

## ORIGINAL ARTICLE

# *In vitro* INHIBITORY EFFECT OF ALUMINUM PHTHALOCYANINE TETRASULFONATE CHLORIDE AGAINST *Leishmania (Viannia) Peruviana* AND *Leishmania (Viannia) Braziliensis*

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This study is part of the thesis: Izarra Rojas KV. Actividad fotodinámica in vitro de ftalocianina de aluminio tetrasulfonada clorada (AlPcClS<sub>4</sub>) frente a estadios extracelular e intracelular de *Leishmania (Viannia) peruviana*, *L. (V.) braziliensis* y *L. (Leishmania) amazonensis*, [Thesis]. Lima: Universidad Nacional Mayor de San Marcos; 2018.

## ABSTRACT

**Objectives:** To evaluate the in vitro photodynamic activity of aluminum phthalocyanine tetrasulfonate chloride (AlPcClS<sub>4</sub>) on promastigotes and amastigotes of *Leishmania (Viannia) peruviana* and *Leishmania (Viannia) braziliensis*. **Materials and methods:** The activity of photodynamic therapy using AlPcClS<sub>4</sub> on *Leishmania* promastigote and amastigotes was determined by the Methyl Thiazole Tetrazolium (MTT) colorimetric method and quantitative PCR, respectively. **Results:** Photodynamic treatment showed an inhibitory effect on promastigotes, particularly on *Leishmania (V.) peruviana*, to a lesser extent on *Leishmania (V.) braziliensis* and also on intracellular forms of both species. At 24 hours post-radiation, using concentrations of 200 µM and 350 µM, the inhibitory effect on *Leishmania (V.) peruviana* was 72.9% and 73.9% respectively; at 96 hours the inhibitory effect was of 78.8% and 80.6%, respectively. Regarding intracellular forms, the inhibitory effect on *Leishmania (V.) peruviana* amastigotes was 57.8% at 72 hours post-treatment, using a concentration of 200 µM. The IC<sub>50</sub> was 56.5, 50, 44 and 39.7 µM, at 24, 48, 72 and 96 hours post-radiation, respectively. **Conclusions:** Photodynamic therapy using AlPcClS<sub>4</sub> against *Leishmania* species showed encouraging results, mainly on *Leishmania (V.) peruviana*, suggesting a potential use as an alternative or complement to the usual treatment of tegumentary leishmaniasis. However, new trials are still required to determine the selectivity index for the intracellular form of the parasite, and to develop methods to facilitate the efficient entry of the molecule into the host cell and the parasite.

**Keywords:** Tropical Diseases; Cutaneous Leishmaniasis; Photodynamic Therapy Photosensitizers; Alternative Treatment (source: MeSH NLM).

## INTRODUCTION

Leishmaniasis is a vector-borne disease caused by a protozoan of the *Leishmania* genus; in the Americas, it is transmitted to mammals, including humans, by a phlebotomine of the genus *Lutzomyia* known as “white blanket” or “Titira” <sup>(1)</sup>. Depending on the infecting *Leishmania* species and the host immune system, this disease produces ulcerative lesions, mucosal metastasis, and liver, spleen and pancreas damages, which can cause death when not treated in a timely manner <sup>(2)</sup>.

The Peruvian Ministry of Health reported 5,808 cases of tegumentary leishmaniasis in 2018, of which 94% and 6% represented cutaneous and mucocutaneous cases, respectively, caused mainly by *Leishmania (Viannia) peruviana*, *Leishmania (V.) braziliensis*, *Leishmania (V.) guyanensis*, and *Leishmania (Leishmania) amazonensis*. Other unusual clinical forms

**Cite as:** Izarra-Rojas KV, Rojas-Palomino N, Gonzales-Medrano JL, Minaya-Gómez G, Berrocal-Huallpa A, Santiago-Contreras J, et al. *In vitro* inhibitory effect of aluminum phthalocyanine tetrasulfonate chloride against *Leishmania (Viannia) peruviana* and *Leishmania (Viannia) braziliensis*. Rev Peru Med Exp Salud Publica. 2020;37(3):462-70. doi: <https://doi.org/10.17843/rpmesp.2020.373.4465>

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**Received:** 16/04/2019  
**Approved:** 17/06/2020  
**Online:** 31/07/2020

reported in our country are disseminated cutaneous leishmaniasis caused by species such as *Leishmania (Viannia) braziliensis*, *Leishmania (V.) guyanensis*, *Leishmania (V.) peruviana*, and *Leishmania (L.) amazonensis*, and diffuse cutaneous leishmaniasis caused by *Leishmania (L.) amazonensis*. In Peru there are no reports of visceral leishmaniasis cases, which can be lethal if not detected on time.

The first line therapy for all *Leishmania* species are pentavalent antimonials, and the second line is amphotericin B therapy, both administered parenterally in long sessions. Due to the nature of these drugs, their use causes adverse effects, such as nausea, vomiting, myalgia, nephrotoxic effects, etc., which could result in treatment abandonment and increase the reporting of therapeutic failures due to incomplete or inadequate doses. Furthermore, their use is not recommended in pregnant women or in patients with arrhythmias<sup>(3-5)</sup>. Likewise, miltefosine used as an oral treatment and recommended by the World Health Organization also presents side effects, in addition to increasing the cost of treatment for this disease<sup>(5,6)</sup>. These limitations have stimulated the search for more effective therapeutic alternatives with fewer side effects.

Photodynamic therapy (PDT) can induce cell death by apoptosis and necrosis mediated by singlet oxygen and highly reactive toxic radicals (reactive oxygen species), which are produced by the interaction of the photosensitizing agent, a specific wavelength and molecular oxygen<sup>(7)</sup>; these characteristics make it a potential alternative for the treatment of infectious diseases, including leishmaniasis<sup>(8-10)</sup>. This study aims to evaluate the *in vitro* effect of a new photosensitizer against the promastigote and amastigote stages of *Leishmania (V.) peruviana* and *Leishmania (V.) braziliensis*, species of epidemiological relevance for public health in Peru.

## MATERIALS AND METHODS

A descriptive observational study was carried out in the laboratories of Microbial Ecology (Faculty of Biological Sciences) and Organic Chemistry (Faculty of Chemistry) of the Universidad Nacional Mayor de San Marcos, and in the National Reference Laboratory of Leishmaniasis of the Instituto Nacional de Salud del Perú (INS).

### Macrophages and promastigotes cell line

The cell line trials were performed in 24-well plates (Corning, Costar Cat. COR-3524), to which a circular plate was added at the base. The DH82 dog macrophage cell line was provided by the INS Cell Culture Laboratory, cultured in

## KEY MESSAGES

**Motivation for the study:** Pentavalent antimonials used in the treatment of leishmaniasis cause therapeutic failures due to incomplete or inadequate doses, or treatment abandonment. Therefore, it is necessary to look for new less toxic therapeutic agents to replace or complement the current treatment.

**Main findings:** In an *in vitro* model, the photosensitizer aluminum phthalocyanine tetrasulfonate chloride (ALPcClS<sub>4</sub>) inhibits the growth of promastigotes and amastigotes of *Leishmania (V.) peruviana* and *Leishmania (V.) braziliensis*.

**Implications:** The inhibitory effect of photodynamic therapy on ALPcClS<sub>4</sub> against *Leishmania* motivates to keep researching on the selectivity index through new formulations and to evaluate the appropriate vector that allows the highest absorption in *in vivo* models.

a minimum essential medium (MEM) (Gibco Cat. 61100-061), supplemented with 15% inactivated fetal bovine serum (IFBS) (Gibco, Cat. 1079255) and antibiotics (Gibco, Cat. 15240-062), incubated at 37 °C, 5% CO<sub>2</sub> and maintained by successive tapping every 72 hours.

Cultures of *Leishmania (V.) peruviana* and *Leishmania (V.) braziliensis* strains, cryopreserved at  $-70 \pm 5$  °C, were also used. For reactivation, *Leishmania* strains were defrosted and immediately transferred to a biphasic medium on blood agar with 15% rabbit blood, then to Schneider's *Drosophila* liquid medium at pH 6.65-6.75 (Gibco, Cat. 21720024) supplemented with 20% IFBS (11.12), plus 150 µg/mL gentamicin and then incubated at  $24 \pm 1$  °C, to obtain higher parasite mass. The reactivated strains were kept in Schneider's medium at 10% with IFBS. The *in vitro* infection process in macrophages was carried out with metacyclic promastigotes obtained by induction at an acid pH of 5.5 with 10% IFBS and antibiotics.

The process of macrophage infection took place in 24-well plates of  $1 \times 10^5$  macrophages/mL, in MEM with IFBS at 15%, and incubated at 37 °C and 5% of CO<sub>2</sub> for 15 hours. Metacyclic promastigotes were added in a 20:1 ratio (parasite: macrophage) and incubated at 33 °C and 5% of CO<sub>2</sub> for 5 hours. Finally, three washes were performed with 2% MEM and IFBS to eliminate non-phagocytic parasites.

### Inhibitory activity assessment

A solution of chlorinated tetrasulfonate aluminum phthalocyanine (ALPcClS<sub>4</sub>) photosensitizer (Frontier Scientific) was

prepared at an initial concentration of 1 mM plus 0.5% dimethyl sulfoxide (DMSO) (Applichem) in phosphate buffer saline (PBS) at pH 7.4 and filtered at 0.45  $\mu\text{m}$ .

To determine the mean inhibitory concentration ( $\text{IC}_{50}$ ) of  $\text{AlPcClS}_4$ , two independent trials were conducted, each one of them in triplicate. Regarding promastigotes, they were evaluated at the compound concentrations of 0; 25; 50; 75; 100; 200 and 350  $\mu\text{M}$  in 3 mL of liquid medium with  $5 \times 10^5$  parasites ( $T_0$ ) and incubated during 24 hours at 26 °C in a dark room. At the end, LED light was irradiated once at a specific wavelength of 675 nm, 30  $\text{J/s} \cdot \text{m}^2$  of power and at a distance of 10 cm for 30 minutes, allowing an irradiation power of approximately 5.4  $\text{J/cm}^2$ . Also, the inhibitory effect of phthalocyanine PDT on *Leishmania* promastigotes at 0, 24, 48, 72 and 96 hours was indirectly determined by the colorimetric MTT (Methyl Thiazole Tetrazolium) assay<sup>(13)</sup>.

Regarding intracellular amastigotes, after the infection and washing process, 1 mL of MEM with IFBS at 15% and  $\text{AlPcClS}_4$  at concentrations of 50, 75, 100 and 200  $\mu\text{M}$  was added, then the samples were incubated for 24 hours at 37 °C and 5% of  $\text{CO}_2$ . Afterwards, they were irradiated with LED light and incubated again for 72 hours post-irradiation. The effect of the compound on the amastigote forms was determined by quantitative PCR.

The effect of  $\text{AlPcClS}_4$  with and without exposure to light was independently evaluated. Promastigotes and DH82 macrophages were used as growth control; and stibogluconate (Pentostam) at concentrations of 100, 200 and  $2 \times 10^4$   $\mu\text{M}$  for promastigote trials and 200  $\mu\text{M}$  for intracellular amastigotes, 0.01% triton was used as treatment control.

### MTT colorimetric assay

The MTT colorimetric assay was developed according to the methodology described by Mesa *et al.*<sup>(14)</sup> with the following modifications: the promastigotes treated at different concentrations of phthalocyanine and then irradiated were collected in sterile conical tubes of 1.5 mL; the parasites were centrifuged at 3,500 rpm; the supernatant was carefully discarded; then 450  $\mu\text{L}$  of liquid medium and 50  $\mu\text{L}$  of MTT (5 mg/mL) were added, and then the samples were incubated for 4 hours at 32 °C. Finally, to stop the formation of formazan crystals, a 10% solution of sodium dodecyl sulfate (SDS), 0.01 N HCl<sup>(14)</sup> was added to the wells. The samples were read at an absorbance of 570 nm in the Eon microplate spectrophotometer (Biotek).

### DNA extraction and quantification of the parasitic load

The infected and treated macrophages, 72 hours post-irradiation, were washed with sterile PBS at pH 7.2 to

remove remnants from the culture medium and from the DNA extraction. 100  $\mu\text{L}$  of trypsin-EDTA was added to the well and incubated for 10 minutes to detach the cells from the slides. Then, 100  $\mu\text{L}$  of PBS was added, the cell suspension was collected and placed in 1.5 mL conical tubes to extract nucleic acids, by using the PureLink® Genomic DNA Kit (Invitrogen, Cat. K1820-02), and the samples were eluted in a final volume of 60  $\mu\text{L}$ .

The inhibitory effect of photodynamic treatment in infected macrophages was measured by quantifying the parasitic load by qPCR<sup>(15,16)</sup>. Oligonucleotides Leis.L1 5'-GACGCACCCCTC-CAA-3' and Leis.U1 5'-AAGTGCTTCCCATCGCAACT-3' were used as PCR initiators, and Leis.P1 FAM 5'-CGGTTCG-GTGTGTGCGCC-3' TAMRA as a marked probe; the methodology is described in Wortmann *et al.*<sup>(15)</sup>

The standard curve was made using *Leishmania* (*V. braziliensis*) genomic DNA at a concentration of 415.75 ng equivalent to  $5 \times 10^6$  parasites/ $\mu\text{L}$  (120 parasites = 10 pg of DNA) and dilutions equivalent to  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ ,  $10$ ,  $10^{-1}$  parasites. The final volume was 20  $\mu\text{L}$ , and consisted of 1X Kapa Probe Fast qPCR Master Mix (Kapa Biosystems, Cat. KK4701), 0.2  $\mu\text{mol}/\mu\text{L}$  of each primer, 0.04  $\mu\text{mol}/\mu\text{L}$  of the probe and 5  $\mu\text{L}$  of DNA from the samples. DNA was amplified by an initial denaturation of 95 °C for 30 seconds to activate the enzyme, followed by 40 denaturation repeats at 95 °C for 30 seconds and hybridization/extension at 60 °C for 30 seconds.

### Statistical analysis

The results mean and standard deviation were determined with Microsoft Excel 2010. Likewise, the inhibition percentage was calculated from the optical densities found in the MTT assay, using the formula<sup>(17)</sup>:

$$\text{Growth inhibition (\%)} = \frac{(\text{OD}_{570} \text{ control sample} - \text{OD}_{570} \text{ Tx}_i \text{ sample})}{(\text{OD}_{570} \text{ nm control sample})} \times 100$$

OD = optical density 570 nm

Additionally, the QQ plot and Shapiro-Wilk test were done to evaluate the normality and homogeneity of the residual values of the data using a scatter plot. FDT measurements were compared according to 0, 25, 50, 75, 100, 200 and 350  $\mu\text{M}$   $\text{AlPcClS}_4$  concentrations on *Leishmania* (*V.*) *peruviana* and *Leishmania* (*V.*) *braziliensis* using ANOVA and Tukey's test, with a  $\alpha = 0.05$ .  $\text{IC}_{50}$  was also determined against *Leishmania promastigote*.

## Ethical criteria

The Ethics Committee of the Instituto Nacional de Salud approved this study, with RD No. 397-2016-OGI-TT-OPE/INS.

## RESULTS

The effect of PDT on *Leishmania* was quantified by the MTT assay. For *Leishmania (V.) peruviana*, control therapy presented an OD<sub>570</sub> of  $0.27 \pm 0.04$ ; for 100  $\mu\text{M}$  the OD<sub>570</sub> was  $0.22 \pm 0.05$ , for 200  $\mu\text{M}$  the OD<sub>570</sub> was  $0.15 \pm 0.03$ , and for 350  $\mu\text{M}$  the OD<sub>570</sub> was  $0.14 \pm 0.02$  (Figure 1). After 24 hours of 50  $\mu\text{M}$  photosensitizer radiation, this species presented a 26% inhibition of parasitic growth compared to the control sample; at concentrations of 100, 200 and 350  $\mu\text{M}$  it showed a parasitic growth inhibition of 65.6%, 72.9% and 73.9%, respectively.

At 96 hours post-radiation the inhibition percentage reached 68.9%; 78.8% and 80.1%, respectively. At 24, 48, 72 and 96 hours was, the IC<sub>50</sub> was 56.5; 50; 44; and 39.7  $\mu\text{M}$  respectively (Figure 2). Regarding the intracellular form, at 72 hours

post-exposure the photosensitizer concentrations of 50, 75, 100 and 200  $\mu\text{M}$  reached an inhibition of the *Leishmania (V.) peruviana* amastigotes of 20.5%; 46.5%; 65.2% and 57.8%, respectively (Table 1).

The values regarding the PDT effect on *Leishmania* promastigotes were analyzed independently for each species. Each assay was composed by 4 evaluation times performed in triplicate at 7 different concentrations, a total of 28 treatments per species were analyzed. Data obtained for *Leishmania (V.) peruviana* and *Leishmania (V.) braziliensis* presented a normal distribution ( $p = 0.1041$  and  $0.1036$ , respectively). Comparison of means by ANOVA allowed to determine that there is a statistically significant difference between treatments in both *Leishmania* species ( $p = 0.0001$ ).

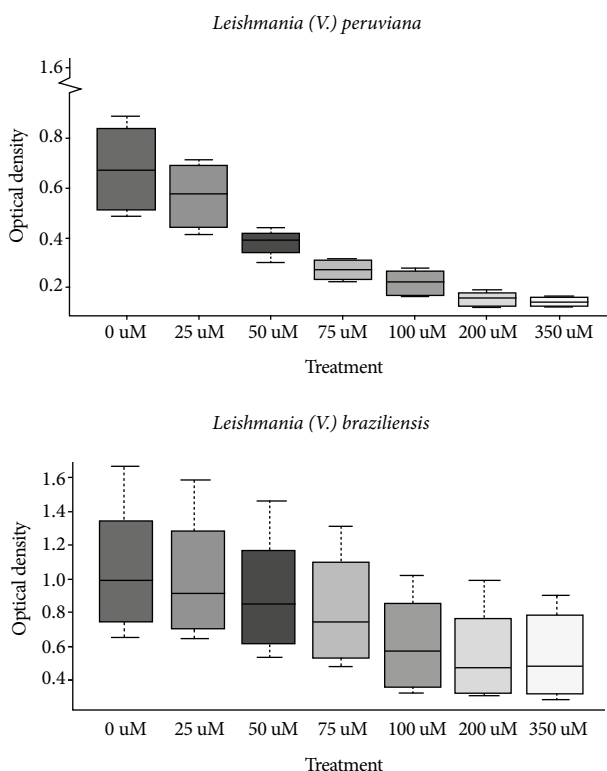
PDT in *Leishmania (V.) peruviana* with 200 and 350  $\mu\text{M}$  of AlPcCl<sub>4</sub> did not present statistically significant differences ( $p > 0.05$ ) by using Tukey's test ( $\alpha = 0.05$ ), evaluating each one at 24 and 48 hours. Similarly, these same concentrations evaluated at 72 and 96 hours post-exposure did not present statistically significant differences either ( $p > 0.05$ ) (Figure 3).

Treatment only with irradiation at 670 nm and at 5.4 J/cm<sup>2</sup>, with and without exposure to the photosensitizing agent, did not show any phototoxic effect compared to the growth control.

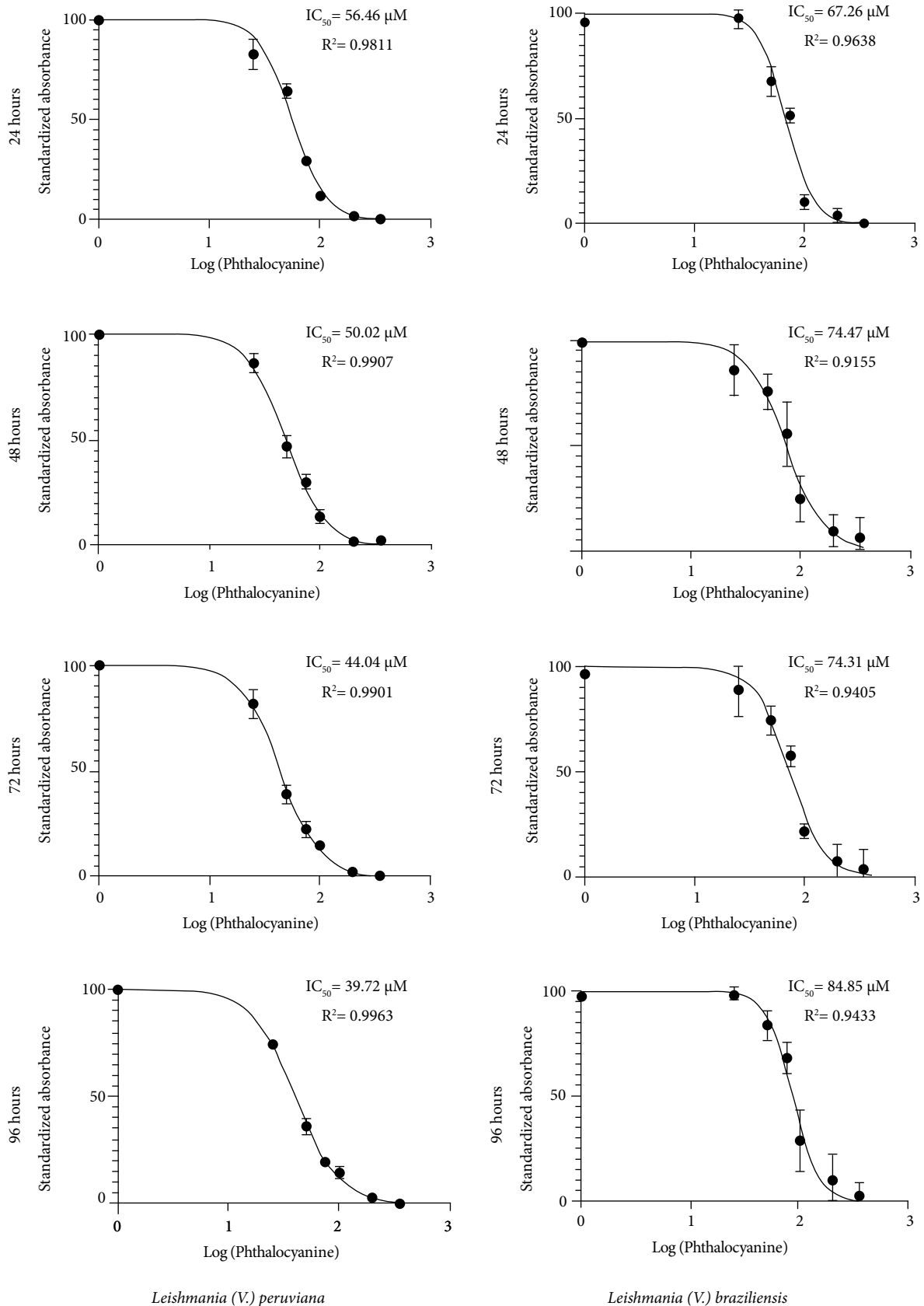
## DISCUSSION

This study evaluated the phototoxic effect of aluminum phthalocyanine tetrasulfonate chloride in an in vitro phototherapy assay on *Leishmania (V.) braziliensis* and *Leishmania (V.) peruviana* promastigotes, by using the MTT assay, which allowed the quantification of formazan crystals as a result from the enzymatic activity of the mitochondrial dehydrogenase enzyme of the living parasites<sup>(13)</sup>. The photodynamic treatment consisted in LED light irradiation at a wavelength of 670 nm and potency of 5.4 J/cm<sup>2</sup> on *Leishmania* promastigotes in culture medium with AlPcCl<sub>4</sub> concentrations of 25; 50; 75; 100; 200 and 350  $\mu\text{M}$ , according to the methodology described by Amin *et al.*<sup>(7)</sup> on neoplastic cells. Regarding amastigotes, the assessment of the effect by PDT was carried out with quantitative PCR.

Phthalocyanines are chemically stable organic compounds that have received particular attention in phototherapy as a sensitizing agent mainly in the treatment of infectious diseases due to their low toxicity and their biochemical properties that improve the humoral and cellular response to the infectious agent or target cells in case of neoplasia.



**Figure 1.** Mean optical density and standard deviation of the formazan crystals' quantification according to the MTT (Methyl Thiazole Tetrazolium) assay, at different concentrations of phthalocyanine in *Leishmania (V.) peruviana* and *Leishmania (V.) braziliensis*



**Figure 2.** Mean inhibitory concentration ( $IC_{50}$ ) of aluminum phthalocyanine chloride in photodynamic therapy on *Leishmania (V.) peruviana* and *Leishmania (V.) braziliensis*, evaluated at 24, 48, 72 and 96 hours post-irradiation.

**Table 1.** Determination of mean inhibitory concentration (IC<sub>50</sub>) and selectivity index of aluminum phthalocyanine tetrasulfonate chloride and sodium stibogluconate against promastigote and amastigote stages of *Leishmania (V.) peruviana* and *Leishmania (V.) braziliensis*.

| Species                      | Hours | Inhibition % |       |       |        |        |                    | IC <sub>50</sub> | 95% CI        |
|------------------------------|-------|--------------|-------|-------|--------|--------|--------------------|------------------|---------------|
|                              |       | 25 μM        | 50 μM | 75 μM | 100 μM | 200 μM | 350 μM             |                  |               |
| <b>Phthalocyanine</b>        |       |              |       |       |        |        |                    |                  |               |
| Promastigotes                |       |              |       |       |        |        |                    |                  |               |
| <i>L. (V.) peruviana</i>     | 24    | 12.89        | 26.09 | 52.47 | 65.60  | 72.88  | 73.90              | 56.46            | 53.49 - 59.36 |
|                              | 48    | 10.24        | 40.89 | 53.53 | 66.15  | 75.97  | 76.51              | 50.02            | 48.30 - 51.75 |
|                              | 72    | 14.53        | 48.67 | 61.81 | 67.71  | 78.40  | 79.77              | 44.04            | 42.42 - 45.69 |
|                              | 96    | 20.86        | 51.33 | 64.86 | 68.91  | 78.78  | 80.61              | 39.72            | 38.75 - 40.70 |
| <i>L. (V.) braziliensis</i>  | 24    | 0.81         | 16.45 | 25.68 | 49.60  | 53.43  | 55.50              | 67.26            | 63.60 - 70.94 |
|                              | 48    | 5.43         | 14.12 | 26.13 | 44.00  | 52.93  | 54.70              | 74.47            | 67.86 - 81.47 |
|                              | 72    | 2.73         | 11.24 | 19.84 | 37.75  | 44.87  | 47.00              | 74.31            | 69.13 - 79.66 |
|                              | 96    | 3.21         | 10.09 | 17.40 | 34.75  | 42.56  | 44.38              | 75.88            | 71.74 - 80.07 |
| DH82 Macrophages             |       |              |       |       |        |        |                    |                  |               |
| <i>L. (V.) peruviana</i>     | 72    | ND           | 20.52 | 46.45 | 65.16  | 57.78  | ND                 | 60.28            | 56.78 - 63.80 |
| <i>L. (V.) braziliensis</i>  | 72    | ND           | 4.15  | 9.04  | 20.31  | 36.90  | ND                 | 94.92            | 90.80 - 99.63 |
| <b>Sodium stibogluconate</b> |       |              |       |       |        |        |                    |                  |               |
| Promastigotes                |       |              |       |       |        |        |                    |                  |               |
| <i>L. (V.) peruviana</i>     | 24    | ND           | ND    | ND    | 35.75  | 45.70  | 98.13 <sup>a</sup> | 216.4            | 196.3 - 240.8 |
|                              | 48    | ND           | ND    | ND    | 35.25  | 41.94  | 98.36 <sup>a</sup> | 250.1            | 215.4 - 295.6 |
|                              | 72    | ND           | ND    | ND    | 33.25  | 48.58  | 98.43 <sup>a</sup> | 202.1            | 182.7 - 228.3 |
|                              | 96    | ND           | ND    | ND    | 46.45  | 49.04  | 98.35 <sup>a</sup> | 152.3            | 127.4 - 181.3 |
| <i>L. (V.) braziliensis</i>  | 24    | ND           | ND    | ND    | 21.40  | 31.73  | 96.88 <sup>a</sup> | 389.4            | 325.2 - 471.2 |
|                              | 48    | ND           | ND    | ND    | 21.39  | 30.38  | 96.02 <sup>a</sup> | 408.4            | 288.4 - 604.2 |
|                              | 72    | ND           | ND    | ND    | 27.01  | 37.19  | 96.36 <sup>a</sup> | 310              | 257.9 - 381.3 |
|                              | 96    | ND           | ND    | ND    | 30.93  | 33.24  | 97.52 <sup>a</sup> | 355.1            | 276.0 - 470.8 |
| DH82 Macrophages             |       |              |       |       |        |        |                    |                  |               |
| <i>L. (V.) peruviana</i>     | 72    | ND           | ND    | ND    | ND     | 54.56  | ND                 | ND               | ND            |
| <i>L. (V.) braziliensis</i>  | 72    | ND           | ND    | ND    | ND     | 50.23  | ND                 | ND               | ND            |

<sup>a</sup> Evaluated at  $2 \times 10^4$  μM pentavalent antimonials.

IC<sub>50</sub>: mean inhibitory concentration; 95% CI: 95% confidence interval.

ND: not determined.

They also participate in the production of oxidative molecules such as reactive oxygen species and singlet oxygen, which act directly on amino acid residues of proteins and enzymes, such as cysteine, methionine, tryptophan, among others, related to the virulence of the pathogen<sup>(10)</sup>. Studies related to PDT as an alternative to leishmaniasis treatment have found promising levels of IC<sub>50</sub> when evaluating several photosensitizers such as zinc phthalocyanine (ZnPc), aluminum phthalocyanine (AlPc) and aluminum tetrasulfonate (AlPcS<sub>4</sub>), among others, on amastigotes and promastigotes of *Leishmania (V.) braziliensis*, *Leishmania (V.) panamensis*, *Leishmania (L.) amazonensis*, *Leishmania (L.) chagasi*, *Leishmania (L.) major* and *Leishmania (L.) tropica*<sup>(8,9,18-20)</sup>. These species cause cutaneous and mucocutaneous lesions<sup>(1)</sup>.

Pinto *et al.*<sup>(9)</sup> found that *in vitro* photodynamic therapy evaluated on the fourth day post-irradiation, that used AlPcClS<sub>4</sub> concentrations of 1 and 10 μM and irradiated at a length of 659 nm on *Leishmania (V.) braziliensis* promastigote, achieved an inhibition of approximately 42% and 49%, respectively. On the ninth day post-irradiation, an inhibition of 34% and 41.3%, respectively, was achieved. In *Leishmania (L.) major*, evaluated under the same conditions, the inhibition effect at the fourth day post-irradiation was 50% for both concentrations, while at the ninth day post-irradiation inhibition was 32% and 41% corresponding to concentrations of 1 and 10 μM, respectively.

Hernandez<sup>(21)</sup> evaluated aluminum phthalocyanine chloride (AlPcCl) and aluminum phthalocyanine disulfonate

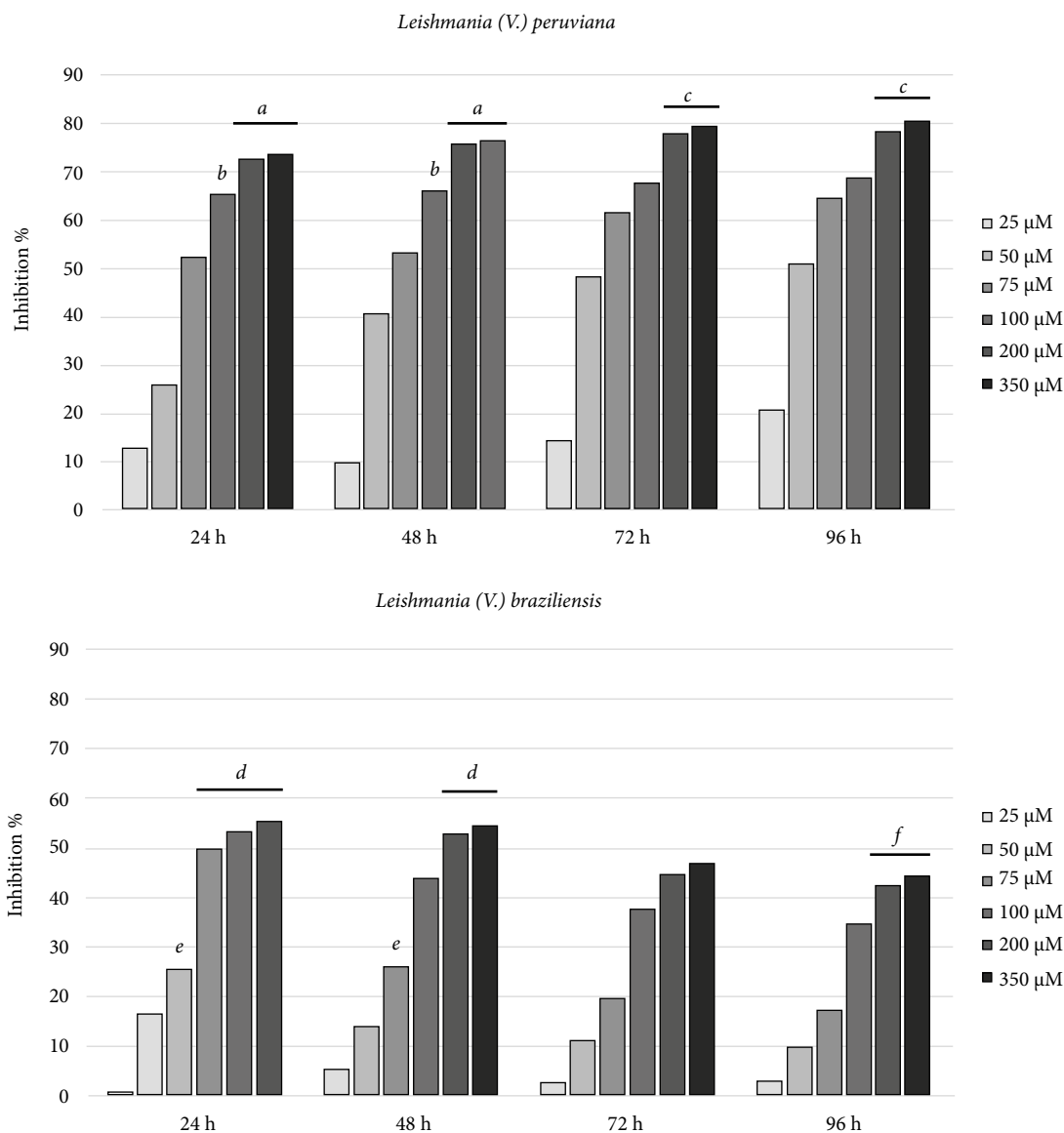


Figure 3. Inhibition of *Leishmania* parasitic growth, by exposure to photodynamic therapy with aluminum phthalocyanine tetrasulfonate chloride (a, b, c, d, e, and f, represent treatments that do not present statistically significant differences, Tukey p test > 0.05).

chloride (AlPcCl<sub>2</sub>) on *Leishmania (L.) amazonensis* by using an irradiation of 3 J/cm<sup>2</sup> and reported an IC<sub>50</sub> of 0.046 μM ± 0.018 and an IC<sub>50</sub> of 4.101 μM ± 0.136, respectively. Likewise, Escobar *et al.* (20) used AlPcCl with an irradiation of 10.0 J/cm<sup>2</sup> at 670 nm and, at 24 hours post-irradiation, and reported an IC<sub>50</sub> of 0.0033 and 0.17 μM in promastigotes of *Leishmania (L.) chagasi* and *Leishmania (V.) panamensis*, respectively. Also, Zinc phthalocyanine (ZnPc) under the same conditions and with the same evaluated species, presented an IC<sub>50</sub> of 6.45 μM and 6.05 μM, respectively. In our study, we have used the same time interval and the IC<sub>50</sub> found was 56.5 μM and 67.26

μM for *Leishmania (V.) peruviana* and *Leishmania (V.) braziliensis*, respectively.

It is likely that the heterogeneous PDT results are due to the time and fluency of irradiation, the wavelength used, the nature of the compound used as photosensitizer (isomers), the physicochemical properties related to cell internalization and molecular charge, these variables together with the quantum yield of singlet oxygen, the subcellular location, the sulfonation degree, the size of the molecule, the variety of metal ions, the absorption of light, the affinity for the target tissue, the selectivity by cellular compartments, such as the mitochondria and the irradiation power achieved in the cells, could influen-

ce the ability of the compound to cross the lipid bilayer of the parasite's cell membrane and reduce the effects and efficiency of the photodynamic treatment<sup>(22)</sup>.

In this study, similar to Amin *et al.*<sup>(7)</sup> description, the irradiation alone had no effect on promastigotes, infected macrophages or free macrophages. Likewise, the photosensitizer without exposure to LED light radiation was not toxic in any of the concentrations used, which is consistent with the principle of PDT, where the independent application of the compound or irradiation has no effect on cell viability<sup>(23)</sup>.

It was not possible to determine the selectivity index (SI), a useful parameter to estimate the effectiveness of a compound on a given pathogen<sup>(24)</sup>, neither the mean lethal concentration (LD<sub>50</sub>) on the cell line containing the intracellular form of *Leishmania*, important parameter for the determination of the SI, which constitutes the main limitation of the study.

Likewise, PDT could improve the performance of conventional therapy, which implies several possibilities regarding the management of tegumentary leishmaniasis, such as dose reduction, application time of conventional therapies, species-specific therapy, etc.; as a result, side effects could be reduced<sup>(25,26)</sup>. However, it is still necessary to carry out studies that determine the risk of disease reactivation or relapse and the inadequate response to treatment; it is also important to conduct studies related to evaluating strategies that allow improving the efficiency of treatment through exposure to more than one cycle of irradiation. In this study, a single irradiation with LED light for 30 minutes was used, which is probably not sufficient to affect all parasites. In addition, it is important to carry out research studies related

to formulating, developing, and evaluating the effectiveness and selectivity index in cell lines and animal models to estimate the compound efficiency on the parasite.

In conclusion, ALPcClS<sub>4</sub> as a PDT photosensitizer showed a constant anti-*Leishmania* effect mainly on *Leishmania (V.) peruviana* promastigotes and, in a smaller proportion in *Leishmania (V.) braziliensis*, the latter started a recovery process after 72 hours post-treatment, unlike *Leishmania (V.) peruviana* that showed a higher sensitivity to treatment, without a recovery process until 96 hours post-treatment. In the amastigote forms the phototoxic effect was lower, but the response of DH82 macrophages infected with *Leishmania (V.) peruviana* was higher compared to macrophages infected with *Leishmania (V.) braziliensis*.

**Acknowledgements:** To the National Innovation Program for Competitiveness and Productivity - INNOVATE-PERU (Agreement No. 177-PNICP-PIAP-2015) for funding this research. To the National Public Health Center of the Instituto Nacional de Salud, for the facilities provided.

**Authorship contributions:** KVIR, NRP, JLGM and ABH participated in the conception, research design, data collection, results, analysis, interpretation and writing of the article. NRP, GMG, ABH, JSC and JLQ participated in the conception, design, work direction, critical review of the article, approval of the final version, and contribution of study materials. JSC and JLQ participated in obtaining funding.

**Funding source:** The study was funded by the National Innovation Program for Competitiveness and Productivity - INNOVATE-PERU (Agreement No. 177-PNICP-PIAP-2015) of the Ministry of Production, Lima, Peru.

**Conflicts of interest:** The authors declare no conflict of interest.

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