ORIGINAL PAPER

Assessment of *Leishmania major* and *Leishmania braziliensis* promastigote viability after photodynamic treatment with aluminum phthalocyanine tetrasulfonate (AlPcS$_4$)

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Abstract: Cutaneous leishmaniasis is an infectious disease caused by protozoans of the genus *Leishmania*, which is transmitted through the bite of hematophagous insects of the genus *Lutzomyia*. This study aimed at testing *in vitro* the phototoxic effect of aluminum phthalocyanine tetrasulfonate (AlPcS$_4$) on the viability of *Leishmania major* and *Leishmania braziliensis*. Stationary phase promastigote forms were treated with AlPcS$_4$ at 1.0 μM and 10.0 μM and incubated for one hour. Then 659 nm laser was applied at 5 and 10 J/cm². Parasite viability was determined by differential count using the trypan blue dye exclusion method and by monitoring growth curves for nine days. Trypan blue exclusion assay showed a significant reduction of viable parasites compared to controls, *L. major* seemed more sensitive to the toxic effects of AlPcS$_4$ in the dark. The most effective photodynamic therapy (PDT) was obtained with AlPcS$_4$ at 10.0 μM and 10 J/cm² whereas *L. braziliensis* showed the highest mortality rate after treatment.

Key words: cutaneous leishmaniasis, antiparasitic agents, *Leishmania major*, *Leishmania braziliensis*, AlPcS$_4$, photodynamic therapy.

INTRODUCTION

American cutaneous leishmaniasis (ACL) is an infectious zoonotic disease caused by a protozoan of the *Leishmania* genus, transmitted through the bite of infected phlebotomine sand flies belonging to the *Lutzomyia* genus (1-3).

Cutaneous leishmaniasis is endemic in more than 70 countries worldwide and is a public health threat on four continents (the Americas, Europe, Africa, and Asia). The World Health Organization (WHO) considers ACL as one of the six main infectious diseases and one of the most common public health problems in the Americas, because of its high incidence, wide geographic distribution, and ability to produce deformities (3). From 1985 to 2005, in Brazil, an annual average of 28,568 autochthonous cases was recorded. In the 1980's, ACL was recorded in 19 states, and in 2003, autochthony was confirmed in all Brazilian states (4). According to the Center of Epidemiological Surveillance, 432 cases of leishmaniasis were registered in São Paulo state in 2009, with a further 162 cases in the first half of 2010 (5).

ACL is characterized in its cutaneous form by a single or multiple painless ulcerated lesions, mainly on exposed parts of the body including the face and limbs (3).

Conventional treatment is with pentavalent antimonials, first-line drugs, for at least 20 days. Antimony is indicated for treatment of all forms of cutaneous leishmaniasis, although mucosal forms require more care and may have slower response times and a higher relapse rate (6). The patient must be monitored for side effects; also, pentavalent antimonials cannot be administered to pregnant women or patients

with renal or hepatic failure, cardiac arrhythmia, and Chagas' disease (3). Because conventional aggressive chemotherapy treatments are often unsatisfactory and produce undesirable side effects, the development of alternative treatments is of great importance.

Photodynamic therapy (PDT) has potential application in various areas of medicine, such as against cancer, skin diseases, and antimicrobial activity. PDT is currently being studied as an alternative treatment for ACL (7-17). Castano *et al.* (9-11) conducted an extensive review to clarify how sensitizers interact with target cells as reactive oxygen species are formed, how they are able to destroy tumor cells, and the mechanisms by which cell death may be triggered to help understand the processes caused by photodynamic therapy.

The effects of PDT in the *in vitro* and *in vivo* treatment of cutaneous leishmaniasis have been investigated. Several photosensitizers have been tested at different concentrations and different laser parameters, such as application time, wavelength, etc. Phthalocyanines, second generation photosensitizers for photodynamic therapy, such as zinc, chloroaluminum phthalocyanine and chloroaluminum phthalocyanine tetrasulfonate ($AlPcS_4$) have been studied and identified as promising agents (15, 18-20). $AlPcS_4$ displays low toxicity, *in vivo* and *in vitro* studies, without high irradiation and more photodynamic-dependent cytotoxicity and anti-tumor effect in the red area of the spectrum (> 660 nm) where tissue penetration is high, showing considerable promise as a photosensitizer for PDT (19). The few clinical reports using PDT for treatment of cutaneous leishmaniasis have also shown promising results. Patients treated with ALA-PDT achieved a greater than 90% cure rate without evidence of recurrence (21, 22). Side effects from PDT observed in humans are transient burning sensation during illumination and subsequent hyperpigmentation (21, 23). PDT is less aggressive than conventional treatments which sometimes require hospitalization for patient monitoring, pushing up treatment costs. Also, the esthetic effects of PDT are superior to conventional treatments (21, 22).

In light of the promising findings using PDT to treat leishmaniasis, this study aimed to evaluate the phototoxic effect on viability and growth for *Leishmania major* and *Leishmania braziliensis* promastigotes treated with chloroaluminum phthalocyanine tetrasulfonate as the photosensitizer.

MATERIALS AND METHODS

Parasites

Leishmania major (MRHO/SU/1959/P) and *Leishmania braziliensis* (MHOM/BR/96/LSC96-H3) were cultured in M199 medium and Schneider medium, respectively, supplemented with 10% heat-inactivated FBS, 2.5 µg/mL of hemin at 26°C.

Effect of $AlPcS_4$ on *L. major* and *L. braziliensis* Promastigotes

Tests were performed with stationary phase promastigotes and all three independent assays were performed in triplicate. The photosensitizer chloroaluminum phthalocyanine tetrasulfonate ($AlPcS_4$) (Porphyrin Products Inc., USA) was previously prepared in PBS at 1 mM and further diluted in the test concentrations. The experimental groups were divided as follows:

→ **Group 1: controls**
- Untreated promastigotes maintained under regular culture.
- PS 1.0 µM: promastigotes incubated with the photosensitizer at 1.0 µM without irradiation.
- PS 10.0 µM: promastigotes incubated with the photosensitizer at 1.0 µM without irradiation.

→ **Group 2: promastigotes irradiated with energy densities of 5 J/cm²**
- Light control: promastigotes irradiated without photosensitizer.
- PDT 1.0 µM: promastigotes incubated with 1.0 µM of $AlPcS_4$ and irradiated.
- PDT 10.0 µM: promastigotes incubated with 10.0 µM of $AlPcS_4$ and irradiated.

→ **Group 3: promastigotes irradiated with energy densities of 10 J/cm²**
- Light control: promastigotes irradiated without photosensitizer.
- PDT 1.0 µM: promastigotes incubated with 1.0 µM of $AlPcS_4$ and irradiated.
- PDT 10.0 µM: promastigotes incubated with 10.0 µM of $AlPcS_4$ and irradiated.

The promastigotes, 1 x 10^6 parasites/well, were distributed in 24-well cell culture plates. For the tests, parasites were incubated with $AlPcS_4$ at 1.0 µM and 10.0 µM for 60 minutes at 26°C. The contents of each well were then centrifuged and

sediment resuspended in fresh medium. Cells were then irradiated with a near infrared (λ 659 nm, 40 mW) GaAlAs laser diode semiconductor (Kondortech Equipamentos Odontológicos Ltda, Brazil). Equipment calibration was measured by an Espectra Pro 2500i spectrometer (PiAction, USA).

Cell viability was determined by differential count of dead and live parasites using the trypan blue dye exclusion method (15). Parasite survival after treatment was monitored by growth curve. Growth was microscopically determined by daily counts of parasite numbers in a hemocytometer for nine days. Results correspond to three independent assays performed in triplicate. Inhibition of parasite growth was determined by comparison to untreated controls.

Statistical Analysis

Data were analyzed using ANOVA (BioStat 5.0® program, Brazil) and results were considered significant when $p < 0.05$.

RESULTS

Phototoxic Effect of AlPcS$_4$ in *L. major* and *L. braziliensis* Promastigotes

There were no differences in cell viability between *L. major* promastigotes treated with photosensitizer without irradiation and submitted to PDT, except for the highest photosensitizer concentration and highest irradiation level 10 J/cm². The viability of *L. major* promastigotes treated only with photosensitizer statistically differed from the control group (Figure 1 – A), thus indicating a toxic effect from the photosensitizer. The opposite was observed in *L. braziliensis* where the groups treated with photosensitizer showed no significant difference to controls (Figure 1 – B). These results suggest that AlPcS$_4$ is more toxic to *L. major* than to *L. braziliensis*. The highest percentage of deaths were observed (about 99%) in *L. braziliensis* promastigotes, with the higher photosensitizer concentration of 10.0 µM and irradiation of 10 J/cm² (Figure 1 – B).

Ours results show a significant reduction in parasite survival by the trypan blue dye exclusion method ($p < 0.01$) compared to controls for groups receiving photodynamic therapy in both *Leishmania* species (Figure 1 – A and B) except for *L. braziliensis* treated with 1.0 µM and 5 J/cm² (Figure 1 – B). Promastigotes irradiated without phthalocyanine showed no significant difference
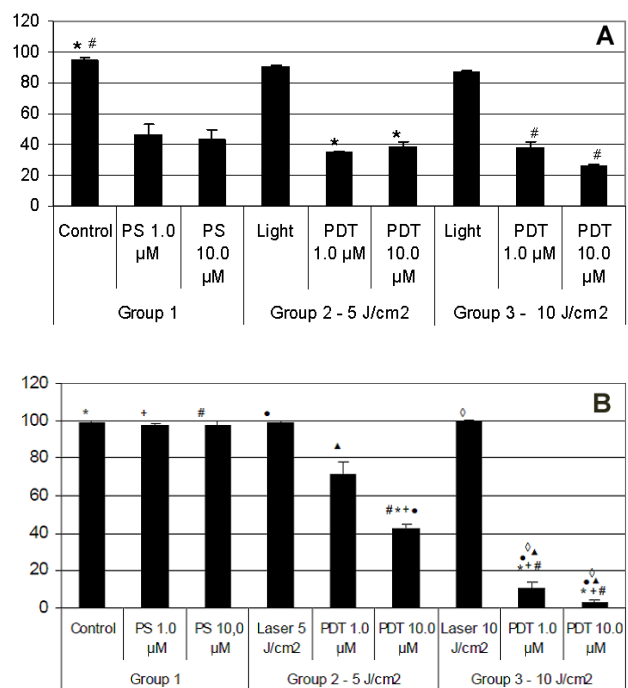

Figure 1. Promastigote viability analyzed by the trypan blue dye exclusion method after photodynamic therapy with AlPcS$_4$. (**A**) *Leishmania major*, (**B**) *Leishmania braziliensis*. The same symbols represent statistical differences between groups at a significance level of $p > 0.05$ to *Leishmania major* and $p > 0.01$ to *Leishmania braziliensis*.

to their control groups (Figure 1 – A and B) indicating that the laser energy densities tested do not reduce promastigote viability.

Survival of Promastigotes in Culture after PDT

Analysis of *L. major* growth curves (Figure 2 – A and B) shows that there were no significant differences in growth for the control group and groups treated with both laser energy densities (Table 1). A significant difference in parasite growth ($p < 0.01$) was observed between the control group and the groups which received only AlPcS$_4$ in concentrations of 1.0 µM and 10.0 µM. This was expected because parasite death was seen after incubation of promastigotes with phthalocyanine only (Table 1).

Parasite growth was significantly different ($p < 0.01$) between the control group and groups treated with 1.0 µM and 10.0 µM AlPcS$_4$ photodynamic therapy at both energy densities (5 J/cm² and 10 J/cm²). Growth curves for parasites treated with phthalocyanine and those submitted
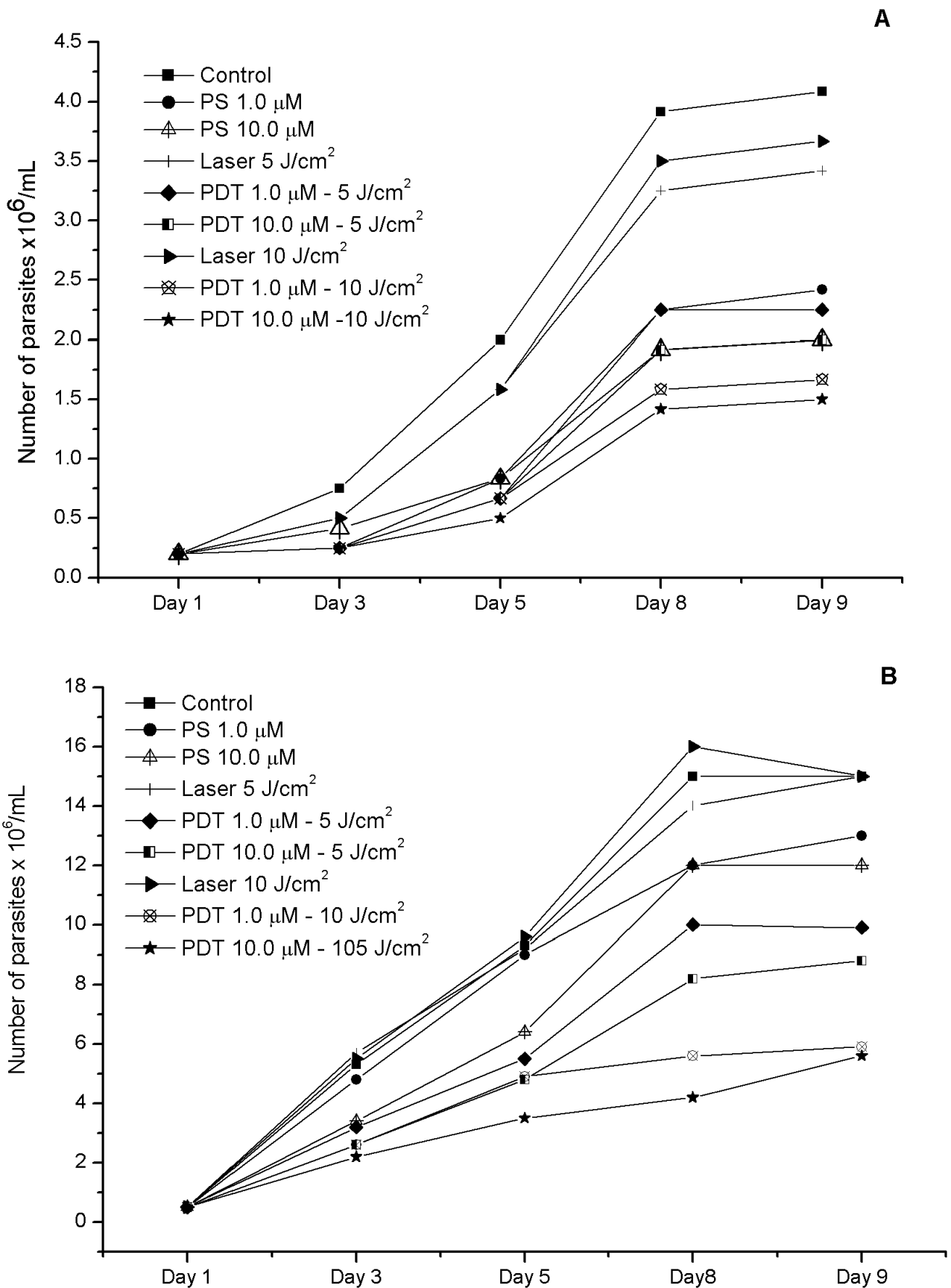
Figure 2. Growth curves for promastigotes after photodynamic treatment. (**A**) Growth curves for *Leishmania major* after PDT with AlPcS$_4$ and laser at 5 J/cm^2 and after PDT with laser at 10 J/cm^2. (**B**) Growth curves for *Leishmania braziliensis* after PDT with AlPcS$_4$ and Laser at 5 J/cm^2 and after PDT with AlPcS$_4$ and laser at 10 J/cm^2.

to PDT with 5 J/cm² were similar, suggesting that the tested AlPcS$_4$ doses are toxic to *L. major*. The most promising results were obtained with 10.0 µM phthalocyanine and 10 J/cm² irradiation.

For *L. braziliensis* (Figure 2 – B) no significant differences were observed between promastigote growth in the control group and the groups treated with laser without phthalocyanine. However, for *L. major* a significant difference was observed ($p < 0.05$) in promastigote growth over nine days compared to the control group and those which received AlPcS$_4$ treatment without

laser irradiation ($p < 0.05$). This corroborated the viability data, where the tested concentrations of AlPcS$_4$ were not toxic to *L. braziliensis* promastigotes (Table 1).

The lowest survival rates were observed when promastigotes were irradiated with 10 J/cm² at both photosensitizer concentrations, suggesting that energy dose is important for *L. braziliensis* promastigotes where even at the lowest photosensitizer concentration, the photo-antimicrobial effect was more intense than in *L. major*.

Table 1. Comparative results for *Leishmania major* and *Leishmania braziliensis* promastigotes growth after PDT with AlPcS$_4$

Treatments			Number of parasites x 10⁶/mL	
			L. major	*L. braziliensis*
Group 1	Untreated cells		4.1 ± 0.4*	15 ± 2.2*
	PS	1.0 µM	2.4 ± 0.3†	13 ± 3.5†
		10.0 µM	2.0 ± 0.3#	12 ± 3.5#
Group 2	5 J/cm²	Light	3.4 ± 0.6§	15 ± 1.8§
		1.0 µM	2.3 ± 0.3	9.9 ± 1.9*†#§+
		10.0 µM	2.0 ± 0.6*†#§	8.8 ± 1.5*†#§+
Group 3	10 J/cm²	Light	3.7 ± 0.6+	15 ± 4.8+
		1.0 µM	1.7 ± 0.1	5.9 ± 2.2*†#§+
		10.0 µM	1.5 ± 0.3*†#§+	5.6 ± 1.7*†#§+

Data represent the number of parasites after nine days of cultivation. The same symbols represent statistical differences between groups *$p < 0.05$ vs control; † $p < 0.05$ vs PS 1.0 µM; # $p < 0.05$ vs PS 10.0 µM; § $p < 0.05$ vs laser at 5 J/cm²; and + $p < 0.05$ vs laser at 10 J/cm².

DISCUSSION

This work demonstrated the phototoxic effect of chloroaluminum phthalocyanine tetrasulfonate (AlPcS$_4$) on *Leishmania major* and *Leishmania braziliensis* promastigotes. Similar results were demonstrated by Escobar *et al.* (24) with *L. panamensis* and *L. chagasi* using aluminum phthalocyanine chloride zinc phthalocyanine as the photosensitizer to reduce cell viability and inhibit growth *in vitro*. Aluminum phthalocyanine chloride at 0 to 10 µg/mL was also phototoxic to intracellular and extracellular forms of *Leishmania amazonensis* when irradiated with 3.6 J/cm² with no effects on non-irradiated parasites or macrophages (15).

Phototoxic effects against promastigotes

depend on factors such as phthalocyanine type, concentration, and charge as well as *Leishmania* species (25). In this study, *L. major* was more sensitive to the toxic effects of AlPcS$_4$. Even in the dark, *L. major* viability decreased, whereas no cytotoxicity was observed in *L. braziliensis* without illumination.

Phthalocyanine type and concentration, and laser strength (J/cm²) are also important factors which require consideration in antimicrobial photochemotherapy. Some molecules do not exhibit the desired phototoxic effect as demonstrated when zinc phthalocyanine stabilized in sol-gel TiO$_2$ was not phototoxic to *L. chagasi* or *L. panamensis* promastigotes (26).

Studies by Akilov *et al.* (25) suggest that molecular charges and structure are important

efficiency factors in PDT against *Leishmania* because cationic benzophenoxazine analogs were more effective than anionic photosensitizers. Also, the same compound can exhibit different degrees of toxicity depending on species tested. The same effect was observed in classical antitumor PDT, where the effectiveness of therapy was related to appropriate photosensitizing, tumor type, photosensitizer concentration, and irradiation (9-11).

Although no studies were found on use of AlPcS$_4$ as photosensitizer in *Leishmania* PDT, anti-parasitic mechanisms are still unknown. In a study using PDT with AlPcS$_4$ for *T. foetus* amitochondrial protozoa, the type of cell death induced by PDT was not necrosis and the authors suggested it may have been apoptotic or autophagic cell death (27). In a previous work, a MTT assay showed that PDT with 10.0 μM of AlPcS4 and 5 and 10 J/cm^2 reduced *L. major* mitochondrial activity. It is therefore possible that cell death in *Leishmania* is related to apoptosis mediated by photosensitizing action on mitochondria. Subcellular AlPcS$_4$ fluorescence has been correlated with granules predominantly located at one cytoplasm site in the vicinity of the nucleus, with an initial increase in fluorescence intensity followed by photobleaching (28, 29). In Hep-2 cells, PDT with AlPcS$_4$ led to significant alterations in mitochondria, causing potential membrane loss, alteration in cardiolipin distribution, and cell death, thereby suggesting an initially faster process of the intrinsic death pathway, considerably decreasing cell viability in a short time (29, 30). For carbaporphyrin ketals used in *Leishmania tarontelae*, the presence of reactive oxygen species, such as singlet oxygen and superoxide radicals, was associated with superoxide oxidation of the probe in the mitochondria with an increase in compound concentration (31).

In assays with other photosensitizers such as 3.7-bis (di-n-butylamino) phenothiazin-5-ium bromide PPA904, fluorescence was observed immediately after treatment application and parasite death occurred in the initial minutes of treatment; after that parasite load remained unchanged (32, 33). Topically applied PPA904 cream in the ears of mice to treat cutaneous leishmaniasis was irradiated with a wavelength of 665 ± 15 nm at 50 J/cm^2 (50 mW/cm^2) and decreased parasite load (32).

Ours results are consistent with other studies showing that PDT alone was not sufficient to eradicate parasites (34). We observed reduced cell viability, but some parasites remained alive and were able to grow.

Changes in photosensitizer delivery system might improve phototoxic action. Acenaphthoporphyirins combined with artificial liposomes of dimyristoyl phosphatidylcholine (DMPC) and artificial liposomes from a mixture of DMPC, cholesterol, and distearoyl phosphatidylglycerol (DSPG) were able to carry porphyrins to *Leishmania* tarentolae promastigotes and liposome associated compounds were effective against *in vitro Leishmania panamensis* amastigotes, but zinc phthalocyanine stabilized in TiO$_2$ sol-gel was not phototoxic to *L. chagasi* or *L. panamensis* promastigotes (26, 34).

Experiments with application of aminolevulinic acid (ALA) irradiated at 635 nm and 50 J/cm^2 efficiently eradicated the parasite and initiated a rigorous inflammatory response (21). However, phototoxicity in this case is more associated to a macrophage death triggered response (21).

The results presented in this study reinforce the possible use of PDT in the treatment of American cutaneous leishmaniasis. Despite a favorable result, AlPcS$_4$ has the limitation of being toxic to *L. major* promastigotes without irradiation at the tested concentrations. An ideal compound would only damage the cell after interaction with light. An aspect that should be considered in PDT for ACL treatment is absence of resistance, as aspects of the host-parasite relationship in *Leishmania* spp. can be neutralized by photodynamic antimicrobial treatments (35). These findings suggest that it is valid to test and develop new photosensitizers to determine whether they are directly effective against amastigotes and other *Leishmania* species, and to select those with greatest therapeutic potential for use in treating cutaneous leishmaniasis. PDT tests with *in vivo* models should be conducted to better understand the mechanisms and therapeutic potential of PDT in treating cutaneous leishmaniasis patients.

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SUBMISSION STATUS

CONFLICTS OF INTEREST

There are no conflicts of interest.

FINANCIAL SOURCE

ETHICS COMMITTEE APPROVAL

This study was approved by the Ethics Committee of Vale do Paraíba University (UNIVAP), process number I034CEP/2009.

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