PHOTODYNAMIC THERAPY CAN KILL SEVERAL CANDIDA SPECIES IN IN VITRO AND IN VIVO MODELS

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ABSTRACT

Candidiasis is an infection caused by the encapsulated yeast Candida species and the most afflicted sites are skin, lung, renal and nervous systems. The aim of this study was to investigate the ability of methylene blue (MB) combined with a low-power red laser to inactivate four Candida species in \textit{in vitro} and \textit{in vivo} experimental models. To perform the \textit{in vitro} study, suspension of \textit{Candida} tested (10\textsuperscript{6} cfu/mL) was used. The light source was a laser (diode laser with wavelength 660 nm) (Photon Lase III, DMC, Sao Carlos, Brazil) emitting at 660nm with output power of 200 and 250mW for 5 and 10 min of irradiation, resulting fluences of 200 mw/cm\textsuperscript{2} equal 60 and 120 J/cm\textsuperscript{2} at 5 and 10 min respectively as well as 250 mw/cm\textsuperscript{2} had fluences at 5 and 10 min equal 75 and 150 J/cm\textsuperscript{2}, respectively. As photosensitizer, methylene blue (MB) 0.1 mg/ml was used. For the \textit{in vivo} study, Twenty male Wistar rats (Rattus norvegicus) inoculated with the most effect Candida species (10\textsuperscript{6}cfu/mL). Twenty-four hours after inoculation, PDT was performed using MB and the laser power exhibited the most effective on Candida species. Histopathology of mice wound tissue was examined after 3, 5 and 7 days. PDT was efficient \textit{in vitro} against \textit{Candida} species. In the \textit{in vivo} experiment, PDT was also effective; however, its effect was less expressive than in the \textit{in vitro} study. In conclusion, PDT seems to be a helpful alternative to treat dermal candidiasis; however, more effective parameters must be found in \textit{in vivo} studies.

KEYWORDS: Antifungal; \textit{Candida}; Diode laser; Methylene Blue; Photodynamic therapy; Photosensitizers.
INTRODUCTION
Photodynamic therapy (PDT) is a phototherapy based on the utilization of substances that can photosensitize biological tissues and are capable of being activated in the presence of light. The cells that are considered therapeutically targets are stained with the photosensitizing agent and irradiated with light (Wainwright, 1998 and Prates et al., 2007). The photodynamic process rapidly generating reactive oxygen species (ROS) as for instance peroxides, hydroxyl radicals, superoxide ions and singlet oxygen, the last being implicated as the major causative agent of cellular damage in photodynamic process. In addition, this technique has been shown to have effects against a range of pathogens and also against drug-resistant microorganisms (Wilson, 2004).

The main light sources used in antimicrobial PDT are lasers, specially the red and near-infrared lasers, because they produce monochromatic light of a known wavelength, light dosimetry is easy to calculate and the light can be passed down an optical fiber for localized treatment (Monfrecola et al., 2004). Since the conventional light sources have been changed for lasers, new photosensitizers have been researched with strong absorption in the range from 600 to 700 nm, which correspond to wavelengths emitted by commercial lasers.

The literature suggests the fluence as the most important light parameter to obtain a higher eradication of microbial cells in photodynamic therapy (Munin et al., 2007). However, the influence of others light parameters as time of irradiation and irradiance are not frequently reported. Moreover, sometimes neither wavelength is correctly presented (Oliveira et al., 2007). It makes the idea that fluence leads the range of parameters to compare different microorganism photoinactivation studies.

Candida genus yeasts are common occurrence in the oral cavity of healthy individuals and Candida albicans is the most prevalent species, totaling 60–70% of the isolates, followed by Candida tropicalis and Candida glabrata (Stenderup, 1990), other non-albicans Candida species and species of other genera have also been reported as causative agents of invasive infections. These microorganisms are usually commensal but among certain individuals and under specific situations they can transform into parasitary form, causing oral candidosis (Junqueira et al., 2005).

In the last decades, candidosis became a human disease of increasing importance due to the high number of immunocompromised patients associated to acquired human
immunodeficiency virus (HIV), use of immunosuppressants after organ transplantations and antineoplastic therapies. Oropharyngeal candidosis is the most common manifestation of HIV infection, occurring among 84% of the patients (Allen, 1994). Most of the oral infections caused by C. albicans are treated by the simple topical application of polyene antifungals, such as nystatin and amphotericin B. while, in HIV-associated oral candidosis, these agents may be toxic to human and not effective and the administration of ketoconazole, fluconazole or itraconazole may be necessary (Johnson et al., 1995).

The widespread use of topical and systemic antifungals among HIV-infected patients resulted in an alarming increase in the number of isolates resistant to this therapy. This resistance is mainly associated to severe immunossuppression, recidivating of infections and long-period treatment. Approximately 81% of all AIDS patients are colonized by antifungal resistant Candida. Considering this, it is necessary the development of alternative therapies for oral candidosis associated to HIV virus. Promising studies have been performed with a new method of treatment: the photodynamic therapy (PDT) (Teichert et al., 2002).

The aim of this study was to investigate the ability of methylene blue (MB) combined with a low – power red laser to inactivate five Candida species in in vitro and in vivo experimental models for use as safe cure for many infected human.

MATERIALS & METHODS

Organisms

Five Candida species used in this study (C. kefyr, C. dubliniensis, C. pulcherrima, C. albicans and C. glabrata) were previously isolated and identified in the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University.

Inoculum preparation

Yeast were sub-cultured from vial stock onto Sabouraud dextrose agar (Difco, Detroit, USA) in air atmosphere for 48h at 37°C. The inoculum initial quantification was confirmed by cells growing in the control group.

Irradiation source

A GaAlAs diode laser (Photon Lase III, DMC, São Carlos, Brazil) with wavelength 660 nm was used in this study. Yeast strains were continually irradiated from the top of a flat-bottomed micro-titer plaque and the laser beam passed through all the suspensions at 1.0 cm²
spot size, which was coincident for all groups. Fluence rates of 200 and 250 mW/cm² were compared at 5 and 10 min of irradiation. 200 mW/cm² had fluences at 5 min equal 60 while at 10 min equal 120 J/cm² as well as 250 mW/cm² had fluences at 5 and 10 min equal 75 and 150 J/cm², respectively.

**Photosensitizer**

For the sensitization of *Candida* species the solution of methylene blue dye was (0.1 mg/ml) was used as photosensitizer. The solution of methylene blue was prepared by the dissolution of the powder (Sigma Chem. Co., St. Louis, USA) in physiologic solution (0.85% NaCl). Then, the solution was filtered through a sterile filter membrane (0.22 µm Millipore, Sao Paulo, Brazil) After filtration, the photosensitizer Solutions were stored in the dark (*Prates et al.*, 2008).

**Photodynamic therapy in vitro and colony-forming units (CFU) determination**

Yeasts were harvested onto Sabouraud dextrose agar and then suspended in phosphate buffer saline (PBS) at a concentration of approximately 10⁶ cfu/mL. This cell density was chosen because higher yeast cells concentration may inhibit photodynamic effect (*Prates et al.*, 2009). The suspension strain was divided into four groups. The control group (L-PS-) was untreated by either laser or photosensitizer (PS). In the laser groups (L+PS-), the yeast suspensions were treated with laser in the absence of the photosensitizer. In the PS group (L-PS+), the yeast suspension were treated with photosensitizer in the absence of laser. In the PDT groups (L+PS+), the yeast suspensions were treated with laser in the presence of the photosensitizer.

In a sterile 96 well flat-bottomed microtiter plate, 0.1 ml of Candida suspension and 0.1 ml of the photosensitizer or physiologic solution were added. After that, the containing of each well was irradiated according to the previously described groups in fluence rates of 200 and 250 mW/cm² at 5 and 10 min. of laser illumination; thereafter, they were serially diluted in PBS to generate dilutions of 10⁻¹ to 10⁻⁴ times the original concentration (*Prates et al.*, 2008). 10µl aliquots of each dilution were streaked onto a Sabouraud agar plate in triplicate and incubated to allow colony formation (*Agren et al.*, 1997).

**In vitro statistics**

The yeast colonies were counted and converted into CFU for analysis. All samples were submitted to this process and statistical analysis of the experimental data was performed using
one-way analysis of variance (ANOVA). Mean comparisons were carried out with the Tukey’s test, which retains the overall significance level at 5% (P<0.05) (Pfaller et al., 1988).

Photodynamic therapy in vivo

Animals

Twenty male Wistar rats (Rattus norvegicus), weighing 150 to 180g, from (College of Veterinary Medicine, Cairo University). During the experiment, the animals were allocated in individual cages cleaned daily and maintained in an environment with temperature (22°C ±2°C), humidity (60-70%) and illumination (12-h light/dark cycles) controlled. Water and standard rat chow were provided ad libitum. The experiment was conducted according to the Guiding Principles in the Use of Animals Ethics Committee CEP/Univale (registration 18/2002).

Experimental design

The animals were anesthetized with ketamine and xylazine via intra-peritoneal injection. Trichotomy was then performed on the dorsolateral region of all animals, and one circular secondary intention wounds of 10 mm in diameter were made in the dorsolateral region of the animals by performing a surgical incision in the skin and subcutaneous cell tissue using a scalpel.

The area of the wounds was previously marked using crystal violet and checked using an analogical pachymeter (Mitutoyo Suz Americana Ltda®, Brazil). The depth of the surgical incision was controlled by removing the epithelial tissue until the dorsal muscular fascia was exposed. The most effective Candida species from in vitro experiment was harvested from Sabouraud dextrose agar and then suspended in PBS at a concentration of approximately 10^6 cfu/mL, which 0.1ml of the suspension was slowly inoculated in the wounds of the mice. Following second day of the inoculation, the mice were divided into four groups each had five mice: The control group PS-); (L+PS-); (L-PS+) and (L+PS+). A GaAlAs diode laser (Photon Lase III, DMC, São Carlos, Brazil) had a power output of 250 mW at 10 min, continuous wave, infrared spectrum (invisible), a collimated beam with a spot area of (1cm^2 ) 0.0035 cm^2, and had been previously calibrated by the manufacturer. Both laser groups were treated at a power density (250 mW/cm2) on the wound surface. The wounds were cleaned daily with 0.9% saline solution immediately prior to application of the laser treatment. All therapies were initiated 6 hours after surgery and repeated daily for week of the experiment (Mandarin et al., 2010).
Histomorphometric Analysis
The animals were examined every (1 day) for week, an interval coinciding with the removal of wound fragments after 3, 5 and 7 days. The fragments removed for histological analysis were preserved in 10% buffered formaldehyde for 24 h. The material was processed for paraffin embedding by ethanol dehydration, diaphanization with xylene, infiltration and embedding in paraffin wax. Cuts of 4 μm were obtained with a rotary microtome (Reichert-Jung 2045 Multicut®, Germany) and stained with picrosirius (Sirius red F3B, Mobay Chemical Co., Union, New Jersey, USA). Images of histological sections were captured with a polarization microscope (Olympus AX-70®, Brazil), four random images from different parts of the field were obtained from each sample(Gonçalves et al., 2010).

RESULTS AND DISCUSSION
Photodynamic therapy in vitro and colony-forming units (CFU) determination
The reduction of colony forming units in each of the tested groups is tabulated for each of the fluence rates used in this study. Table (1) shows the mean of standard deviation values of the logarithm of colony forming units per millimeter (cfu/mL) at the fluence rate 200mW/cm² of The wavelength 660 diode laser at 5 and 10 minutes. The effect of laser (diode laser) at the fluence rate 250mW/cm² is shown in Table (2). A statistically significant decrease on yeast viability was observed for all groups and the same fluence in different exposure time presented statistically significant differences on yeast cell inactivation.

It was clear from the results recorded in Table (1) that, the group irradiated with laser at the fluence rate of 200 mW/cm² at 5 min. in the presence of photosensitizer (group L+PS+) was more effective than the group irradiated with laser only (L+PS-). The reduction of the tested yeast species viability exposed to laser in the presence of photosensitizer in relation to other groups was higher for C. kefyr followed by C. pulcherrima, C. albicans, C. dubliniensis and then C. glabrata. In addition, ten minutes of light delivery at the fluence rate of 200mW/cm² was sufficient time to eradicate completely C. kefyr followed by C. pulcherrima, C. dubliniensis, C. glabrata and C. albicans, respectively.
Table 1. Mean of standard deviation values of the logarithm of colony forming units per millimeter (cfu/ml) at the fluence rate of 200mW/cm² for the following studies groups: L+P+ = group treated with laser in the presence of photosensitizer (n = 3); L+P- = group treated only with laser (n = 3); L-P+ = group treated only with photosensitizer (n = 3); L-P- = group treated neither with laser nor with photosensitizer (n = 3).

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<th>Yeast species</th>
<th>Viable count colony forming unit CFU(log)/ml</th>
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<td>C. glabrata</td>
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<td>C. albicans</td>
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<td>C. kefyr</td>
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<td>C. pulcherrina</td>
<td>6.93 ±0.06</td>
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<td>C. dubliniensis</td>
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A, B, C, D, E and F: order of statistically significant difference from higher to lower (Tukey's test: p <0.05)

Table (2) declared that, the reduction of yeast cells in the tested PDT groups was more effective in the fluence rate at 250mW/cm² in relation to the group irradiated with laser at the fluence rate of 200mW/cm². In addition, five and ten minutes of light delivery was sufficient time to eradicate completely C. glabrata, C. kefyr and C. Pulcherrima as well as, C. dubliniensis showed reduction more than C. albicans.

Table 2. Mean of standard deviation values of the logarithm of colony forming units per millimeter (cfu/ml) at the fluence rate of 250mW/cm² for the following studies groups: L+P+ = group treated with laser in the presence of photosensitizer (n = 3); L+P- = group treated only with laser (n = 3); L-P+ = group treated only with photosensitizer (n = 3); L-P- = group treated neither with laser nor with photosensitizer (n = 3).

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<td>C. dubliniensis</td>
<td>6.96 ±0.24</td>
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A, B, C, D, E and F: order of statistically significant difference from higher to lower (Tukey's test: p <0.05)

Several studies that were performed with different Candida species contribute to the development of treatment options for different types of infectious diseases that can occur in different sites of the human body, and for a better comprehension of the transmissibility of that fungus. The treatment of Candida infections is troublesome because the effects of antifungal drugs are usually species-dependent. Several studies pointed to photodynamic therapy as a treatment alternative for several infectious (Sobel and Vasquez, 2003).
In vitro studies showed that the use of laser associated with a photosensitizer is very effective against bacteria, yeasts, virus and parasite (Wainwright, 1998). However, a great number of variables may influence the number of microorganisms affected by this technique including: type and concentration of the photosensitizer, microorganisms physiologic stage, photosensitizer incubation period before the irradiation, exposure period and density of laser energy (Wilson and Mia, 1993).

This study showed that, five minutes of light delivery at the fluence rate of 200mW/cm\(^2\) was sufficient time to eradicate completely only C. kefyr while at 250mW/cm\(^2\) at the same time showed the complete inhibition on three Candida species in the tested PDT groups C. glabrata, C. kefyr and C. Pulcherrima in the presence of photosensitizer. Sandra et al. (2006) exhibited that, C. krusei was the species that presented higher percentage of cfu/mL vreduction (91.6%) after the photo activation of methylene blue at a concentration of 0.1 mg/ml with 685 nm laser light (28 J/cm\(^2\)), followed respectively by C. albicans (88.6%), C. dubliniensis (84.8%) and C. tropicalis (82.3%): On the other hand, a reduction of 42% of C. albicans cfu/mL has been reported by Wilson and Mia (1993) when 0.1 mg/ml methylene blue and 660 nm laser light with an energy dose of 2.04 J/cm\(^2\) was used. Possibly, the differences between these results can be attributed to the parameters used for the laser irradiation. The effects of photodynamic therapy on different Candida species have also been studied by others. After the photo activation of toluidine blue with the He Ne (632.8 nm) laser, the authors in reference found higher cfu/mL reduction percentage for C. albicans (77%), followed respectively by C. tropicalis (65%), C. stellatoidea (63%) and C. kefyr (40%). Moreover, Bliss et al., 2004 used photofrin as photosensitizer in photodynamic therapy, verified similar reduction of the metabolic activity of C. albicans and C. krusei. However, C. glabrata showed resistance to this type of therapy.

Different microorganisms seem to present different susceptibility to antimicrobial PDT under similar conditions (Brue et al., 2005). It can be caused by the intrinsically particularities in each species metabolism, for instance, enzymatic apparatus against reactive oxygen species and structural composition on cellular wall and membrane. The irradiation protocols in antifungal PDT are very diverse, with typical output powers used in the range 10–100 mW, and fluences being between 10 and 200 J/cm\(^2\) (Prates et al., 2008). In some cases, the light parameters may be higher than those used in antibacterial PDT.
On the other hand, the amount of photons passing through a microbial suspension is more efficient to inactivate cells in a longer time of illumination. Thus, an adequate fluence rate should result in the eradication of the microorganisms treated. It has been reported that, an increase in the fluence rate enhances the microbial damage, although it seems to have an upper limit of photons to observe this effect. In fact, if the number of photons is higher than this limit, consequently the antimicrobial effect will decrease because the dye in the suspension will not absorb all the excess of light. In addition, a higher fluence rate could promote an increase in photo-bleaching (Renato et al., 2010).

**Photodynamic therapy in vivo**

*C. glabrata* exhibited the most effective candida species to Photodynamic therapy. It was harvested from Sabouraud dextrose agar and then suspended in PBS at a concentration of approximately $10^6$ cfu/mL, which 0.1ml of the suspension was slowly inoculated in mice left paw. The reduction in the size of the wound on the 3rd days was beginning to decrease and significantly greater in the groups (L+PS+) treated with Laser and Methylene blue followed by Laser treated without photosensitizer (L+PS-) as compared with control while group (L-PS+) and (L-PS-) showed inflammation of wounds. On seven days, all animals in all groups had almost healed wounds. The analysis of the region of the wound epithelium showed that there was a greater proliferation of epithelial cells L+PS+ groups, since the thickness of the epithelium was higher in both groups compared to the others. Regarding the surface density, the (L+PS+) group epithelium showed higher values compared to the other groups at the end of 7 days. In addition to proliferation in the epithelial layer, an increase in the keratin cell layer can also be observed (Figure 1).
After 3rd, 5th and 7th days from rats wounded the wound fragments removed for histological analysis.

**Methylene Blue and laser experiment**

After 3rd, 5th and 7th days from rats wounded the wound fragments removed for histological analysis.

**slide (1) after 3rd day**

Control group (L-PS-): Early vascular and cellular phase characterized by hemorrhage (black arrow) and neutrophilic inflammation reaction (red arrow) X 20. Laser group (L+PS-): Subsiding of congestion and hemorrhage in association with proliferation phase which
characterized with angiogenesis and granulation tissue formation (black arrow) X 20. MB group (L-PS+): Prominent edema and epithelization (arrow) under the scab, minimal inflammatory changes X 20. Laser and MB group (L+PS+): Marked proliferative phase characterized by angiogenesis, fibroplasias and granulated tissue formation (black arrow) with minimal epithelization (red arrow) X 20 (Figure 2 and Table 3).

**Slide (2) after 5\(^{th}\) day**

Control group (L-PS-): Inflammatory phase characterized by neutrophils and macrophages infiltration (black arrow). Slight congestion and hemorrhages X 20. Laser group (L+PS-): Inflammatory phase characterized by macrophages and mononuclear cells infiltration X 20. MB group (L-PS+): Marked proliferative phase and decline inflammatory phase characterized by macrophages, angiogenesis (black arrow) and fibroplasias X 20. Laser and MB group (L+PS+). Inflammatory and beginning of proliferation phase, notice the presence of macrophages, hemorrhages, angiogenesis (black arrow) and slight fibroplasias (fibroblast) X 20 (Figure 3 and Table 4).

**Slide (3) after 7\(^{th}\) day**

Control group (L-PS-): That scab and inflammatory phase X 10; Laser group (L+PS-) Decline of inflammatory phase and marked proliferative phase characterized by fibroplasias, epithelization, collagen deposition and contraction. X 10. MB group (L-PS+) Subsiding of proliferative phase and marked maturation and remodeling (black arrow) X 10; Laser and MB group (L+PS+): Inflammatory phase characterized by creeping epithelium, inflammatory cells especially polymorphs and macrophages (black arrow) X 10 (Figure 4 and Table 5).

The findings of the present investigation demonstrate the actions of low-level laser therapy in reducing the size of wounds, associated with a greater speed in the wound healing process. There are various reports in the literature on the benefits of laser treatment in different phases of the healing process (*Bayat et al., 2010*). This therapy has been shown to have positive effects on angiogenesis, collagen deposition, fibroblast proliferation and tissue epithelium (*Zhang et al., 2003*). Other studies have shown the benefits of oils and ointments derived from natural products in stimulating the healing of skin wounds (*Gungormus and Akyol, 2009*). Although the use of these oils and ointments is common in clinical practice, it remains unclear which aspects related to the healing process are most stimulated by this therapy.
Table 3. Effect of methylene blue (MB), laser and methylene blue with laser on wound infected with yeast cells in the 3rd day: (+) mild, (++) moderate, (+++) Sever and (-) no lesion. group (L-PS-) was untreated by either laser or photosensitizer (PS). Group (L+PS-), the mice were treated with laser in the absence of the photosensitizer. Group (L-PS+), the mice were treated with photosensitizer in the absence of laser. Groups (L+PS+), the mice were treated with laser in the presence of the photosensitizer.

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Figure (3): Histopathologic features of wound rat tissues after inoculated with C. glabrata for 5 days and treated with Photodynamic therapy: group (L-PS-) was untreated by either laser or photosensitizer (PS). Group (L+PS-), the mice were treated with laser in the absence of the photosensitizer. Group (L-PS+), the mice were treated with photosensitizer in the absence of laser. Groups (L+PS+), the mice were treated with laser in the presence of the photosensitizer.

Table 4. Effect of methylene blue (MB), laser and methylene blue with laser on wound infected with yeast cells in the 5th day: (+) mild, (++) moderate, (+++) Severy and (-) no lesion. group (L-PS-) was untreated by either laser or photosensitizer (PS). Group (L+PS-), the mice were treated with laser in the absence of the photosensitizer. Group (L-PS+), the mice were treated with photosensitizer in the absence of laser. Groups (L+PS+), the mice were treated with laser in the presence of the photosensitizer.

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Table 5. Effect of methylene blue (MB), laser and methylene blue with laser on wound infected with yeast cells in the 7th day: (+) mild, (+++) moderate, (+++) severe and (-) no lesions. Group (L-PS-) was untreated by either laser or photosensitizer (PS). Group (L+PS-), the mice were treated with laser in the absence of the photosensitizer. Group (L-PS+), the mice were treated with photosensitizer in the absence of laser. Groups (L+PS+), the mice were treated with laser in the presence of the photosensitizer.

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Figure (4): Histopathologic features of wound rat tissues after inoculated with *C. glabrata* for 7 days and treated with Photodynamic therapy: group (L-PS-) was untreated by either laser or photosensitizer (PS). Group (L+PS-), the mice were treated with laser in the absence of the photosensitizer. Group (L-PS+), the mice were treated with photosensitizer in the absence of laser. Groups (L+PS+), the mice were treated with laser in the presence of the photosensitizer.
Animals treated by PDT presented reduction of *Cryptococcus neoformans* viability. The overall finding was that MB-stained infected tissue was able to kill yeast cells in the mice afflicted paw following red laser irradiation. The parameters used *in vivo* presented a moderated fungicidal effect. In addition, *C. neoformans* recover showed a statistically reduction after 9 min. of illumination, and the microbial reduction was about 1 log (Renato et al., 2010). Also, Fuchs et al. (2007) showed that *C. neoformans* is susceptible to photoinactivation and their results showed until 6 logs reduction of this yeast. Furthermore, cell wall structure of their samples had injuries caused by the lack of ROM2 locus. In this work, the inactivation degree was changed by the illumination parameters. At 300mW/cm², it was found about 6 logs reduction, which shows that this capsulate yeast can be damaged by PDT. Although phototoxic effect on *C. neoformans* following PDT has been demonstrated in the literature, to the best of our knowledge it is the first application in animal model to treat dermal cryptococcosis.

Previously, *in vitro* studies have demonstrated that fibroblast proliferation can be stimulated by Chemical stain (Amaral et al., 2001). Hence, the action of the laser therapy could have had a direct biostimulatory effect on these fibroblasts and triggered the production of collagen, improving its structural strength (Gonçalves et al., 2012). There are several reports in the literature about the benefits of different plant extracts in different stages of inflammation and oxidation that act directly on tissue repair (Carvalho et al., 2011). Previous studies have shown that compounds with antioxidant activity are capable of down-regulating the inflammatory process and reducing functional and morphological cell damage (Park et al., 2010). As the preliminary phytochemical analysis of *Brassica oleracea* extract indicated the presence of antioxidant compounds such as alkaloids and flavonoids, the potential benefits of this extract in fibroblast proliferation (toxic agents; the results showed significant action of the polyphenols present in the extract in promoting recovery and restructuring of hepatic tissue cells, with decreased necrosis and inflammation (Gonçalves et al., 2013). Further pharmacological evaluations are essential to elucidate the detailed mechanism of action of this extract, which might give it great potential for the treatment of skin wounds.

Another interesting factor to be considered in the process of cutaneous wound repair is the rate of re-epithelialization of the tissue. According to Fukuka et al. (2002), a higher number of cell divisions, associated with increased expression of growth factors and their receptors, can facilitate recovery of the epithelium and thereby accelerate the repair process in the skin.
Stimulation of re-epithelialization of the tissue represents an important step towards resolution of the lesion and strengthening the scar. In this study, Laser and Balsam *Brassica oleracea* were effective in promoting epithelialization of the tissue at the end of 21 days of treatment. However, differences were found in the surface density of the epithelium, with the best results found in the group treated with Balsam.

**CONCLUSION**

In conclusion, PDT seems to be an alternative to treat dermal candidasis; however, more effective parameters must be found for *in vivo* studies.

**REFERENCES**


