Photodynamic effect of light-emitting diode light on cell growth inhibition induced by methylene blue

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The aim of this study was to propose the use of red light-emitting diode (LED) as an alternative light source for methylene blue (MB) photosensitizing effect in photodynamic therapy (PDT). Its effectiveness was tested against *Staphylococcus aureus* (ATCC 26923), *Escherichia coli* (ATCC 26922), *Candida albicans* (ATCC 90028) and *Artemia salina*. The maximum absorption of the LED lamps was at a wavelength of 663 nm, at intensities of 2, 4, 6 and 12 J.cm^{-2} for 10, 20, 30 and 60 min of exposure, respectively. Assays with and without LED exposure were carried out in plates containing MB at concentrations of 7 to 140.8 μM for microorganisms and 13.35 to 668.5 μM for microorganisms or microcrustaceans. The LED exposure induced more than 93.05%, 93.7% and 93.33% of growth inhibition for concentrations of 42.2 μM for *S. aureus* (D-value=12.05 min) and 35.2 μM for *E. coli* (D-value=11.51 min) and *C. albicans* (D-value=12.18 min), respectively after 20 min of exposure. LED exposure for 1 h increased the cytotoxic effect of MB against *A. salina* from 27% to 75%. Red LED is a promising light device for PDT that can effectively inhibit bacteria, yeast and microcrustacean growth.

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1. Introduction

Photodynamic therapy (PDT) has been successfully used for the treatment of several diseases involving abnormal cell growth such as cancer, rheumatoid arthritis, vitiligo, pathological myopia, age-related macular degeneration and arteriosclerosis (Levy 1995; Sternberg and Dolphin 1996; Sternberg *et al* 1998). The greatest advances have been in cancer treatment, but PDT has also shown effectiveness as an antibacterial and antifungal agent (Wainwright 1998; Phoenix *et al* 2003).

PDT is based on the interaction of visible light and a photosensitizer agent, which under photo-activation generates short-lived cytotoxic species *in situ*. Under excitation, the photosensitizer is converted from singlet to triplet state by an intersystem crossing process which, in turn, reacts with surrounding molecules to yield radical species and hydrogen peroxide – known as the type 1 mechanism, or transfers its energy to molecular oxygen ($^3O_2$) to produce singlet oxygen ($^1O_2$) – known as the type 2 mechanism. Both types of PDT result in localized cell death; however, the principal photodynamic mechanism has been shown to be the type 2 mechanism, which generates the highly toxic singlet oxygen (Weishaupt *et al* 1976).

Clinically, once the photosensitizer drug reaches its maximum level in the target tissue, a carefully regulated light dose is shone to activate the compound and start the photodynamic attack. For superficial skin diseases the light is applied directly to the affected tissue, while for internal diseases the light source is connected to an optical fibre catheter, which allows it to reach the target organ.

High-potency laser sources are preferable, but their high costs make PDT inaccessible for many institutions and therefore make the dissemination of this technology

Keywords. LED light; methylene blue; microorganism; photodynamic therapy

Abbreviations used: CFU, colony-forming unit; IC50, inhibition of 50% growth; LED, light-emitting diode; MB, methylene blue; MHB, Mullen–Hinton broth; %T, percentage of transmittance; PDT, photodynamic therapy

difficult in most countries. The low cost of lasers based on diode technology is very attractive and has been employed for PDT, although they are still expensive for poor nations (Machado 2000; Mang 2004). Recently, however, various alternative light sources have been researched to replace the laser in PDT. One of the most interesting of these is the light-emitting diode (LED) because of its low price. It is possible to find different colours of LED light in the market, with radiations covering almost all of the visible electromagnetic spectrum, including red light (650 nm region). This alternative, despite having a lower potency than laser, has several economic advantages and can be used to optimize the investigation of new compounds for PDT (Mang 2004).

The high cost of PDT in clinical application does not result only from the price of the equipment, but also from the high cost of the drugs used for PDT. Therefore, it is desirable to develop new medicines, whose properties should include high efficiency, low side-effects and reduced costs. Following these requirements, some organic dyes have been proposed as PDT candidates. Methylene blue (MB, structure shown in figure 1), a well-known dye with high light absorption at 665 nm, is effective in PDT, showing ability to generate $^1O_2$ and photodynamic activity for clinical applications against several diseases (Tardivo *et al* 2004, 2005). Even without light, MB has natural antifungal and antibacterial activity (Wainwright and Crossley 2002; Calzavara-Pinton *et al* 2005), whose toxic potential can be increased by light activation.

Bacteria and fungi are commonly used in preclinical studies for modelling cell growth. Another bioassay that may be useful for the investigation of new PDT compounds is the *in vitro* assay of lethality using brine shrimp (*Artemia salina*). This experiment is quick and inexpensive, and can be adapted for the evaluation of photodynamic activity. *Artemia salina* is a tiny crustacean used in many physiological and bioassay studies, including the general toxicity of polluted water, investigation of bioactive plant extracts, mycotoxins, dinoflagellate toxins, anaesthetics, toxicity of oil dispersants, di-n-alkyl phthalates and co-carcinogenic phorbol esters (Kinghorn *et al* 1977). The eggs of brine shrimp are readily available commercially at low cost and remain viable in their dry state for years. This bioassay has a good correlation with cytotoxic activity in some human solid tumours and with pesticide activity (McLaughlin *et al* 1998; Hostellmann 2001).

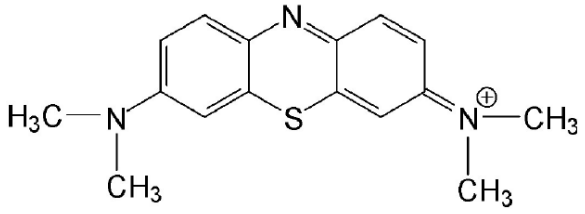


Figure 1. Structure of methylene blue.

Therefore, the aim of the present paper is to contribute to PDT development by researching alternative light sources using LED light and its effect on the photodynamic activity of MB in biological experiments. The investigation of the effectiveness of PDT with MB and a LED device was carried out on cultures of *Staphylococcus aureus*, *Escherichia coli* (prokaryotic cells), *Candida albicans* and *A. salina* (eukaryotic cells).

2. Materials and methods

2.1 *Photosensitizer and light*

MB (Vetec, $C_{16}H_{18}ClN_3S{\cdot}3H_2O$, MW 373.90 g mol^{-1}) and all other reagents and solvents used were of analytical grade and used without purification. The MB stock solution of 2.67×10^{-2} mol l^{-1} was prepared in deionized water and then diluted by the appropriate volume to obtain the test solutions. The LED (EverLight Co., Taiwan, ROC) light system was constructed using 4 units (in series) that emitted red light, and their individual potency was determined using a handheld laser power meter (Edmund Optics Inc., Barrington, NJ, USA). The fluorescence emission spectrum was obtained in a SPEX Fluorolog2, model 1680. The absorption spectra were obtained in a Cary 50 spectrophotometer (Varian Inc., Palo Alto, CA, USA).

2.2 *Bacterial strains and culture conditions*

Staphylococcus aureus (ATCC 26923), *E. coli* (ATCC 26922) and *C. albicans* (ATCC 90028) were used in the experiments. The bacteria were kept in tryptic casein agar and the yeast in Sabouraud dextrose agar in stock culture plates in a fridge at –4°C. Before the experiments, the microorganisms were grown in Mullen–Hinton broth (MHB) at 37°C for 15 h, then harvested by centrifugation and suspended in 0.85% saline at a stock concentration of (1–2)10^8 colony-forming units (CFU)/ml.

2.3 *Assay with bacteria and yeast*

All experiments were carried out in two groups: one group exposed to the light and other kept in the dark. Two control groups were prepared: one by mixing 400 μl of inoculum + 100 μl of sterile saline (positive control), and the other by mixing 100 μl of MB at different concentrations + 400 μl of MHB without microorganisms (negative control). The test groups were prepared by adding 100 μl of MB solution in different concentrations to 400 μl of the calibrated inocula

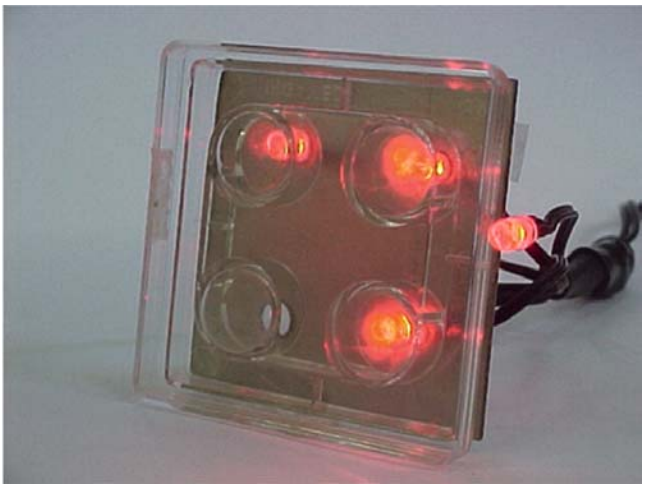


Figure 2. LED system with 4 units connected to a 4-well plate.

in 4-well (15 mm diameter) sterile polystyrene plates, giving final concentrations varying from 7 to 140.8 μM. The light sources were placed vertically above the wells containing the samples (figure 2) and the plates were illuminated (one LED light for each well), under shaking at 25ºC for 30 min. After illumination, all the samples were transferred to tubes, diluted with MHB to a final volume of 3.5 ml and incubated at 37ºC. The growth rate was quantified by a spectrophotometer and the percentage of transmittance (%T) at 580 nm measured at 0, 2, 4 and 6 h until confluent growth of the positive control group was obtained. The results were expressed in %T as a function of turbidity resulting from microbial cell growth. At time 0, all test tubes were 100%T, and increased turbidity was therefore inversely proportional to the %T.

Inhibition of 50% (IC50) of the microbial cell growth for each microorganism was calculated from the exact correlation between MB concentration and the percentage of growth inhibition, obtained from the %T of each treatment in comparison with the %T of the positive control group, considering that 100%T indicated 0% cell growth (no turbidity), and 20%T indicated 100% cell growth, or no inhibition at all.

To calculate the killing rate and D-value (defined as the time in minutes required to reduce the population by 1 log-fold), a concentration of 35.2 μM MB lower than the IC50 determined from the previous experiment was used and the illumination time was varied from 10, 20 to 30 min. Immediately after light exposure, the cells were harvested and washed with saline by centrifugation and diluted 10, 100 and 1000 times with saline, and 100 μl of each dilution was then plated in tryptic casein agar, incubated for 24 h at 37ºC and the number of CFUs counted. All experiments were carried out in triplicate.

2.4 *Assays with* A. salina

The *A. salina* eggs were hatched in saline water (NaCl 3.8 g l^{-1}). After 48 h at room temperature, 20 mature crustaceans were transferred to test-tubes containing 1.5 ml of aqueous MB solution and 1.5 ml of saline, with the final MB concentrations in the tubes being: 13.35, 67, 133.5, 343 and 668.5 μM. The samples were separated into two identical groups, with one group being illuminated (1 h) and the other group, including the control tubes without MB, being kept in the dark for 24 h. The numbers of live and dead crustaceans were then counted. All experiments were carried out in triplicate.

2.5 *Statistical analysis*

Comparison of the data was carried out using an ANOVA one-way test with the software Statistica® followed by a Tukey test, using the final %T value and number of CFUs.

The slopes of the killing curves were measured by regression analysis and D-values were obtained from the slope of the killing curve line.

3. Results

The constructed LED system (4 x LED units – *see* figure 2) proved to be very effective and easy to use. The emission spectrum of red LED light and the aqueous MB absorption spectrum are shown in figure 3. The maximum absorption band of the dye, centred at 665 nm, is almost coincident with the maximum light emission of the red LED (figure 3). The measured potency of each LED was around 5 mwatts at 663 nm. As the illuminated area used in this work was around 1.5 cm^2, the energy dose calculated for 30 min

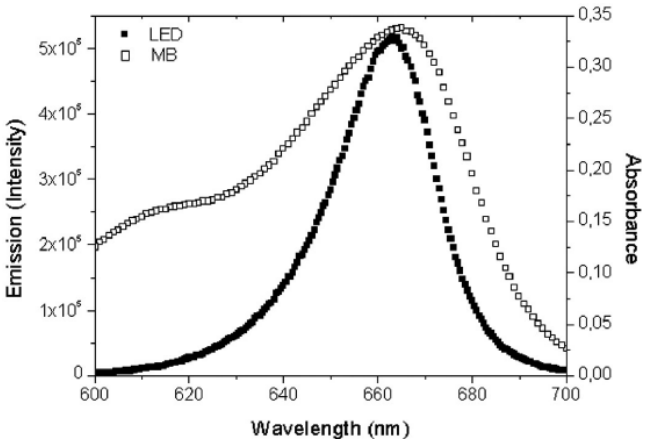


Figure 3. Spectrum of the red LED light source system (■) and the absorbance spectrum of the MB-water solution (□).

illumination at this wavelength was 6 J cm^{-2}; for 20 min it was 4 J cm^{-2} and for 10 min it was 2 J cm^{-2}.

In the microorganism assay (figure 4), confluent growth (approximately 20–40%T) was reached after 4 h of incubation for *S. aureus* and *E. coli*, and after 6 h for *C. albicans* in MHB (positive control group).

In a second experiment, MB was added to the cells under the same experimental conditions. The percentage growth rate of the microorganisms decreased proportionally as the MB concentration increased, as could be seen by the decrease in turbidity of the medium and the increase in %T values (figures 4A, B and C). The inhibition of 50% (IC50) of the microbial cell growth for each microorganism was 43.35 ± 6.75 μM for *S. aureus*, 56.40 ± 11.48 μM for *E. coli* and 63.20 ± 11.30 μM for *C. albicans*.

Then, after the IC50 for each microorganism with MB was determined, in a third experiment using the same experimental conditions and the same concentrations of MB as described above, the cultures were submitted to LED light for 30 min. When the LED light irradiated the cultures without MB, no effect was seen on the growth of any of the microorganisms. However, for the cultures treated with MB, after 30 min of light activation, significant growth inhibition could be seen through the unchanged %T values for all the microorganisms from the beginning to the end (4 or 6 h of incubation) of the assay (figures 4D, E and F).

The effect of exposure time to LED irradiation was also investigated for the cultures with MB. A concentration lower than the IC50 was chosen because of the remarkable photosensitizing effect seen at lower concentrations for all the microorganisms tested. The cultures were illuminated for 10, 20 and 30 min (figure 5).

It can be seen that inhibition is dependent on exposure time to the LED. The response to MB and LED light calculated by the killing-rate curve and D-value was similar for all microorganisms.

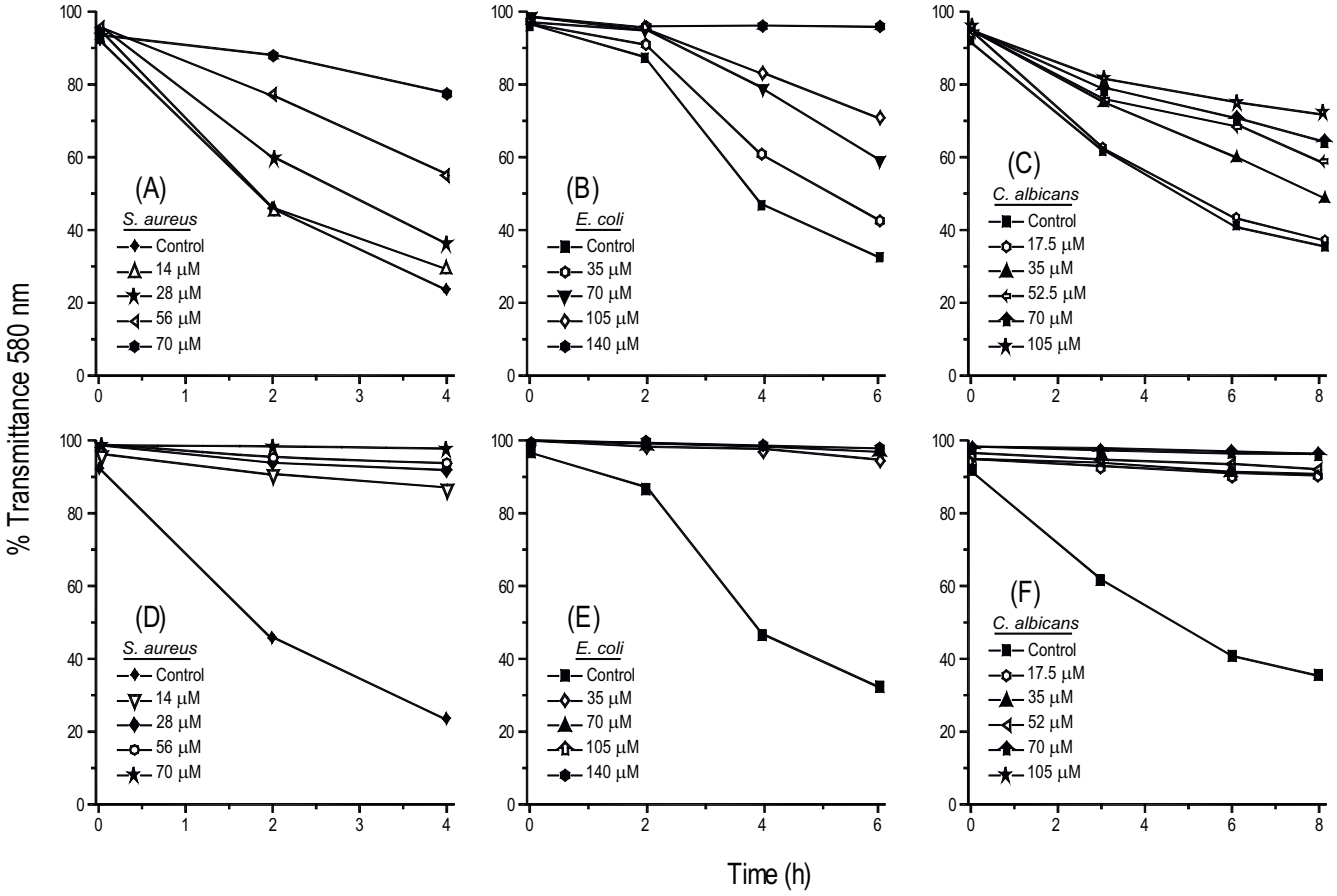
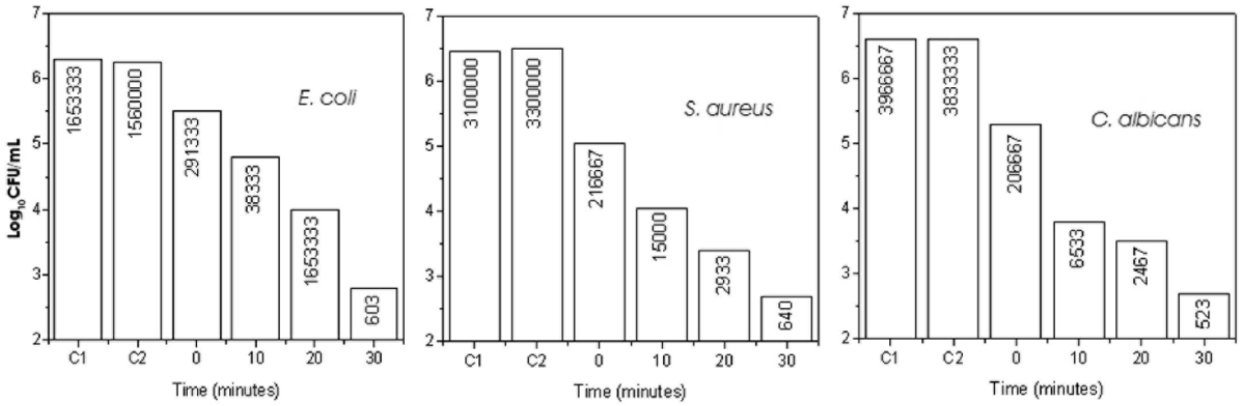


Figure 4. Effect of LED light and different concentrations of MB on microbial cell growth. The *S. aureus, E. coli* and *C. albicans* cells were cultured with MB at various concentrations, and the growth rates of non-irradiated samples (**A**, **B** and **C**) and samples irradiated with LED light for 30 min (**D**, **E** and **F**) were measured by transmittance at 580 nm. The values are shown in %T but to calculate the IC50 the %T were transformed to % of growth inhibition using the control group as reference for 0% of inhibiton. The concentrations of MB used are shown in the figure. Each point represents the mean ± SD of triplicate measurements.



	E. coli	*S. aureus*	*C. albicans*
r^2	0.9872	0.9800	0.9287
$k(h^{-1})$	0.2001	0.1911	0.1891
D-value (minutes)	11.51	12.05	12.18

Figure 5. Effect of LED time exposure on the recovery of viable counts for *E. coli, S. aureus* and *C. albicans* in the presence of methylene blue (35.2 μM). The control groups C1 and C2 are microorganism cultures with and without LED exposure for 30 min, respectively. Time 0 is the time the microorganisms were cultured with 35.2 μM MB, and times 10, 20 and 30 min are the times the microorganism cultures were in the presence of MB and LED exposure. Kill rates (k) are derived from the recovery curve of time 0 to 30 min of LED exposure, and the D-values are calculated from the reduction of 1 log-cycle from the same curve. Each point represents the mean of triplicate measurements.

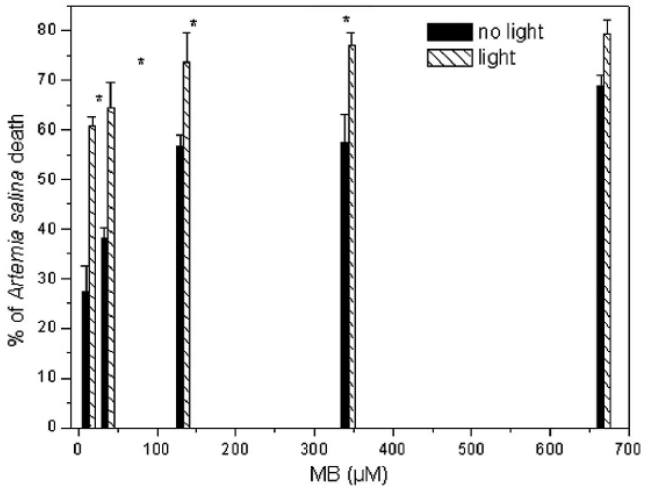


Figure 6. Effect of LED light and different concentrations of MB on *A. salina*. The cytotoxic test was carried out with the microcrustacean *A. salina*, which was cultured with MB at various concentrations, and the effect in non-irradiated samples and samples irradiated with LED light for 1 h was measured by the death percentage. The concentrations of MB used are shown in the figure. Each point represents the mean ± SD of triplicate measurements. (*) $P < 0.05$ compared to the control group.

Additionally, to demonstrate that the photoactivation effect of LED light on MB solution is not exclusive to microorganisms, the photodynamic effect on another live organism, the crustacean *A. salina*, was evaluated. The experiments with *A. salina* were modified for PDT assays and the results are shown in figure 6.

MB alone showed a toxic effect on the crustaceans similar to that observed for microorganisms in the non-exposed samples, and the combination of LED light on MB solution increased the death percentage of *Artemia*, confirming that the LED light was effective in PDT for both the microorganisms and the crustaceans.

4. Discussion

In this study, it was found that red LED light increased the inhibitory PDT effect of MB in prokaryotic and eukaryotic cells. The photosensitizing mechanism of the LED light on MB follows the same pattern as the majority of other light sources that generate singlet oxygen and act against a wide range of target cell molecules, including membrane lipids, cytoplasmic enzymes and nucleic acids (Wainwright 1998).

MB has no difficulty in crossing bacterial cell walls. Because of its cationic charge, it binds easily to the negative charge of the lipopolysaccharides of Gram-negative bacteria (Livermore 1990). Gram-positive bacteria have only capsular material and peptidoglycan outside of the cytoplasmic membrane, enabling MB to cross it easily as well (Zeina *et al* 2001). Its penetration into *C. albicans* cells seems to be more difficult, as studies carried out with cationic porphyrin have

shown that it can enter the cytoplasm only after membrane damage has been induced by light (Lambrechts *et al* 2005), or under the action of antifungal agents (Sud and Feingold 1981). In the present study, no difference was observed in the ability of the combination of MB and red LED light to inhibit and kill the three types of microorganism cells tested.

No photodynamic study has been carried out with the microcrustacean *A. salina* until now, but this model has been extensively used for investigating plant compound cytotoxicity, and a good relationship has been found between brine shrimp lethality and detection of antitumoral compounds in terrestrial plant extracts (McLaughlin *et al* 1998; Hostettmann 1991; Carballo *et al* 2002).

The IC50 of MB for the non-irradiated samples in this study increased in the following order: *S. aureus* <*E. coli* <*C. albicans* <*A. salina* (figures 4–6), and it was observed that increasing concentrations of MB resulted in proportionately increased effects in all the experiments.

Studies carried out by Zeina *et al* (2001) suggest that the source of the light is not important for PDT in cases where the wavelength covers the absorption maxima. For instance, Zeina *et al* did not observe a difference in photodynamic effect between sunlight and a projector light. Our results, using LED light, were comparable with those of studies using lasers, and partially support Zeina´s conclusion.

The results showed that LED light was as effective against *S. aureus, E. coli* and *C. albicans* as those of experiments using laser (Wilson and Mia 1993; Usacheva *et al* 2001) or an Exal light box (Wainwright *et al* 1997). Concentrations of 42 μM MB exposed to diode laser (665 nm) with a potency of 15 J cm^{-2} (Usacheva *et al* 2001) and 0.90 μM MB exposed to an Exal light box with a potency of 6.3 J cm^{-2} led to the total death of *S. aureus* (Wainwright *et al* 1997). In this experiment, MB concentrations of 7 and 14 μM exposed to red LED light (potency of 6 J cm^{-2}) for 30 min led to 79% and 84% inhibition, respectively, against *S. aureus.* For *E. coli*, a MB concentration of 180 μM exposed to diode laser (665 nm) with a potency of 40 J cm^{-2} (Usacheva *et al* 2001) and 90 μM MB exposed to an Exal light box (6.3 J cm^{-2}) (Wainwright *et al* 1997) led to total cell death. In the present study, a concentration of 35.2 μM MB exposed to LED light at intensities of 2 and 4 J cm^{-2} led to 88.8% and 93.7% inhibition, respectively, and at a concentration of 70.4 μM MB with 6 J cm^{-2} , induced 96% inhibition. Experiments on *C. albicans* using MB at 130 μM exposed to: (i) a laser light (685 nm) intensity of 28 J cm^{-2} induced 88.6% inhibition (Souza *et al* 2006) and (ii) a laser light (660 nm) of 2.04 J cm^{-2} induced 42% inhibition (Wilson and Mia 1993). In the present study, the LED light (6 J cm^{-2}) with MB at 70.4 μM induced 95.14% inhibition.

The experiments with *A. salina* using red LED light plus MB produced the same effects as those seen for the microorganisms (figure 6). For samples using a concentration of 13.35 μM of MB, the light increased deaths by 126%, while at a higher concentration (668.5 μM) the effect was only 15% greater than that on the non-illuminated cells. This result may be explained by the fact that MB alone, at high doses, already induces a high death percentage (Wainwright and Crossley 2002; Calzavara-Pinton *et al* 2005). Another possible explanation is that, at higher concentrations, MB may undergo a self-aggregation process in water media, thereby diminishing the singlet oxygen yield (Sternberg *et al* 1998). Furthermore, aggregated monomer and dimer forms of MB (whose peaks appear at 610 nm and 664 nm, respectively) can show photodynamic activity via type I PDT, which is less effective than type II PDT (Usacheva *et al* 2003; Panda and Chakraborty 1997; Borissevitch and Gandini 1998).

Since MB is known to be slightly toxic to normal cells at high concentrations, it is important to emphasize that increased toxicity occurs only when it is irradiated by the LED, hence this therapy will be of use mainly for local and topical applications. For usage by the intravenous route, it is important to know the exact site where the drug will accumulate in the body, and then expose that site to LED irradiation.

LED light appears to be a promising light source for PDT and is not just a monochromatic form of radiation, a special characteristic compared with lasers. This is advantageous for PDT because it increases the overlay of the spectrum of LED irradiation and the light absorption by MB (figure 3), which means that MB absorbs high doses of light from this source.

In the present study, the dose of the light energy from the LED was calculated by considering only the maximum peak of light absorption, without adding the smaller doses of light absorbed in wavelengths lower than 650 nm and higher than 670 nm (figure 3).

The investigation of the light exposure time indicates that the balance between MB concentration and illumination time can control the efficiency of PDT. For non-perfused tissues where the accumulation of MB is poor, the irradiation time should be prolonged to allow a similar therapeutic effect.

Exposure time is fundamental to PDT activity, as can be observed by the effects on cell death rate at different lengths of exposure.

Compared with other light sources, the LED appears to be a very good option for PDT because of its high efficacy and very low cost.

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References

Borissevitch I E and Gandini S C M 1998 Photophysical studies of excited-state characteristics of *meso*-tetrakis (4-*N*-methyl-pyridiniumyl) porphyrin bound to DNA; *J. Photochem. Photobiol. B: Biol.* **43** 112–120

Calzavara-Pinton P G, Venturini M and Sala R 2005 A comprehensive overview of photodynamic therapy in the treatment of superficial fungal infections of the skin; *J. Photochem. Photobiol. B: Biol.* **78** 1–6

Carballo J L, Hernández-Ida Z L, Pérez P and García-Grávalos M D 2002 A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products; *BMC Biotechnology* **472** 2–17

Hostettmann K 1991 Assays for bioactivity; in *Methods in plant biochemistry* (eds) P M Dey and J B Harbarne (London: Academic Press) pp 6–9

Kinghorn A D, Harjes K K and Doorenbos N J 1977 Screening procedure for phorbol esters using brine shrimp (*Artemia salina*) larvae; *J. Pharm. Sci.* **66** 1362–1363

Lambrechts S A G, Aalders M C G and Van Marle J 2005 Mechanistic study of the photodynamic inactivation of *Candida albicans* by a cationic porphyrin; *Antimicrob. Agents Chemother.* **49** 2026–2034

Levy J G 1995 Photodynamic therapy; *Trends Biotechnol.* **13** 14–18

Livermore D M 1990 Antibiotic replace and transport by bacteria; *Scand. J. Infect. Dis.– Suppl.* **74** 15–22

Machado A E H 2000 Photodynamic therapy: principles, potential of application and perspectives; *Química Nova* **23** 237–243

Mang T S 2004 Lasers and light sources for PDT: past, present and future; *Photodiagnosis Photodynamic Therapy* **1** 43–48

McLaughlin J L, Rogers L L and Anderson J E 1998 The use of biological assays to evaluate botanicals; *Drug Inf. J.* **32** 513–524

Panda K and Chakraborty A K 1997 Studies on the interaction of bacterial lipopolysaccharide with cationic dyes by absorbance and fluorescence spectroscopy; *J. Photochem. Photobiol. A: Chem.* **111** 157–162

Phoenix D A, Sayed Z, Hussain S, Harris F and Wainwright M 2003 The phototoxicity of phenothiazinium derivatives against *Escherichia coli* and *Staphylococcus aureus*; *FEMS Immunol. Med. Microbiol.* **39** 17–22

Souza S C, Junqueira J C, Balducci I, Koga-Ito C Y, Munin E and Jorge A O C 2006 Photosensitization of different *Candida* species by low power laser light; *J. Photochem. Photobiol. B: Biol.* **83** 34–38

Sternberg E D and Dolphin D 1996 Pyrrolic photosensitizers; *Curr. Medicinal Chem.* **3** 293–324

Sternberg E D, Dolphin D and Bruckner C 1998 Porphyrin-based photosensitizers for use in photodynamic therapy; *Tetrahedron* **54** 4151–4202

Sud I J and Feingold D S 1981 Heterogeneity of action mechanisms among antimycotic imidazoles; *Antimicrob. Agents Chemother.* **20** 71–74

Tardivo J P, Giglio A D, Paschoal L H C, Ito A S and Baptista M S 2004 Treatment of melanoma lesions using methylene blue and RL50 light source; *Photodiagnosis Photodynamic Therapy* **1** 345–346

Tardivo J P, Giglio A D, Oliveira C S, Gabrielli D S, Junqueira H C, Tada D B, Severino D, Turchiello R F and Baptista M S 2005 Methylene blue in photodynamic therapy: from basic mechanisms to clinical applications; *Photodiagnosis Photodynamic Therapy* **2** 1–17

Usacheva M N, Teichert M C and Biel M A 2001 Comparison of the methylene blue and toluidine blue photobactericidal efficacy against Gram-positive and Gram-negative microorganisms; *Lasers Surg. Med.* **29** 165–173

Usacheva M N, Teichert M C and Biel M A 2003 The role of the methylene blue and toluidine blue monomers and dimers in the photoinactivation of bacteria; *J. Photochem. Photobiol. B: Biol.* **71** 87–98

Wainwright M 1998 Photodynamic antimicrobial chemotherapy (PACT); *J. Antimicrob. Chemother.* **42** 13–28

Wainwright M and Crossley K B 2002 Methylene blue – a therapeutic dye for all seasons; *J. Chemother.* **14** 431–443

Wainwright M, Phoenix D A, Marland J, Wareing D R A and Bolton F J 1997 A study of photobactericidal activity in the phenothiazinium series; *FEMS Immunol. Med. Microbiol.* **19** 75–80

Weishaupt K R, Gomer C J and Dougherty T J 1976 Identification of singlet oxygen as the cytotoxic agent in photoinactivation of a murine tumor; *Cancer Res*. **36** 2326–2329

Wilson M and Mia N 1993 Sensitization of *Candida albicans* to killing by low-power laser light; *J. Oral Pathol. Med.* **22** 354–357

Zeina B, Greenman J, Purcell W M and Das B 2001 Killing of cutaneous microbial species by photodynamic therapy; *Br. J. Dermatol.* **144** 274–278

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