

Efficacy of Photogem[®] (Hematoporphyrin Derivative) as a Photoactivatable Larvicide against *Aedes aegypti* (Diptera: Culicidae) Larvae

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Abstract: Dengue vector is responsible for millions of deaths every year and has caused disastrous impacts on health systems. The continuous use of chemical insecticides, such as carbamates, pyrethroids and organophosphates generates resistant populations of the mosquito, therefore, new control methods must be investigated. The joint action of the population and guidelines for preventing the reproduction of the mosquito associated with the use of photoactivatable insecticides can be the alternative for the control of epidemiological outbreaks in affected regions. In this study, the photo-larvicidal activity of Photogem[®](PG), a derivative of hematoporphyrin, was investigated against 2nd–early 3rd instar of *Aedes aegypti* larvae (Diptera: Culicidae) under different lighting conditions (artificial lighting system and sunlight). The dynamics of PG accumulation was characterized by CLSM (confocal laser scanning microscopy) and total time PG elimination in solution was investigated by ultraviolet-visible spectrophotometry. The maximum photo-activity of PG was observed in 0.5 h under sunlight exposure which achieved 100% larval mortality. Fluorescence images showed a uniform distribution of PG along the digestive tract. PG remained stable in the sunlight for 48 h and in an artificial lighting system for longer periods, therefore, it can be used for the control of *Aedes aegypti* larvae as a new alternative to chemical insecticides. The method is considered environmentally friendly due to its rapid degradation in the presence of light. Further studies are required, so that the potential of the technique can be explored in real breeding places.

Key words: Photoactivatable larvicide, PG, hematoporphyrin derivative, *Aedes aegypti*, natural lighting, artificial lighting.

1. Introduction

Mosquitoes that belong to the *Culicidae* family are responsible for diseases transmission, such as malaria, yellow fever, chikungunya, Zika and dengue [1]. In particular, dengue fever is an infectious disease caused by arboviruses of the *Flavivirus* family, classified into four antigenically distinct serotypes, namely dengv-1, dengv-2, dengv-3 and dengv-4 [2]. Infections by one of the viral serotypes provide no cross-protective immunity, therefore, populations living in dengue endemic areas are subjected to exposure to the four virus serotypes [3]. Dengue fever is one of the major

worldwide public health problems that has affected humans aggressively and its symptoms comprehend high fever, severe headache, myalgia, arthralgia and prostration [4]. In many cases, dengue hemorrhagic fever (a more severe form of the disease) can be fatal, especially in patients with chronic diseases, such as diabetes mellitus, hypertension, and uremia [5].

The latest global estimate of dengue cases showed that 390 million people had been infected, which has high impacts on public health systems [6]. Dengue can cause a loss of up to 3.5 billion dollars a year in the Americas, which exceeds the costs estimated for other viral diseases, as HPV (human papillomavirus) and infections by rotavirus [7]. In Brazil, approximately 1,500,000 cases of dengue were notified in 2015,

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which represents a 300% increase in comparison to the same period in 2014 [8].

Aedes aegypti (Linnaeus, 1762) is currently also associated with the transmission of two viruses, namely chikungunya and Zika. The number of chikungunya cases increased 600% in 2015 in comparison to 2014, as approximately 18,000 cases were notified [8]. Infections by Zika virus reached around 18 Brazilian states in 2015 and caused population to be on alert due to their relationship with outbreaks of microcephaly, which hit mainly the Northeastern region.

The integrated mosquito control is still a major tool for combating dengue and other diseases caused by *Ae. aegypti*, since vaccines are not available yet. Most widely used insecticides are powerful chemicals of three different classes, namely pyrethroids, carbamates and organophosphates, all are neurotoxic action. Some restrictions have been imposed on their use, since they contaminate the environment and present risks to human health [9, 10]. Moreover, their prolonged use causes the appearance of resistant populations of *Ae. aegypti*, compromising their effectiveness [11]. In view of such problems, alternatives must be investigated, minimizing the selection of resistant organisms and providing none or minimal environment impacts.

The use of compounds activated by visible light, known as PS (photosensitizers) or PI (photoactivatable insecticides) presents a great potential as an alternative for the mosquito control. Their mechanism of action is characterized by the production of highly reactive oxygen species ($^1\text{O}_2$) responsible for the oxidation of a biologic target after activation by suitable light doses [12]. Porphyrins and their derivatives have shown high effectiveness against a broad spectrum of insect vectors. A major advantage of such compounds is that they can absorb most wavelengths emitted by visible light, which increases their efficiency in the production of reactive

oxygen species. Furthermore, the use of porphyrins as PI is comparably advantageous and safer than conventional chemicals, since they exert no or low environmental impacts and cause no mutagenic effects, which minimize the risk of photo-resistant cell clones [13].

The phototoxic effects of a meso-substituted porphyrin meso-tri (N-methylpyridyl), meso-mono (N-tetradecylpyridyl) porphine (C14) were tested against *Ae. aegypti* [14]. Larvae were irradiated with fluorescent lamps in a 400-800 nm spectral range at 4.0 mW/cm² light intensity. C14 porphyrin exhibited high potential as larvicide in this condition. Another study reported the effect of a novel porphyrin, namely meso-tri (N-methyl-pyridyl), mono (N-dodecyl-pyridyl) porphine (C12) associated with two specifically selected carriers (i) Eudragit® 100 (Evonik Industries, Essen, Germany), and (ii) cat food pellets (Friskies®). Both C12-porphyrin formulations were tested against *Anopheles gambiae* and *An. arabiensis* larvae under natural lighting conditions (30-110 mW/cm²) from 30 minutes to 3 hours. Such conditions were highly efficient in the photoinactivation of the organisms and led to approximately 100% mortality rate after lighting [15].

Due to the increasing number of diseases transmitted by vector *Ae. aegypti*, other porphyrins should be investigated as an alternative for mosquito control. In this study, we investigated the effectiveness of PG, a hematoporphyrin derivative—HpD, as a photoactivatable larvicide for the elimination of *Ae. aegypti*. In a previous study, it was demonstrated that the larvae in the 2nd instar had the capability of accumulating porphyrin and the main pathway of the PS introduction is via ingestion [16]. Photolarvicidal activity and photodegradation studies of PG were performed in the presence of sunlight (natural light source) and fluorescent lamps (artificial light source), and the dynamics of PG distribution was characterized in *Ae. aegypti* larvae.

2. Materials and Methods

2.1 Insects

An *Ae. aegypti* colony was established from eggs kindly donated by Bioagri laboratory (Mérieux Nutrisciences, Charqueada- SP, Brazil). The spawning was maintained at the Biophotonics Laboratory, at São Carlos Institute of Physics, in plastic containers with 4 liters of deionized water, at (27 ± 2) °C and under a photoperiod of 12 hours in the dark and 12 hours in light. The larvae were fed once a day with Alcon BASIC® MEP 200 Complex (Alcon, Camboriú-SC, Brasil) fish food, containing approximately 44% protein, 5% fat, 5% carbohydrates, minerals and vitamins.

2.2 Photosensitizer

Photogem®, a hematoporphyrin derivative obtained by Photogem LLC Company in Moscow, Russia and approved for clinical use by the Brazilian National Health Surveillance Agency (ANVISA) tested in this study. Five milligrams of Photogem were dissolved in 1 mL of distilled water for the obtaining of a 5 mg/mL stock solution, from which 20, 40 and 80 µg/mL experimental concentrations were prepared. These concentrations were established according to Nogueira [17].

2.3 Lighting Source

Two light sources, i.e. an artificial lighting system and natural light, were used for the irradiation of the larvae. The artificial lighting was performed by four fluorescent tubular lamps (Sylvania®, F-20 W T-12) at 1.2 to 2.3 mW/cm² irradiance. The samples were distributed and aligned 20 cm from the lamps for ensuring a uniform light distribution. Natural lighting samples were irradiated with sunlight in two different periods: (i) period 1, with 30 to 60 mW/cm² irradiance range and (ii) period 2, with 5 to 20 mW/cm² irradiance range. All experiments involving natural lighting were conducted in a standardized external area of the Biophotonics Laboratory. The irradiance

measurements were obtained by an Ocean Optics USB 2000 UV-VIS (Ocean Optics, USA) calibrated spectroradiometer.

2.4 Experimental Setup for Phototoxicity

Groups of 30 *Ae. aegypti* larvae (2nd–early 3rd) were distributed on transparent plastic trays containing 30 mL of dechlorinated water, at pH 7.0. All assays were performed in triplicate sub-samples, with 20, 40 and 80 µg/mL photosensitizer (PG) concentrations, as previously established. After exposure to PG, the larvae were fed with 100 mg of fish food and stored in the dark for a 12-hour incubation. The trays were then exposed to an artificial lighting system or the sunlight. The PG phototoxic potential was evaluated after 30 minutes, 2, 12, 24 and 48 h of exposure. The percentage of mortality was verified from the average of the three replicates. Two control groups, namely dark + PG + larvae and light + larvae were established.

2.5 Anatomic site of Photogem® Accumulation

Larvae samples were observed under a fluorescence confocal microscope (Zeiss LSM780, Jena, Germany) with appropriate optical filters (excitation 405 nm) for the analysis of the PG accumulation anatomic site in *Ae. aegypti*. All larvae were incubated overnight in water with 80 µg/mL PG (the highest concentration used in the phototoxicity assays) and then immersed in a container with PG-free water for 1 minute, allowing PG excess removal.

2.6 Photostability of Photogem®

Ultraviolet and visible absorption spectroscopies of the PG samples were obtained before and after exposure to light, and registered by a Cary UV-VIS spectrophotometer (Varian, Australia) at 20 µg/L in water, for the obtaining of the total PG degradation time in the presence of light. The PS solution was placed in a plastic cuvette (volume of 4 cm³) coated with transparent PVC film, avoiding sample

evaporation. The cuvette was irradiated by a system of fluorescent lamps and sunlight (30-60 mW/cm²). The absorbance of the samples was monitored at regular time intervals (0, 12, 24, 36 and 48 hours).

2.7 Statistic Treatment

Results are reported as arithmetic mean values \pm SD (standard deviation). Mortality data were analyzed by one-way ANOVA followed by Tukey's test at a 5% level of significance. All graphs were generated with OriginPro V. 9 (OriginLab Corp.).

3. Results

3.1 Phototoxicity Assays

Photogem® revealed no detectable dark-toxicity, an expected behavior of porphyrin derivatives and hematoporphyrin [13]. In contrast, the groups exposed to an artificial lighting system showed high mortality in the presence of all concentrations of the PS (Fig. 1). *Ae. aegypti* larvae subjected to an 80 μ g/mL PG concentration showed 100% mortality in the first 24 hours of exposure to artificial light. The analysis of the behavior of surviving larvae in the 48-hour treatment at low concentrations revealed a slower development in comparison to the control group. Moreover, swimming difficulty and low mobility of the larvae were observed when they were touched by histological needles. Larvae mortality rates at 20, 40 and 80 μ g/mL of PG and artificial lighting are shown in Fig. 1.

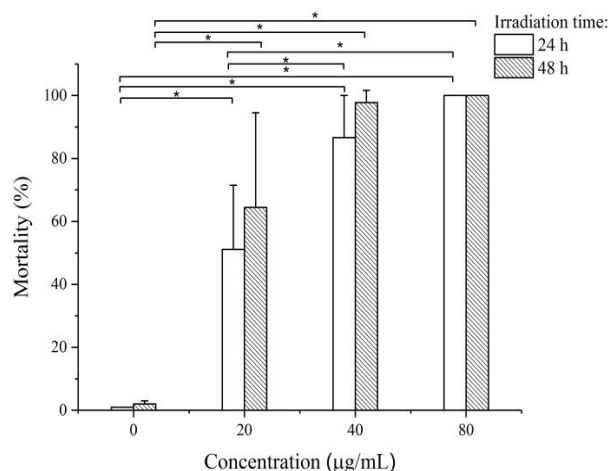


Fig. 1 Photolavical activity of Photogem® activated by an artificial lighting system.

Mortality of *Ae. aegypti* larvae incubated for 12 hours in the dark and irradiated with a system of fluorescent lamps at 1.2 to 2.3 mW/cm² irradiance. The larval mortality was monitored throughout the illumination period and recorded at 24 and 48 hours. Data are presented as mean \pm SD. Asterisk indicates significant differences ($p < 0.05$) between treatments.

Two natural lighting conditions (periods 1 and 2) were employed for the analysis of their influence on the mortality rates of larvae exposed to PG. Fig. 2a brings the high PG phototoxic effect in *Ae. aegypti* larvae under lighting conditions of period 1 (30 to 60 mW/cm²). Mortality rates of 71%, 91% and 100% were observed for PG at 20, 40 and 80 μ g/mL, respectively, after 30 minutes of sunlight exposure. After 2 hours of natural lighting, 100% mortality was detected for all PG concentrations. Fig. 2b shows the photoactivity of PG against *Ae. aegypti* larvae under low sunlight intensity (period 2). Almost 100% of the larvae had died in exposure to 20 and 40 μ g/mL PG after 24 hours of lighting conditions of period 2 (5 to 20 mW/cm²). Furthermore, the higher PG concentration (80 μ g/mL) induced total mortality after 24-hour irradiation. In this condition, the larvae mortality was PG dose-dependent. All treated groups showed statistical differences when compared to the control group.

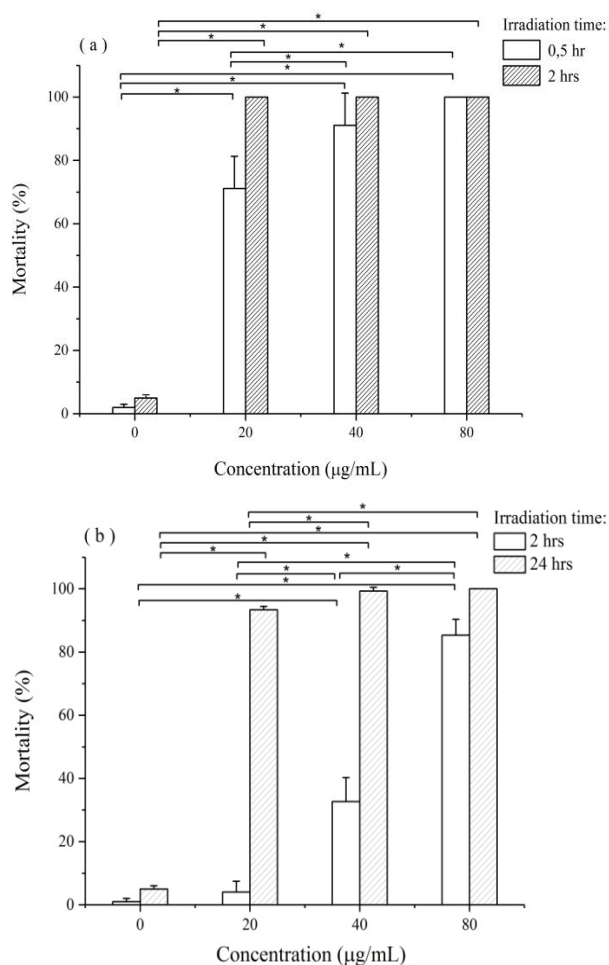


Fig. 2 Photolarvicidal activity of Photogem® activated by sunlight.

Fig. 2a shows mortality of *Ae. aegypti* larvae incubated for 12 hours in the dark and irradiated with sunlight at 30 to 60 mW/cm² irradiance range (period 1); Fig. 2b shows mortality of *Ae. aegypti* larvae incubated for 12 hours in the dark and irradiated with sunlight at 5 to 20 mW/cm² irradiance range (period 2). The larval mortality was monitored throughout the illumination period and recorded at 0.5 and 2 hours for period 1 and at 2, 12 and 24 hours for period 2. Data are presented as mean \pm SD. Asterisk indicates significant differences ($p < 0.05$) between treatments.

3.2 Confocal Microscopy

The fluorescence images show the distribution of the higher PG concentration (80 µg/mL) in larvae kept

in the dark for 12 hours (incubation period). Fig. 3 presents the microscopic image of the *Ae. aegypti* larva. The PG fluorescence (emission: 600-700 nm) was observed throughout the larvae digestive system (Fig. 3b). In all treatments, a greater accumulation of PG was observed in the region of the gastric caeca and midgut. According to the images (Fig. 3d, arrow), PG is also distributed outside the lumen and reached other midgut compartments. Such red fluorescence did not appear in the control.

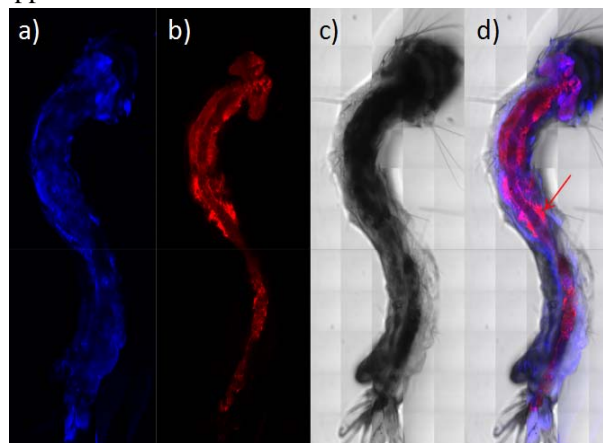


Fig. 3 Fluorescence images of *Ae. aegypti* larvae.

Fig. 3a shows larva fluorescence (emission: 500-600 nm); Fig. 3b shows PG fluorescence (emission: 600-700 nm) distributed throughout the intestines of the larva; Fig. 3c shows bright field image; Fig. 3d shows image overlay with the fluorescence of PG and larva. The red arrow (in Fig. 3c) represents the PG fluorescence outside the lumen. Scale bars: 500 µm.

3.3 Photostability of Photogem®

The photodegradation profile of PG under natural and artificial radiation was characterized by decay time of five typical absorption bands (Fig. 4) [18]. The largest absorption band around 370 nm corresponds to a Soret band, while the other four lower bands correspond to Q-bands, with absorption at 507, 540, 570, 620 nm. During the irradiation time, all bands decreased and a new absorption band was formed in

the 640 nm region in function of the increasing irradiation time (Figs. 4a and 4b). The new band (640 nm) remained visible for 48 hours under irradiation by artificial lighting, whereas under irradiation by sunlight, the same band was totally consumed in 48 hours.

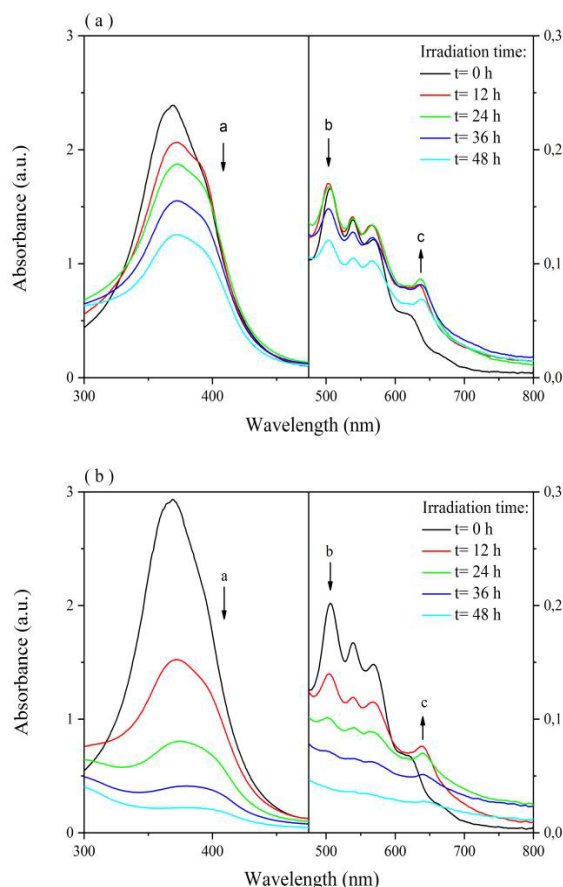


Fig. 4 Optical absorption spectra of Photogem®.

Fig. 4a shows photodegradation in an artificial lighting system (1.2 to 2.3 mW/cm² fluence rate); Fig. 4b shows photodegradation in sunlight (30 to 60 mW/cm² irradiance range). Photodegradation of Photogem® in water at 0-48 hour irradiation time. Arrows represent the decay of the Soret band and Q-bands (Figs. 4a and 4b, respectively), accompanied by the appearance of a photoproduct at 640 nm (arrow c).

4. Discussion

The control of *Ae. aegypti* is often based on the

elimination of the vector aquatic phase through the application of chemical larvicides in the breeding place. However, the effectiveness of such products is uncertain due to their ability to select resistant populations [19]. An approach that complements the *Ae. aegypti* control strategies is the release of genetically modified mosquitoes in the environment by the SIT (Sterile Insect Technique) [20]. It consists of the release of sterile males into the environment for copulation with natural females, which results in non-viable eggs. As this is a new strategy, the environmental effects caused by such transgenic insects cannot be predicted, which reinforces the need for investigations into alternative methods for the control of *Ae. aegypti*.

PG, which has been widely used in photodynamic therapy for the treatment of cancer and in photodynamic inactivation of pathogenic microorganisms (fungi and bacteria) [21-24] has shown high potential for the elimination of *Ae. aegypti* larvae. Our results show an almost complete mortality was observed 24 hours after light exposure by an artificial lighting system and natural illumination (periods 1 and 2) with porphyrin at 80 µg/mL. All concentrations induced significant reductions in the larval survival and the minimum mortality was observed 48 h after artificial light treatment with 20 µg/mL PG (64% mortality).

Different conditions of illumination were fundamental for the evaluation of the PG effectiveness under lighting despicable conditions. The increase in the photodynamic activity of porphyrins is directly related to some parameters, such as concentration and light dose, since mortality increases proportionally to those factors (this behavior was also observed in the case of PG). Our results clearly indicate that under low lighting conditions (artificial lighting system and period 2), PG is effective against *Ae. aegypti* larvae, at irradiance values range between 1 and 20 mW/cm². Such a characteristic highlights the benefits of PG application in breeding places located in environments

with artificial light sources and household environments that receive lower natural illumination.

The association of peculiar carriers with PS, such as powdered pet food, can help the intake and accumulation of PS in the intestinal tissues of larvae, improving the palatability of the carrier-PS complex [15]. Previous studies from our group have shown that the efficiency of carriers depends largely on the particles shape and size. The association of PG with small carriers (average diameter $\leq 50\mu\text{m}$) promoted a better photodynamic response in the *Ae. aegypti* larvae, while larger particles (average diameter $\geq 150\mu\text{m}$) reduced PG efficiency in 50% (data not shown). This difference in mortality may be explained by the larvae indiscriminate quantitative ingestion, indicating that feeding is oriented mainly by the dimensions of the particles [1].

Fluorescence images presented a uniform distribution of PG along the digestive tract (gastric caeca, anterior and posterior midgut). The arrow in Fig. 3d shows PG exceeded the peritrophic barrier and was distributed in ectoperitrophic space. The same distribution dynamics was observed in *Ae. aegypti* larvae incubated in C14 porphyrin [14]. Due to their amphiphilic character, porphyrin molecules cross lipid barriers efficiently and act on different intracellular structures, as mitochondria and endoplasmic reticulum [13].

The strategy based on photosensitive compounds can be considered a safe and low-cost alternative for the elimination of *Ae. aegypti* larvae, since sunlight is one of the fundamental keys to its success. Moreover, sunlight accelerates the degradation process of PS and avoids compound permanence in the environment. In this study, the PG photodegradation occurs markedly by sunlight within 48 hours. In artificial lighting, the photodegradation is slower and the formed photoproduct is not consumed in 48 hours (Fig. 4).

Such PS is known to yield low environmental risk when applied at artificial breeding places. However, their use must be reconsidered for applications at

natural breeding places, such as lakes, rivers, streams and wetlands (unusual for *Ae. aegypti*, but very suitable for other species of vectors). Due to the lack of research in this area, further studies are required for the characterization of the toxicity degree of those substances in aquatic organisms, such as fish, microcrustaceans and algae.

5. Conclusions

PG presented phototoxicity to 2nd to early 3th instar of *Ae. aegypti* larvae. Photolarvicidal activity was observed under typical breeding place conditions with both artificial and natural lighting. Fluorescence images revealed PG accumulation in the gastric caeca, midgut and outside the lumen, and reached other midgut compartments. Regarding PG photostability, the results show a fast photodegradation process, which promotes the elimination of the residual PG from the environment. PG can be a good larvicide alternative for the control of *Ae. aegypti* larvae with no long residual effect, posing relative environmental safety.

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