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Exploring the *Galleria mellonella* model to study antifungal photodynamic therapy

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ABSTRACT

Background: Antimicrobial photodynamic therapy (aPDT) shows antimicrobial activity on yeast of the genus *Candida*. In aPDT, the depth at which the light penetrates the tissue is extremely important for the elaboration of the treatment. The aim of this study was to evaluate the action of aPDT on experimental candidiasis and the laser impact in the tissue using *Galleria mellonella* as the infection model.

Methods: G. mellonella larvae were infected with different *Candida albicans* strains. After 30 min, they were treated with methylene blue-mediated aPDT and a low intensity laser (660 nm). The larvae were incubated at 37 °C for seven days and monitored daily to determine the survival curve, using the Log-rank test (Mantel Cox). To evaluate the distribution of the laser as well as its depth of action in the larva body, the Interactive 3D surface PLOT of Image J was used. The effects of aPDT on the immune system were also evaluated by the quantification of hemocytes in the hemolymph of *G. mellonella* after 6 h of *Candida* infection (ANOVA and Tukey's test).

Results: In both the ATCC 18,804 strain and the *C. albicans* clinical strain 17, aPDT prolonged the survival of the infected *G. mellonella* larvae by a lethal fungal dose. There was a statistically significant difference between the aPDT and the control groups in the ATCC strain ($P = 0.0056$). The depth of laser action in the insect body without the photosensitizer was 2.5 mm and 2.4 mm from the cuticle of the larva with the photosensitizer. In the larvae, a uniform distribution of light occurred along 32% of the body length for the group without the photosensitizer and in 39.5% for the group with the photosensitizer. In the immunological analysis, the infection by *C. albicans* ATCC 18,804 in *G. mellonella* led to a reduction in the number of hemocytes in the hemolymph. The aPDT and laser treatment induced a slight increase in the number of hemocytes.

Conclusion: Both aPDT and laser treatment positively influenced the treatment of experimental candidiasis. *G. mellonella* larvae were a useful model for the study of light tissue penetration in antimicrobial photodynamic therapy.

1. Introduction

Candida albicans is a pathogenic and opportunistic fungus, and the most common cause of fungal diseases in humans [1,2]. The increasing rate of morbidity and mortality related to fungal infections is associated with an increase in antifungal-resistant strains, which has encouraged the development of research on new forms of treatment for these infections [3]. One of the therapies strengthened by these studies is the antimicrobial photodynamic therapy (aPDT). aPDT requires the administration of a non-toxic photosensitizer, either systemically or locally, followed by illumination using a visible light beam at an adequate wavelength and in the presence of oxygen [4–8]. Its action leads to the formation of reactive oxygen species (ROS), which have the ability to react rapidly with non-specific targets, such as cell membranes and

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proteins, leading to microbial destruction and direct cell death [6].

The use of aPDT in the control of *C. albicans* has proved to be efficient for both microorganisms in planktonic form and those organized in biofilms [9]. The application of aPDT has been found to enhance the activation of specific cells by enhancing the immune response [10]. The wavelength is considered the most important part of the interaction between the light and tissue, because it determines which part of the tissue will be reached as well as its primary action. During irradiation, there is usually a light reflection between 4 and 7%, and the remaining energy is either scattered, transmitted, or absorbed. When tissue structures are irradiated, photons propagate randomly throughout the tissue, where areas that are pigmented tend to be more absorbent than the adjacent areas [11]. The depth of light penetration in the tissue is of extreme relevance for the elaboration of treatment parameters and for reducing the risks involved in the use of lasers [11].

To investigate the activity of aPDT on the immune system, and the laser penetration depth is crucial the use of host models. Due to the similarity between the mechanisms of the invertebrate and mammalian immune systems, *Galleria mellonella* larvae are increasingly being used to investigate new antimicrobial agents against human pathogens, such as *C. albicans* [3,12,13]. When the body of this larvae is invaded, the microorganisms are confronted with an immune system that is composed of both cellular and humoral responses, which is also observed in humans [14,15].

Therefore, this study aimed to evaluate the influence of aPDT and laser treatment on experimental candidiasis and the laser penetration of the tissue using *G. mellonella* as the host model of infection.

2. Materials and methods

2.1. Strains of C. albicans

A clinical strain of *C. albicans* isolated from oropharyngeal candidiasis (strain 17) and a standard strain of *C. albicans* (ATCC 18,804) [16] were used. Both strains were obtained from the Laboratory of Microbiology and Immunology of the Dentistry Course of São Paulo State University (Unesp), Institute of Science and Technology (ICT), and Campus São José dos Campos in Brazil. These strains were stored in frozen stocks in BHI broth, containing 20% glycerol at −80 °C. In order to ensure the absence of contamination, the strains were cultured in CHROMagar Candida, and only green-colored colonies suggestive of *C. albicans* were used in the study. Thereafter, *C. albicans* strains were cultured in Sabouraud Dextrose agar medium (Himedia Laboratories, Mumbai, India) for 48 h at 37 °C. The colonies were then transferred to the Yeast extract Peptone Dextrose Broth (YPD, Difco, Detroit, MI, EUA) and incubated at 37 °C for 24 h.

2.2. Photosensitizer and light source

Methylene blue (M9140; Sigma Aldrich, São Paulo, Brazil) was used with a volume of 10 µL (concentration determined during the study). The dye was dissolved in sterilized distilled water (H_2O) and it was filtered through a sterile 0.22 µm Millipore membrane (São Paulo, Brazil). After filtration, the photosensitizer solution was stored in the dark. For each experiment, a new photosensitizer solution was prepared. The light source was a Gallium Aluminum Arsenide laser (Photon Lase III, DMC, São Carlos/SP, Brazil) emitting at 660 nm (visible red), with an output power of 100 mW (with energy defined during the study) and a spot size of 0.02 cm^2.

2.3. G. mellonella model

The larvae of *G. mellonella* in the final larval phase were used as the host model. The larvae were obtained from the invertebrate laboratory of the Microbiology and Immunology of the Dentistry Course, Unesp, ICT, Campus São José dos Campos. Each experimental group consisted of 15 randomly selected larvae with a body weight between 250 and 300 mg. All of the insects selected were light-colored and free of dark spots and/or pigments on their cuticle, which could indicate the larvae's involvement in an infectious process, influencing the outcome of the experiment. All analyses were performed in duplicate.

For each assay, at least two control groups were always included. The first received 20 µL of PBS in order to evaluate whether *G. mellonella* death would occur due to traumatic injury caused by the injection or as a result of animal manipulation, that is, the group controlled the technique used by the operator. The second group consisted of larvae that did not receive an injection. The purpose of this group was to provide a control for the quality of *G. mellonella* larvae. In cases where at least one of the control groups contained two dead larvae, the trial was discarded and a new trial was carried out.

Hamilton syringes of 10 µL capacity (Hamilton Inc, USA) were used for the injections. The *Candida* strain was inoculated in the last left proleg of the larva. The needle and inside of the syringe were cleaned after every five injections and/or at the beginning of injections using different inoculum.

2.4. Infection of G. mellonella by C. albicans and survival curve analysis

Standardized suspensions of *C. albicans* were prepared according to the methodology described by Fuchs et al. [17]. To obtain suspensions with the desired number of cells for each experiment, adjustment were carried out using a hemocytometer. In order to confirm the number of fungal cells in the inoculum used, the number of colony forming units per milliliter (CFU/mL) was counted. From the serial dilutions of the inoculum, aliquots were seeded in petri dishes containing Sabouraud Dextrose agar. The plates were incubated at 37 °C for 24 h. After incubation, the number of CFU/mL was calculated.

Suspensions of each strain of *C. albicans* containing 10^5 to 10^7 cells/larvae were injected into the larvae to determine the survival curve and the concentration to be used in the study. After inoculation, the animals were placed in disposable petri dishes and they were incubated in the dark at 37 °C. The number of dead larvae was recorded on a daily basis for the survival curve. The larvae were considered dead when they displayed no movement in response to touch. The dead larvae, once identified, were removed from the group.

2.5. Tests to determine the concentration of photosensitizer and laser energy

For the choice of the photosensitizer concentration to be used in the study, the laser output power was measured directly over the sensor or by passing through *G. mellonella*, using a power meter (Laser Check, MM Optics). The larva was placed between two microscopy slides in a ventral position, the power meter was positioned below the lower slide to the larva, and the laser light was focused over the upper slide to reach the body of the larva. The potency of light that passed through the larva with different concentrations of photosensitizer (75, 100, 150, 300, and 600 µM) was measured before and after administration.

To confirm the light absorption capacity of the *G. mellonella* larva, an analysis of the distribution of light in the larvae's body was carried out by analyzing photographs using Image J (NIH, Bethesda, USA). The laser was applied alone or in association with the photosensitizer, as described above.

To evaluate the distribution of the light as well as its penetration into larvae's body, photographs were taken with the larvae in a prone position and the laser irradiation over the dorsal surface. A CCD camera was placed in an orthogonally to the optical path and registered the intensity of the scattered light. The image was recorded as a bitmap file with 8-bit resolution. A standard ImageJ plug-in (lookup tables) transformed each pixel into a gray scale and false color fire scale, corresponding to the light intensity in the area of interest. Values were given between a minimum of 0 for device threshold and 256 for light intensity saturation. The camera captured the side-scattered light,

which is directly proportional to the local light intensity, such that the images correspond to a two-dimensional light intensity distribution model [18].

All the images were analyzed according to the Interactive 3D surface PLOT plugin of the Image J program. This plugin creates interactive surface graphics of all image types, and the luminance of an image is interpreted as height for the graphic (z-axis).

Subsequently, the concentration of the photosensitizer, which allowed the best light distribution over the thickness of the larvae's body after administration was chosen for the aPDT assays.

In relation to the choice of the most effective laser energy to be used in the experiment, the energies of 6 and 15 J were tested. The aPDT was performed with these energy measurements, and the one that induced the best survival rate in the larvae was chosen.

2.6. Antimicrobial photodynamic therapy

The methodology described by Chibebe et al. [13] was used for aPDT implementation in *G. mellonella*, with some modifications. Thirty minutes after infection, the larvae infected with *C. albicans* strains received methylene blue injection in the last right proleg. Thereafter, they were maintained for 30 min in the dark (pre-irradiation time) to provide a good dispersion of the dye across the body.

The laser was then applied according to the previously defined parameters. Each larva was placed between two microscopy slides in a ventral position under a support system, and the laser light was focused over the upper slide to reach the body of the larva. The experiment was carried out in the dark. For each experiment, the following experimental groups were analyzed: "*Candida* + aPDT", "*Candida* + PBS + laser", "*Candida* + PBS", "PBS + PBS", and "No injection", where n = 15, such that there was a total of 90 larvae.

After treatment with aPDT, the larvae were incubated at 37 °C. They were first analyzed after 18 h, then daily for seven days. The data obtained were used to plot the survival curve of *G. mellonella.*

2.7. Quantification of G. mellonella hemocyte

This methodology was based on studies by Fuchs et al. [17], Scorzoni et al. [19], and Zdybicka-Barabas and Cytryńska [20]. *G. mellonella* larvae were infected with *C. albicans* and treated with aPDT according to the previously parameters determined. The following groups were included: "*Candida* + aPDT", "*Candida* + PBS + laser", "*Candida* + PBS", "PBS + PBS".

The larvae were infected with *C. albicans* (10^6 cells/larva) and subjected to aPDT or laser treatment. After 6 h, the hemolymph was collected to carry out a hemocyte count. The larvae were immobilized in an ice-cold petri dish for about 20 min, then cut using a scalpel in the ventral part cephalocaudally and squeezed to remove the hemolymph, which was collected in microtubes of 1.5 mL. Then, 10 µL of each hemolymph was diluted in 990 µl of IPS (sterile anticoagulant buffer; 150 nM sodium chloride, 5 nM potassium chloride, 10 nM tris – HCL pH 6.9, 10 nM EDTA, and 30 nM sodium citrate) in ice-cold microtubes. The hemocytes were counted using a hemocytometer.

2.8. Statistical analysis

For the survival experiments in *G. mellonella*, the survival curve was determined using the Kaplan-Meier method, and the level of significance between the survival curves was calculated using the log-rank test (Mantel-Cox). The results of hemocytes density were submitted to ANOVA analysis followed by the Tukey's test. All the tests were carried out using GraphPad Prism 5.0, where 5% was considered a significant difference.

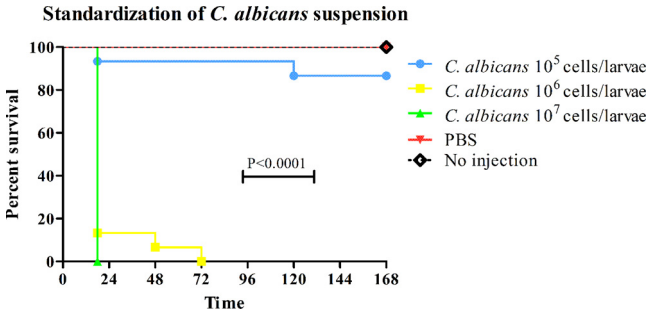

Fig. 1. Survival curve of G. mellonella larvae infected with different concentrations of C. albicans.

3. Results

3.1. Survival curve of G. mellonella infected by different concentrations of C. albicans

By analyzing the survival curve of *G. mellonella* inoculated with different concentrations of *C. albicans* (Fig. 1), we found that the control groups (both PBS and no injection) had survival rates of 100% throughout the seven days of the experiment, which indicated the reliability of the inoculation. Injections of *C. albicans* 10^5 cells/larva showed 13.3% larvae death, while the group infected with *C. albicans* 10^6 cells/larva showed 100% larvae death after 72 h. At a concentration of *C. albicans* 10^7 cells/larva, 100% larvae death occurred after only 18 h. As such, the concentration of 10^6 cells/larva was adopted for all subsequent experiments since it was the only dose that allowed enough time to carry out the different treatments before larvae fatality.

3.2. Effects of laser and photosensitizer on C. albicans non-infected G. mellonella larvae

For each photosensitizer concentration, an average of four measurements of light absorption was obtained at three different moments: before, immediately after, and 30 min after photosensitizer administration. The best response corresponded to the concentration that presented a better absorption of the incident light, indicating that the light could retained by the larva after the application of the photosensitizer. The averages for the different concentrations were analyzed and no significant statistical difference was found between them (Fig. 2). However, the concentration of methylene blue at 100 µM presented an adequate pattern of light absorption over time, and it was chosen for further study.

Light distribution was observed in the larvae with the application of the laser alone or with a photosensitizer (Fig. 3). When the images were analyzed using the Interactive 3D surface PLOT plugin in Image J, it was possible to evaluate the depth of the light distribution in the larvae as well as the average light intensity at each point (Fig. 4). The mean height of the larvae under the two slides corresponded to 3 mm,

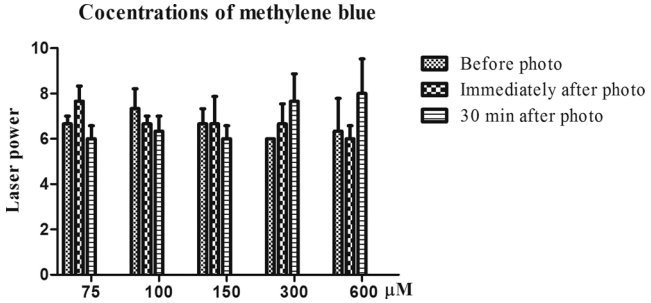

Fig. 2. Analysis of the laser power averages measured with different concentrations of photosensitizer over time. Legend: Photo – photosensitizer.

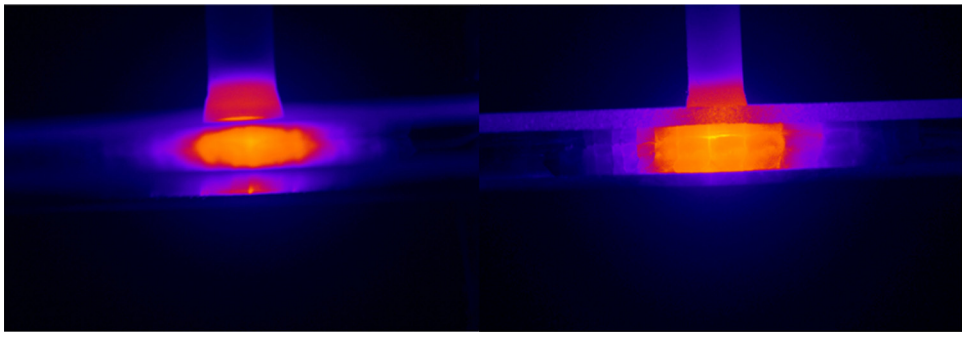

Fig. 3. False color images illustrating light passing through G. mellonella, first image without the use of photosensitizer and the second with 100 μM photosensitizer.

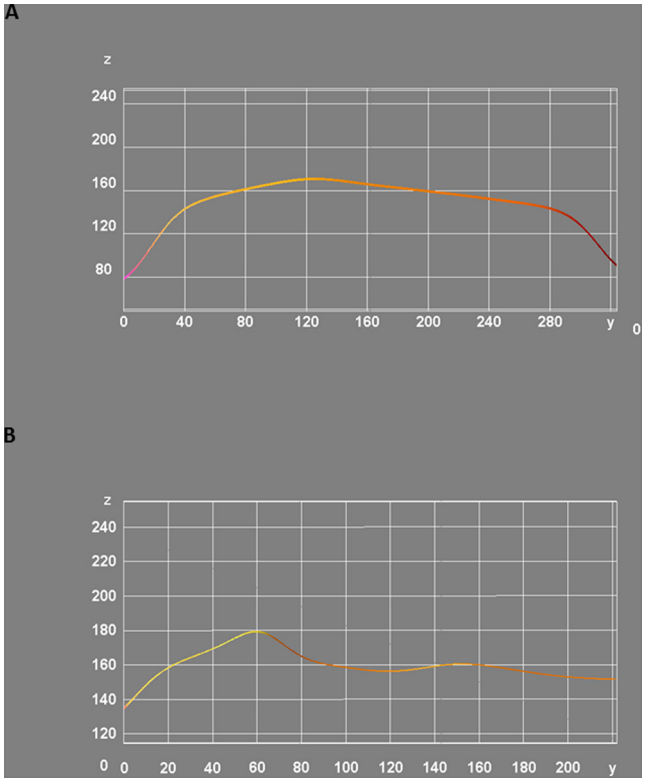

Fig. 4. Z axis corresponds to laser output power, in arbitrary units (255 - 100 mW), axis y corresponds to the height of the larva body, in pixels. A) Plot corresponding to the light distribution in depth on G. mellonella without the use of photosensitizer, y axis (320 - 3 mm larvae height). B) Plot corresponding to the light distribution in depth on G. mellonella with the use of photosensitizer, y axis (220 - 3 mm larvae height).

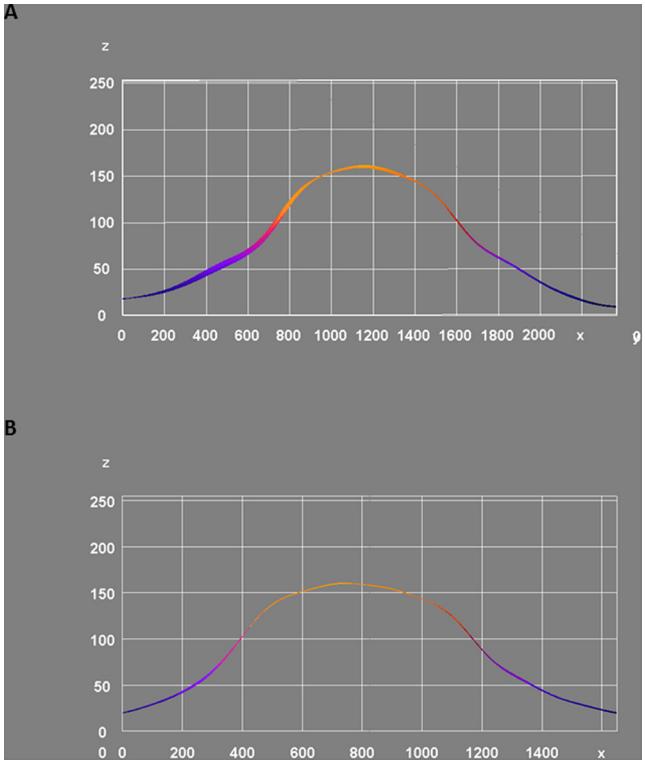

Fig. 5. Z axis corresponds to the laser power (255 - 100 mW), axis y corresponds to the length of the larva body. A) Plot corresponding to the light distribution in the length in G.mellonella without the use of photosensitizer, x axis (2300 - 2cm larvae length). B) Plot corresponding to the light distribution in the length in G. mellonella with the use of photosensitizer, x axis (1700 - 2cm larvae length).

equivalent to 320 pixels in the y-axis of Fig. 4A and 220 of Fig. 4B and 77 mW of power used in irradiation corresponded to 240 in the z-axis of Fig. 4A and 250 in the z-axis of Fig. 4B. Without the photosensitizer, the beginning of a uniform light distribution in the larvae occurred at 0.36 mm, and remained up to 2.5 mm deep in the body of the larva, with a power of 44.91 mW, reaching a peak of 54.54 mW with 1 mm in height. With the photosensitizer, a uniform distribution of light was observed 0.27 mm below the cuticle of the larvae, reaching a power of approximately 49.28 mW, which remained up to 2.45 mm in depth, reaching a peak power of 55.44 mW at 0.81 mm.

In Fig. 5, the light distribution along the length of the body of the larvae was evaluated. The length of the larva was 2 cm, equivalent to 2300 on the x-axis of Fig. 5A and 1700 of Fig. 5B, and 77 mW of power used in irradiation corresponding to 250 in the z-axis of both figures. Without the photosensitizer, a uniform distribution of light occurred for 32% of the larvae's body length. With the photosensitizer, light was found to be distributed in 39.5% of the body of *G. mellonella*.

Next, the survival curve of *G. mellonella* was determined with the isolated application of laser at energies of 6 J and 15 J. This allowed us to evaluate whether the laser incidence was lethal to the larvae. All larvae in the control groups, both PBS and no injection survived the laser energies tested after seven days, such that the survival rate of *G. mellonella* was 100%.

The same result was obtained after aPDT treatment, which consisted of the association of 100 μM of photosensitizer and laser treatment at 6 and 15 J, as well as after treatment with only the photosensitizer. In both case, the survival rate of *G. mellonella* was 100%. Subsequently, the invertebrate model of infection was studied.

3.3. Effects of aPDT and laser treatment on C. albicans ATCC 18804

G. mellonella larvae were infected with *C. albicans* strain ATCC

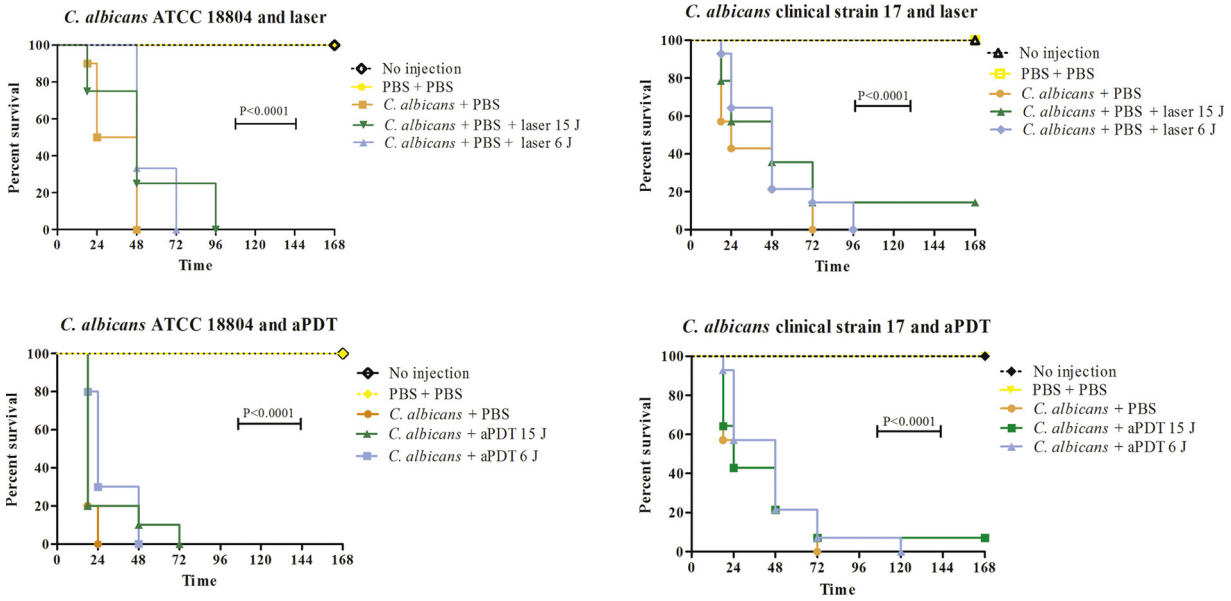

Fig. 6. Survival curve of G. mellonella infected with C. albicans ATCC 18804 and treated with different laser energies. Survival curve of C. albicans infected G. mellonella (ATCC 18804) and treated with aPDT. Survival curve of G. mellonella infected with C. albicans (Clinical strain 17), treated with different laser energies and control groups. Survival curve of G.mellonella infected with C. albicans Clinical strain 17, treated with aPDT.

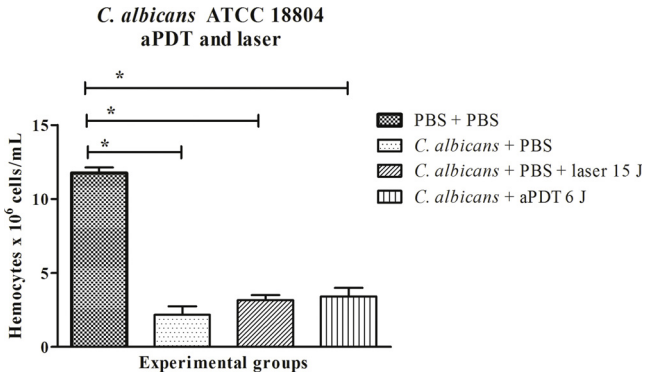

Fig. 7. Analysis of the hemocyte density of G. mellonella infected with C. albicans ATCC 18804 and submitted to laser therapy and aPDT. The use of the asterisk (*) corresponds to the presence of statistical difference between the analyzed groups.

18,804. Then, the larvae were irradiated with a laser at 6 J and 15 J. The laser treatment was found to increase the survival of the larvae compared to the larvae infected with *Candida* that did not receive laser treatment (Fig. 6). In the *Candida* + PBS group, 100% of the larvae died within 48 h, while in the *Candida* + PBS + laser 6 J groups, the larvae died only after 72 h and in the *Candida* + PBS + laser 15 J group after 96 h. Thus, the groups that received treatment showed increased survival rates, however, this increase was not statistically significant.

After aPDT treatment, there was an increase in the survival of the larvae relative to the *Candida* + PBS control group, both for the 6 J and 15 J laser treatments (Fig. 6). A statistically significant difference was found between the control group and the aPDT group after laser treatment at 6 J, which increased the survival rate of the larvae by 48 h.

3.4. Effects of aPDT and laser treatment on C. albicans clinical strain

After testing the *C. albicans* strain ATCC 18,804, next, we analyzed the survival of *C. albicans* clinical strain 17. We found that laser treatments at 6 J and 15 J resulted in a greater survival of the infected larvae than the *Candida* + PBS group (Fig. 6).

When comparing the *Candida* + PBS group to the *Candida* + laser 6 J group alone, we found that there was an improvement in the survival of the larvae, increasing the survival from 72 h to 96 h, respectively, without a significantly statistical difference (P = 0.1769). On the other hand, the *C. albicans* + laser 15 J group experience an increased survival of up to 168 h (P = 0.1890)

After aPDT treatment, we found that the survival of the larvae increased compared to the *Candida* + PBS control group at both 6 J and 15 J (Fig. 6). aPDT increased the survival of larvae at 6 J (P = 0.3201) and 15 J (P = 0.7377).

3.5. Quantification of G. mellonella hemocytes

In order to verify the action of aPDT and laser treatment on the immune system of *G. mellonella,* the number of viable hemocytes in the hemolymph of the larvae was determined after 6 h of infection. All the groups infected by *C. albicans* presented statistically significant differences in relation to the uninfected control group (P < 0.001). The groups subjected to aPDT or laser treatment showed an increase in the number of hemocytes compared to the control group, however, the differences were not statistically significant (Fig. 7).

4. Discussion

With the increasing rate of fungal resistance to available drug therapies, many studies have sought to develop alternative therapies to control infections caused by different pathogens [3]. One of the therapies that has proven to be widely recommended is aPDT, due to its numerous advantages. To the best of our knowledge, aPDT does not induce resistance in microorganisms, which allows for its wide and repeated use, as well as high specificity, without compromising the areas around the treated tissue, and has a broad spectrum of action, serving as a treatment for infections caused by microorganisms or as a cancer therapy [5,6].

Experimental models are necessary for the development of new treatments. A model that is often used is the larvae of *G. mellonella*, an invertebrate that does not have ethical restrictions of use, is available in great quantities, and has low maintenance costs [3,12]. This insect has an immune system that is similar to ours, which is composed of cellular and humoral immune responses. The main cell of its cellular response is the hemocyte, a cell that resembles the neutrophil found in humans

[15,21]. As such, in the present study, we sought to investigate the action of aPDT on the treatment of experimental candidiasis using the animal model of *G. mellonella*.

It is known that applied light can interact with host tissues in different ways, affecting the efficacy of aPDT or laser treatment. The tissue surface can reflect light, however, when the light is not reflected, it penetrates the tissue. In this case, the light can be either absorbed by the tissue, transmitted when it does not interact with the tissue, or scattered when its propagation changes direction. In aPDT treatment, light absorption is the most frequent interaction due to the use of the photosensitizer, which absorbs light photons [22,23]. The activity of aPDT can also be improved by increasing the photosensitizer concentration (methylene blue) and the light energy. However, higher concentrations of methylene blue cause aggregations and the optical shielding phenomenon can restrict the light [24]. This can form dimeric compounds and also reduce the killing of bacterial cells [24,25]. Methylene blue is a metachromatic dye, which, at increasing concentration, experiences changes in the electrostatic and hydrophobic interactions between the molecules of dye. This could result in dye aggregation [25].

We tested five different concentrations of methylene blue in *Galleria mellonella* (75, 100, 150, 300, and 600 μM). The best response corresponded to the concentration that presented a reduction in the average of the power of the incident light, indicating that the light could be retained inside the larva after the application of the photosensitizer, after which it begins the interaction. We did not find a statistically significant difference between the different concentrations, however, the concentration of methylene blue at 100 μM presented an adequate pattern of light absorption over time. As such, it was chosen for subsequent study.

Similar to our study, Garcez and Hamblin [24] tested whether an excessive concentration of photosensitizer could block the light due to high absorption rates at the surface, decreasing the light penetration and aPDT efficiency in the root canals. A quartz cuvette was filled with each solution: 50, 100, 150, and 300 μM, and the laser was delivered perpendicularly to the cuvette surface, emitting at 660 nm and 10 mW. At 50 μM, the light passed through the optical path with no significant attenuation. At 100 μM, the attenuation was 4.5%. At 150 μM, the attenuation was 45%, while at 300 μM, the attenuation was 68% of the initial incident light. These results suggest that a concentration of 50–100 μM is more indicative for use in root canal aPDT.

In order to observe the effects of aPDT on fungal infection, the survival curves of larvae previously infected with *C. albicans* and subject to aPDT treatment and laser irradiation were plotted. After larvae were subject to infection by the *Candida* strain ATCC 18,804, each group was treated as followed: laser at 6 J; laser at 15 J; aPDT at 6 J; and aPDT at 15 J. All groups showed improvement in larval survival, with no statistically significant differences except in the group in which aPDT at 6 J was administered in the untreated *Candida* group. aPDT at 6 J promoted the survival of larvae for up to 48 h compared to 24 h of larvae that did not receive any treatment.

These results are similar to those found by Chibebe et al. [13], who evaluated the effect of aPDT on larvae infected with two strains of *Candida*, and found that aPDT used with methylene blue, as in our study, prolonged larvae survival when the infection was caused by the strain CAN 14.

Merigo et al. [26] studied the use of different laser energy densities with or without different types of photosensitizers in fungal infections *in vitro* and *in vivo*, and also obtained results similar to ours. They used toluidine blue, curcumin, and erythrosine with their respective lasers at different wavelengths (650 nm, 450 nm, and 532 nm) with an energy density of 10 J/cm^2, as well as the use of laser alone, as in our study. All the treated groups showed statistically significant differences compared to the group only infected with *Candida*, with an increase in survival of the larvae. This suggests that both the use of laser irradiation and aPDT are effective in controlling candidiasis in the *G. mellonella* model.

The mechanism of action of aPDT involves the production of ROS when a visible light is associated to a photosensitizer in the presence of oxygen. These reactive species are cytotoxic and are able to reach cell membranes and microbial proteins, leading to direct cell death of microorganisms [5,6].

However, the effects of visible light when associated with the photosensitizer depends on the parameters used in the aPDT, such as the light source wavelength, the energy density reaching the tissue [27], and the concentration of photosensitizer used. In the case of the methylene blue used in our study, when used at very high concentrations, it can be toxic to the host tissue and may exacerbate the inflammatory process, in addition to being able to form an optical barrier during irradiation when in excess. As a result, the passage of light and the action of the aPDT are impaired [28].

Regarding the clinical strain of *Candida* 17, all the groups treated (laser 6 J, laser 15 J, aPDT 6 J, and aPDT 15 J) also showed an increase in the survival of the infected and treated larvae in relation to the control group, where infection did not receive treatment. However, no statistically significant differences were observed between the groups.

Chibebe et al. [13] analyzed the effectiveness of aPDT under a clinical isolate of the oral cavity of patients with oropharyngeal candidiasis resistant to azoles and found an improvement in the survival of the infected larvae. However, they did not observe any statistically significant differences, suggesting that aPDT may not be as effective in clinical strains that show antifungal resistance.

Another hypothesis is related to differences in microorganisms belonging to the same species. Fungi and bacteria may present different patterns of susceptibility to aPDT due to intrinsic metabolic determinants, such as enzymatic defense against reactive oxygen species and efflux pumps that may recognize photosensitizing molecules [29].

Chibebe et al. [13] observed that aPDT treatment in larvae infected with antifungal-resistant clinical isolates associated with the use of antifungals promoted increased survival of larvae when compared to groups where the use of aPDT treatment was isolated. These findings suggest that aPDT combined with antifungal treatment could be effective for infections in *G. mellonella* larvae caused by resistant clinical strains.

The adjustment of the parameters used in aPDT, as well as an understanding of the scope of the light in the applied area, are of great importance in order to achieve good results [11]. In human tissue, visible red irradiation at a wavelength of 660 nm is known to reach about 4–5 mm below the surface of the skin [30]. Although the use of invertebrate models to test the effects of aPDT is increasing, there are no studies that address the distribution of light in the integument of these animal models.

The larval integument of *G. mellonella* is constituted by a layer of epidermal cells and an exoskeleton or cuticle, which is secreted by these cells. This cuticle is essentially formed of chitin, and consists of two segments: the exocuticle and the endocuticle [31]. Proteins and lipids are also constituents of the larval cuticle [32].

In our study, we analyzed the light distribution, with a low power light source emitting at 660 nm, in the integument of *G. mellonella* with Image J software using the Interactive 3D surface PLOT plugin. It was possible to observe that, without the use of photosensitizer, the beginning of the light distribution in the cuticle of the larva occurred at 0.36 mm, and remained for up to 2.5 mm. In association with a photosensitizer, the light distributed occurred at 0.27 mm, and extended up to 2.45 mm below the cuticle. These results suggest that the use of laser irradiation in association with the photosensitizer enhanced light distribution in the cuticle of the larva compared to the laser alone, most likely due to the enhancement of light scattering promoted by the presence of a photosensitizer solution injected into the animal tissue.

Ash et al. [11] studied the variations in the wavelengths applied to human skin and the corresponding light penetration, using the Monte Carlo simulation as a tool. They found that an increased wavelength was associated with an increased depth of penetration in the tissue. At a

wavelength of 650 nm, the depth of penetration in human tissue was about 4.9 mm.

In order to verify the action of aPDT and laser treatment on the immune system of *G. mellonella* after infection with *C. albicans* ATCC 18,804, the number of viable hemocytes in the hemolymph of the larvae was determined. Bergin et al. [33] reported that the fluctuation or alteration in the number of hemocytes can be considered a reliable method to determine the pathogenicity of microorganisms and the level of infection. These authors verified that larvae inoculated with more pathogenic isolates presented a more significant reduction in hemocitary density, whereas larvae inoculated with isolates of low pathogenicity showed only a small change in the density of hemocytes.

In the present study, after 6 h of *Candida* infection, we found a slight increase in the number of hemocytes in the groups treated with aPDT compared to the untreated group, suggesting that this treatment can influence the defense cells of the larvae. Similar to our study, Paziani et al. [34] investigated the effect of aPDT (15 J/cm^2) on the immune response of *G. mellonella* infected with microconidia *Fusarium keratoplasticum* and *Fusarium moniliforme*. The photosensitizers used were methylene blue, new methylene blue N, and the pentacyclic S137. The activity of aPDT was found to increase the hemocitary density of infected larvae, compared to the same treatment under dark conditions. This result was correlated to an increased larval survival and decreased fungal burden. Santos et al. [35] investigated whether treatment with aPDT could attenuate the experimental infection by *P. gingivalis* in *Galleria mellonella.* In the aPDT group, the hemocyte density was found to increase by 9.6×10^6 cells/mL (2.62-fold increase) compared to the infected larvae with no treatment ($p = 0.0175$). As a result, aPDT treatment resulted in a significant reduction of the number of *P. gingivalis* cells in *G. mellonella* hemolymph.

We concluded that aPDT and laser irradiation positively influenced the improvement of *C. albicans* infection in *G. mellonella*. In addition, *G. mellonella* proved to be a potential model host for evaluating the correlation between light tissue penetration and the efficacy of aPDT.

Declarations of interest

None.

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References

[1] P. Uppuluri, A.K. Chaturvedi, N. Jani, R. Pukkila-Worley, C. Monteagudo, E. Mylonakis, J.R. Köhler, J.L. Lopez Ribot, Physiologic expression of the Candida albicans Pescadillo Homolog is required for virulence in a murine model of hematogenously disseminated candidiasis, Eukaryot. Cell 11 (2012) 1552–1556, https://doi.org/10.1128/EC.00171-12.

[2] G.B. Rodrigues, M. Dias-Baruffi, N. Holman, M. Wainwright, G.U.L. Braga, In vitro photodynamic inactivation of Candida species and mouse fibroblasts with phenothiazinium photosensitisers and red light, Photodiagnosis Photodyn. Ther. 10 (2013) 141–149, https://doi.org/10.1016/j.pdpdt.2012.11.004.

[3] M. Arvanitis, J. Glavis-Bloom, E. Mylonakis, Invertebrate models of fungal infection, Biochim. Biophys. Acta Mol. Basis Dis. 1832 (2013) 1378–1383, https://doi.org/10.1016/j.bbadis.2013.03.008.

[4] A. Casas, G. Di Venosa, T. Hasan, Al Batlle, Mechanisms of resistance to photodynamic therapy, Curr. Med. Chem. 18 (2011) 2486–2515, https://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted.

[5] F. Pereira Gonzales, T. Maisch, Photodynamic inactivation for controlling Candida albicans infections, Fungal Biol. 116 (2012) 1–10, https://doi.org/10.1016/j.funbio.2011.10.001.

[6] L.N. Dovigo, J.C. Carmello, C.A. de Souza Costa, C.E. Vergani, I.L. Brunetti, V.S. Bagnato, A.C. Pavarina, Curcumin-mediated photodynamic inactivation of *Candida albicans* in a murine model of oral candidiasis, Med. Mycol. 51 (2013) 243–251, https://doi.org/10.3109/13693786.2012.714081.

[7] A. François, A. Salvadori, A. Bressenot, L. Bezdetnaya, F. Guillemin, M.A. D'Hallewin, How to avoid local side effects of bladder photodynamic therapy: impact of the fluence rate, J. Urol. 190 (2013) 731–736, https://doi.org/10.1016/j.juro.2013.01.046.

[8] A. Sahu, W. Il Choi, J.H. Lee, G. Tae, Graphene oxide mediated delivery of methylene blue for combined photodynamic and photothermal therapy, Biomaterials 34 (2013) 6239–6248, https://doi.org/10.1016/j.biomaterials.2013.04.066.

[9] L.N. Dovigo, A.C. Pavarina, A.P.D. Ribeiro, I.L. Brunetti, C.A. de S. Costa, D.P. Jacomassi, V.S. Bagnato, C. Kurachi, Investigation of the photodynamic effects of curcumin against Candida albicans, Photochem. Photobiol. 87 (2011) 895–903, https://doi.org/10.1111/j.1751-1097.2011.00937.x.

[10] G.S. Rogers, Continuous low-irradiance photodynamic therapy: a new therapeutic paradigm, J. Compr. Canc. Netw. 10 (Suppl 2) (2012) S14–S17 (accessed April 27, 2018), http://www.ncbi.nlm.nih.gov/pubmed/23055207.

[11] C. Ash, M. Dubec, K. Donne, T. Bashford, Effect of wavelength and beam width on penetration in light-tissue interaction using computational methods, Lasers Med. Sci. 32 (2017) 1909–1918, https://doi.org/10.1007/s10103-017-2317-4.

[12] S.M. Cook, J.D. McArthur, Developing Galleria mellonella as a model host for human pathogens, Virulence 4 (2013) 350–353, https://doi.org/10.4161/viru.25240.

[13] J. Chibebe Jr, C.P. Sabino, X. Tan, J.C. Junqueira, Y. Wang, B.B. Fuchs, A.O.C. Jorge, G.P. Tegos, M.R. Hamblin, E. Mylonakis, Selective photoinactivation of Candida albicans in the non-vertebrate host infection model Galleria mellonella, BMC Microbiol. 13 (2013) 217, https://doi.org/10.1186/1471-2180-13-217.

[14] M.S. Lionakis, Drosophila and Galleria insect model hosts: new tools for the study of fungal virulence, pharmacology and immunology, Virulence 2 (2011) 521–527, https://doi.org/10.4161/viru.2.6.18520.

[15] M. Ligeza-Zuber, Mechanisms of Galleria mellonella cellular immune response after infection with entomopathogenic fungus Conidiobolus coronatus, Ann. Parasitol. 58 (2012) 227–228 (accessed April 27, 2018), http://www.ncbi.nlm.nih.gov/pubmed/23914619.

[16] J.C. Junqueira, S.F.G. Vilela, R.D. Rossoni, J.O. Barbosa, A. Carolina, B.P. Costa, V.M.C. Rasteiro, J.M.A.H. Suleiman, A. Olavo, C. Jorge, Oral colonization by yeasts in HIV- positive patients in Brazil, Rev. Inst. Med. Trop. Sao Paulo 54 (2012) 17–24, https://doi.org/10.1590/S0036-46652012000100004.

[17] B.B. Fuchs, E. O'Brien, J.B. El Khoury, E. Mylonakis, Methods for using Galleria mellonella as a model host to study fungal pathogenesis, Virulence 1 (2010) 475–482 (accessed April 28, 2018), http://www.ncbi.nlm.nih.gov/pubmed/21178491.

[18] C.P. Sabino, M.G. Hospital, A.S. Garcez, M.S. Ribeiro, M.R. Hamblin, M.G. Hospital, Real-time evaluation of two light delivery systems for photodynamic disinfection of Candida albicans biofilm in curved root canals vol. 30, (2016), pp. 1657–1665, https://doi.org/10.1007/s10103-014-1629-x.Real-time.

[19] L. Scorzoni, M.P. de Lucas, A.C. Mesa-Arango, A.M. Fusco-Almeida, E. Lozano, M. Cuenca-Estrella, M.J. Mendes-Giannini, O. Zaragoza, Antifungal efficacy during Candida krusei infection in non-conventional models correlates with the yeast in vitro susceptibility profile, PLoS One 8 (2013) e60047, , https://doi.org/10.1371/journal.pone.0060047.

[20] A. Zdybicka-Barabas, M. Cytryńska, Involvement of apolipophorin III in antibacterial defense of Galleria mellonella larvae, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 158 (2011) 90–98, https://doi.org/10.1016/j.cbpb.2010.10.001.

[21] G. Wu, L. Xu, Y. Yi, Galleria mellonella larvae are capable of sensing the extent of priming agent and mounting proportionatal cellular and humoral immune responses, Immunol. Lett. 174 (2016) 45–52, https://doi.org/10.1016/J.IMLET.2016.04.013.

[22] C. Sabino, M. Ribeiro, Dosimetria na terapia fotodinâmica antimicrobiana, in: PDT Ter, FotodinâMica Antimicrob. Na Odontol. (2013) 27–38.

[23] F.P. Gonzales, T. Maisch, Photodynamic inactivation for controlling Candida albicans infections, Fungal Biol. 116 (2012) 1–10, https://doi.org/10.1016/j.funbio.2011.10.001.

[24] A.S. Garcez, M.R. Hamblin, Methylene blue and hydrogen peroxide for photodynamic inactivation in root canal - a new protocol for use in endodontics, Eur. Endod. J. 2 (2017), https://doi.org/10.5152/eej.2017.17023 29–29.

[25] S.F.G. Vilela, J.C. Junqueira, J.O. Barbosa, M. Majewski, E. Munin, A.O.C. Jorge, Photodynamic inactivation of Staphylococcus aureus and Escherichia coli biofilms by malachite green and phenothiazine dyes: an in vitro study, Arch. Oral Biol. 57 (2012) 704–710, https://doi.org/10.1016/j.archoralbio.2011.12.002.

[26] E. Merigo, S. Conti, T. Ciociola, C. Fornaini, L. Polonelli, G. Lagori, M. Manfredi, P. Vescovi, Effect of different wavelengths and dyes on Candida albicans: in vivo study using Galleria mellonella as an experimental model, Photodiagnosis Photodyn. Ther. 18 (2017) 34–38, https://doi.org/10.1016/j.pdpdt.2017.01.181.

[27] A.S. Queiroga, V.N. Trajano, E.O. Lima, A.F.M. Ferreira, A.S. Queiroga, F.A. Limeira, In vitro photodynamic inactivation of Candida spp. by different doses of low power laser light, Photodiagnosis Photodyn. Ther. 8 (2011) 332–336, https://doi.org/10.1016/j.pdpdt.2011.08.005.

[28] L.C. Suzuki, T.M. Yoshimura, I.T. Kato, R.A. Prates, M.S. Ribeiro, Estudo comparativo entre dois comprimentos de onda no efeito fotodinmico em biofilmes de Candida albicans, (2014) (accessed April 28, 2018), http://repositorio.ipen.br:8080/xmlui/bitstream/handle/123456789/23745/20784.pdf?sequence=1&isAllowed=y.

[29] R.A. Prates, I.T. Kato, M.S. Ribeiro, G.P. Tegos, M.R. Hamblin, Influence of multidrug efflux systems on methylene blue-mediated photodynamic inactivation of Candida albicans, J. Antimicrob. Chemother. 66 (2011) 1525–1532, https://doi.org/10.1093/jac/dkr160.

[30] M. Clement, G. Daniel, M. Trelles, Optimising the design of a broad-band light source for the treatment of skin, J. Cosmet. Laser Ther. 7 (2005) 177–189, https://doi.org/10.1080/14764170500344575.

[31] S. Unsal, H. Ozparlak, A. Aktumsek, Effects of diflubenzuron on the integument of fifth instarGalleria mellonella larvae, Phytoparasitica 32 (2004) 43–51, https://doi.org/10.1007/BF02980858.
[32] M. Ligęza-żuber, Mechanisms of Galleria mellonella cellular immune response after infection with entomopathogenic fungus Conidiobolus coronatus 58 (2012), pp. 227–228.
[33] D. Bergin, M. Brennan, K. Kavanagh, Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of Galleria mellonella, Microbes Infect. 5 (2003) 1389–1395, https://doi.org/10.1016/j.micinf.2003.09.019.
[34] M.H. Paziani, L. Tonani, H.D. de Menezes, L. Bachmann, M. Wainwright, G.Ú.L. Braga, M.R. von Zeska Kress, Antimicrobial photodynamic therapy with phenothiazinium photosensitizers in non-vertebrate model Galleria mellonella infected with Fusarium keratoplasticum and Fusarium moniliforme, Photodiagnosis Photodyn. Ther. 25 (2019) 197–203, https://doi.org/10.1016/j.pdpdt.2018.12.010.
[35] J.D. dos Santos, J.A. de Alvarenga, R.D. Rossoni, M.T. García, R.M. Moraes, A.L. Anbinder, A.O. Cardoso Jorge, J.C. Junqueira, Immunomodulatory effect of photodynamic therapy in Galleria mellonella infected with Porphyromonas gingivalis, Microb. Pathog. 110 (2017) 507–511, https://doi.org/10.1016/J.MICPATH.2017.07.045.