light off. Digital images are captured using smartphone's rear camera, without flash or zoom. The images are transferred to a computer in JPEG (Joint Photographic Experts Group) format so that the percentage of surface occupied by yeast can be calculated. Only plates with a homogeneous growth distribution in the Petri dish are considered. To determine the areas of the culture medium and the fungal colony, the image is converted to gray scale and segmented by thresholding. The threshold used is 160, i.e., values above this intensity are considered as colonies and the others as background group.²⁷

Transmission electron microscopy

Cells are fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and postfixed in 1.5% potassium permanganate for 1 hour while protected from light. Samples are dehydrated in an acetone series and embedded in Spurr resin (Ted Pella, Redding, CA). Ultrathin sections were imaged on a 400-mesh copper grid and contrasted in 5% uranyl acetate and 3% lead citrate. Ultrastructural analysis is performed using a Zeiss109 transmission electron microscope at 80 kV.²⁸ Ultrastructural changes in the cytoplasmic organelles as well as the elemental chemical composition of fungal cells will be visualized.

DNA fragmentation assay

The DNA fragmentation assay was performed to evaluate the integrity of the genetic material. The assay was performed according to the protocol described elsewhere by Wi et al., 2012.²¹ Briefly, yeast colonies were suspended in 500 μ L of lysis buffer [100 mM Tris-HCl, pH 8.0, sodium dodecyl sulfate (SDS) 0.5%, 250 nM NaCl, 30 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] and vortex mixed for 10 s, then incubated at 100° C for 15 min. Then, 500 μ L of 3.0 M sodium acetate is added and incubated at -20°C for 10 min. The protein in the supernatant is removed with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). DNA is precipitated with an equal volume of isopropanol, washed with 500 μ L of 70% ethanol, dried, and mixed with 20 μ L of TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA, pH 8.0). DNA samples are analyzed in a 1% agarose gel.

Assessment of reactive oxygen species (ROS)

Cells are harvested, seeded per well in 1 ml supplemented medium, and then exposed to LED application at the indicated fluences. Samples are then washed with sterile saline and incubated with SYTO-61 at a concentration of 0.5 μ M (for DNA staining) and CellROX green probe (for ROS staining) for 30 minutes in the dark. Samples are washed and placed in polypropylene tubes. Cells are resuspended in 1 mL of isotonic diluent. At least 10,000 events are recorded using a BD LSRFortessa™ Cell Analyzer (BD, New Jersey, USA). ROS are identified using the FITC channel (gain of 500 V) and cells identified by SYTO-61

staining are quantified using the APC channel (gain of 450 V). Cells are detected with a two-dimensional dot plot between the APC channel (gain of 562 V) and the FSC channel (gain of 150 V). A second gate is generated by a two-dimensional dot plot between the APC and FTIC channels (gain of 320 V) to select viable cells that are positive for the ROS.

Data analysis

The inhibition rate of each of the interventions (LEDs 61.13 J/cm², 91.70 J/cm², and 183.39 J/cm²) is calculated based on the percentage decrease in the mean value of colonies compared with the negative control. The percentage of occupancy of the plate by yeast is determined from a digital image of the plate by dividing the number of pixels classified as colonies by the total number of pixels on the plate using an image analysis tool (PetriNote).²⁷

The ANOVA test is used to compare the mean percentages of growth inhibition and plate occupancy between the three fluences of LEDs 410 nm \pm 10 nm and the negative control. A P value less than 0.05 is considered significant. Database, descriptive and analytical analysis is performed using GraphPad Prisma 9.4.0 software for Windows.

Estimated Results

The results of this protocol will allow to determine the antifungal effect of the different fluences of LEDs 410 nm \pm 10 nm on *Candida spp.* and *Malassezia spp. in vitro*. The evaluation of other fungal species by this protocol should be investigated.

Author's contribution

Ribeiro RTSK, Santos ES, Dias CMCC, Grassi MFR, Mascarenhas REM, Barros TF, Mancini MW, and Lopes LA participated in the literature survey and discussion of the research project. Ribeiro RTSK, Santos ES, Grassi MFR, Mascarenhas REM, Gomes JMS, Silva JJ, Regis CG, Moreira DRM, Dantas DO, and Andrade BT developed the initial design of the project and participated in its methodological construction. Dias CMCC and Grassi MFR performed the critical review of the project and approved the final text.

Conflicts of interest

No financial, legal or political conflicts involving third parties (government, corporations and private foundations, etc.) have

been declared for any aspect of the submitted work (including, but not limited to grants and funding, advisory board participation, study design, preparation of manuscript, statistical analysis, etc.).

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