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Evaluating the anti-leishmania activity of *Lucilia sericata* and *Sarconesiopsis magellanica* blowfly larval excretions/secretions in an *in vitro* model

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ABSTRACT

Leishmaniasis is a vector-borne disease caused by infection by parasites from the genus *Leishmania*. Clinical manifestations can be visceral or cutaneous, the latter mainly being chronic ulcers. This work was aimed at evaluating Calliphoridae *Lucilia sericata*- and *Sarconesiopsis magellanica*-derived larval excretions and secretions' (ES) *in vitro* anti-leishmanial activity against *Leishmania panamensis*. Different larval-ES concentrations from both blowfly species were tested against either *L. panamensis* promastigotes or intracellular amastigotes using U937-macrophages as host cells. The Alamar Blue method was used for assessing parasite half maximal inhibitory concentration (IC_{50}) and macrophage cytotoxicity (LC_{50}). The effect of larval-ES on *L. panamensis* intracellular parasite forms was evaluated by calculating the percentage of infected macrophages, parasite load and toxicity. *L. sericata*–derived larval-ES *L. panamensis* macrophage LC_{50} was 72.57 μg/mL (65.35–80.58 μg/mL) and promastigote IC_{50} was 41.44 μg/mL (38.57–44.52 μg/mL), compared to 34.93 μg/mL (31.65–38.55 μg/mL) LC_{50} and 23.42 μg/mL (22.48–24.39 μg/mL) IC_{50} for *S. magellanica*. Microscope evaluation of intracellular parasite forms showed that treatment with 10 μg/mL *L. sericata* ES and 5 μg/mL *S. magellanica* ES led to a decrease in the percentage of infected macrophages and the amount of intracellular amastigotes. This study produced *in vitro* evidence of the antileishmanial activity of larval ES from both blowfly species on different parasitic stages and showed that the parasite was more susceptible to the ES than it's host cells. The antileishmanial effect on *L. panamensis* was more evident from *S. magellanica* ES.

1. Introduction

Leishmaniasis covers a group of diseases caused by intracellular parasites from the genus *Leishmania*; it is transmitted by the bite of infected female sand flies from the genus *Lutzomyia* in the New World and the genus *Phlebotomus* in the Old World (De Almeida et al., 2003; Reithinger and Dujardin, 2007). Clinical manifestations may appear as visceral, mucous and/or cutaneous lesions. The latter form's worldwide incidence is the most predominant and it is estimated that 1.5 million new cases occur annually out of a total of 2 million cases for this group of diseases (De Almeida et al., 2003; WHO, 2010). Leishmaniasis (including all its clinical manifestations) is recorded as being prevalent in 95 countries, affecting 12 million people with around 350 million living at the risk of becoming infected (De Almeida et al., 2003; WHO, 2010). The annual incidence of leishmaniasis in Colombia has increased since 2005 (Alvar et al., 2012; Perez-Franco et al., 2016); the *Leishmania* species associated with patients' cutaneous lesions, in order of frequency, are: *L. panamensis*, *L. braziliensis* and *L. guyanensis* (Corredor et al., 1990; Ovalle et al., 2006; Urbano et al., 2011).

Pentavalent antimonials [sodium stibogluconate (sold as pentostam) or meglumine antimoniate (glucantime)] are first-line drugs for treating cutaneous leishmaniasis, having 75% therapeutic efficiency when used at 20 mg/kg/day dose over a 20-day period (Llanos-Cuentas et al., 2008). The medication's main administration route is parenteral, mainly intramuscular (IM), and requires medical supervision due to secondary effects concerning the liver and pancreas and cardiotoxic

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potential (Tuon et al., 2008). Miltefosine, paromomycin, pentamidine isocyanate and amphotericin B are used as therapeutic alternative, but these do not have the same effectiveness for all parasite species, are more expensive and cause toxicity in patients (Antinori et al., 2012; Kaye and Scott, 2011; Pace, 2014). The current treatment status for cutaneous leishmaniasis, patients' lack of adherence to treatment schemes, the need for medical assistance regarding administration and therapeutic failure highlight the need for searching for therapeutic alternatives. Due to the above, and the relatively benign evolution of a percentage of *L. panamensis*-associated cases, the WHO (2010) has considered topical medication acceptable for the treatment of cutaneous leishmaniasis caused by this species (as per clinical judgment) and it recommends the search for local therapies facilitating treatment and control of this disease (WHO, 2010).

Larval therapy (LT) has provided promising results concerning wound healing (Arrivillaga et al., 2008; Cruz-Saavedra et al., 2016; Polat et al., 2012; Polat and Kutlubay, 2014; Sanei-Dehkordi et al., 2016). LT consists of applying sterile fly larvae to chronic wounds (Sherman et al., 2000); it is an old therapy which was used in the 1930s (Baer, 1931; Čeřovský et al., 2010; Whitaker et al., 2007) but became relegated in the 1940s because of the boom in antibiotic use and surgical progress during this period (Robinson and Norwood, 1933). It became resumed at the end of the 1980s as an alternative regarding the emergence of antibiotic resistance and chronic non-healing wounds which did not respond to conventional treatment (Kerridge et al., 2005; Weil et al., 1933). Larvae-induced wound healing occurs through the following mechanisms of action: removing necrotic tissue/debridement (Chambers et al., 2003), stimulating tissue granulation (Chambers et al., 2003; Prete, 1997), inhibiting and eliminating biofilms (Cazander et al., 2009; Van Der Plas et al., 2008) and an antiseptic effect (Bexfield et al., 2004; Mumcuoglu, 2001; Nigam et al., 2006; Robinson and Norwood, 1933).

As *L. sericata* larvae have a cosmopolitan distribution their larvae are used in most studies relating to antibacterial activity involving this blowfly species (Sherman et al., 2000). *L. sericata*-derived larval excretions/secretions (ES) antimicrobial effect has been demonstrated on Gram-positive and Gram-negative bacteria (Kerridge et al., 2005; Thomas et al., 1999), as well as a reduction in biofilm formed by *S. aureus, S. epidermidis* or *P. aeruginosa* (Cazander et al., 2009; Harris et al., 2009; Jiang et al., 2012). There is also evidence that *S. magellanica*-derived ES have more potent and effective antibacterial activity than *L. sericata* (Díaz-Roa et al., 2014) and that they accelerate cicatricial tissue proliferation in chronic wound cases (Díaz-Roa et al., 2016).

The *L. sericata* larvae and *Calliphora vicina* ES anti-leishmanial effect *in vivo* has been demonstrated in *L. amazonensis*- (Arrivillaga et al., 2008) and *L. major*-infected murine models (Sanei-Dehkordi et al., 2016), as well as *in vitro* models using *L. tropica* (Polat et al., 2012) and *L. major* (Sanei-Dehkordi et al., 2016). LT effectiveness has been observed in human meglumine antimoniate-resistant lesions caused by *L. major* (Polat and Kutlubay, 2014). Results have been published recently about New World *S. magellanica* fly species *in vivo* LT effectiveness (using larvae or ES) concerning golden hamster cutaneous lesions produced by *L. panamensis* parasites (Cruz-Saavedra et al., 2016); LT effectiveness with this fly was observed to be equivalent to that of *L. sericata* cosmopolitan species.

The present study's main objective was to evaluate *Lucilia sericata* and *Sarconesiopsis magellanica* blowfly larval excretions/secretions anti-leishmanial activity against *Leishmania panamensis*. This parasite species has the greatest epidemiological relevance in Colombia and Panamá (WHO, 2010). *L. sericata* and *S. magellanica* larval-ES action on *L. panamensis* promastigotes as well as cytotoxic activity on human U937 macrophages were quantitatively evaluated throughout viability assays. *In vitro* infection was analysed for evaluating the effect of both fly species' larval-ES on the parasite's intracellular stage by determining parameters such as infection percentage, parasite load and survival index concerning different larval-ES.

2. Materials and methods

2.1. Obtaining L. sericata and S. magellanica ES

Instar II and III *L. sericata* and *S. magellanica* larvae were taken from previously established colonies (Pinilla et al., 2013; Rueda et al., 2010) and larval-ES were obtained after larval disinfection, as described by Cruz-Saavedra et al. (Cruz-Saavedra et al., 2016). The larval-ES protein concentration to be used in the biological tests was determined by Pierce BCA Protein Assay (No. 23225) kit, following the manufacturer's instructions. A negative glass bead control was used in the biological tests, replacing the fly larvae.

2.2. Maintaining cell cultures and parasite stages

The U937 monocyte cell line was maintained in suspension in RPMI1640 medium (Gibco Life Technologies Inc.) supplemented with 10% foetal bovine serum (FBS) at 37 °C and 5% CO_2 atmosphere. The *L. panamensis* promastigote (MHOM/CO/87/UA140) culture was kept in Schneider's medium with 10% FBS and incubated at 27 °C. U937 cells were seeded at 2×10^5 cells/well on glass coverslips in 24 well-plates and RPMI medium supplemented with 10% FBS for *in vitro* infection and activated for 5 days by adding 100 ng/mL phorbol-12-myristate-13-acetate (PMA) (Minta and Pambrun, 1985). After activation, metacyclic promastigotes which had been previously opsonised were added in a 40:1 parasite/macrophage ratio, incubated at 34 °C with 5% CO_2 for 6 h (Berman and Neva, 1981; Fernandez et al., 2012). Non-internalised parasites were removed by three washings with PBS; infected cells were maintained in RPMI medium supplemented with 10% FBS and incubated at 34 °C for 48 h.

2.3. L. sericata and S. magellanica larval-ES cytotoxicity tests on U937 cells

Larval-ES macrophage cytotoxicity was determined by the Alamar Blue method (Biosource; Invitrogen, CA, USA, Cat. DAL 1100), following the manufacturer's recommendations. Briefly, U937 cells were maintained and activated as described earlier (Minta and Pambrun, 1985). Preliminary experiments (not shown) had revealed that *S. magellanica* larval-ES were more toxic for cells than *L. sericata*-ES; the range of larval-ES used in this experiment thus varied according to the larval species. *L. sericata* larval-ES were added at 20, 40, 80, 160, 320, 640 and 1280 μg/mL concentration and *S. magellanica* larval-ES were used at 10, 20, 40, 80, 160, 320 and 640 μg/mL. Cells with and without treatment were incubated at 37 °C for 24 h (diluted in RPMI medium without FBS); Alamar Blue was added to determine LC_{50} values, incubating for 6 h at 37 °C in a 5% CO_2 atmosphere. Absorbance was measured with a 570/630 nm filter on a xMark BIORAD reader. Each test was done in triplicate, in three independent experiments. A no-treatment point was included in the LC_{50} calculation.

2.4. L. panamensis promastigote susceptibility to L. sericata and S. magellanica larval ES

L. panamensis promastigotes were cultured on 96 well-plates in Schneider's medium, supplemented with 10% FBS, at 8×10^6 parasite/well concentration; after 24 h, larval-ES were added at 20, 40, 80, 160, 320, 640 and 1280 μg/mL concentration for *L. sericata* and 10, 20, 40, 80, 160, 320 and 640 μg/mL for *S. magellanica* (diluted in Schneider's medium without FBS). They were incubated at 27 °C for 24 h. Incubation with Alamar Blue at 12.5 μg/mL concentration was carried out for 6 h at 27 °C; an xMar BIORAD reader with 570/630 nm filter was used for obtaining absorbance values. Each test was done in triplicate, in three independent experiments. A no-treatment point was included for calculating IC_{50} values.

Table 1
Parameters for evaluating amastigote susceptibility to larval ES.

Parameter	Abbreviation	Equation
Infection percentage	%I	(# Infected cells/300 randomly-chosen cells) *100
Decrease in infection percentage	%DI	[(%I no treatment − %I treatment)/%I no treatment] *100
Parasite load	PL	# amastigotes/# infected cells
Survival index	SVI	%I*PL
Selectivity index	SI	CL_{50}/CI_{50}

2.5. Amastigote susceptibility to L. sericata and S. magellanica ES determined by microscope

U937 cells infected for 48 h with *L. panamensis* were incubated for 10 h at 34 °C in a 5% CO_2 atmosphere, using increasing ES concentrations: 10, 20, 40, 60, 80 and 100 μg/mL for *L. sericata* and 5, 10, 20, 40, 60 and 70 μg/mL for *S. magellanica*. A shorter incubation time was used to avoid cell detachment. A no-treatment point (negative control) was included for cytotoxicity calculation and a point treated with glucantime (100 μg/mL) was used as reference value/internal control for amastigote susceptibility (positive control) to determine decreased infection percentage when counting amastigotes. The coverslips were fixed with methanol for staining with 10% Giemsa. Optical microscope reading involved counting 300 cells, the number of fields needed for such amount and determining how many of them were infected (as a percentage); parasite load was calculated by counting the number of amastigotes per cell. Each test was done in triplicate in two independent experiments. Table 1 describes the parameters used for evaluating amastigote susceptibility to larval ES.

2.6. Data analysis

The GraphPad Prism (5.03) dose-response (variable slope) equation [log (inhibitor) *vs.* normalised response] was used for determining *L. sericata* and *S. magellanica* larval-ES LC_{50} and IC_{50} for the U937-macrophage cell line and *L. panamensis* promastigotes; dose-response curves were plotted using non-linear regression. The absorbance values obtained after blank-correction were normalised as viability percentage as follows; the absorbance mean value recorded for the no-treatment point was 100% viability and the absorbance mean value recorded by the highest larval-ES concentration was assumed as 0% viability. The larval-ES concentrations were transformed into base 10 logarithms for analysing and plotting the data. Infection percentage, decreased infection percentage, parasite load, survival index and selectivity index were determined for analysing amastigote susceptibility to larval ES to obtain an ES toxicity percentage for this parasite stage (Table 1). Selectivity index was calculated as LC_{50}/IC_{50}; statistical significance was defined as $p < 0.05$. Data are expressed as average of two or three independent experiments with their respective standard deviations.

3. Results

Microscopic appearance of *L. panamensis* promastigotes and intracellular amastigotes infecting U937 cells previous to treatment is shown in Fig. 1A and B, respectively.

3.1. L. sericata and S. magellanica larval ES macrophage cytotoxicity

Macrophage LC_{50} was evaluated after 24 h exposure to larval ES. *L. sericata*-derived species LC_{50} was 72.57 μg/mL (Log = 1.861), range 65.35 μg/mL–80.58 μg/mL (log = 1.815–1.906), whilst *S. magellanica*-derived species LC_{50} was 34.93 μg/mL (log = 1.543), range 31.65 μg/mL–38.55 μg/mL (log = 1.500–1.586) (Fig. 2A). A statistically significant difference was found regarding LC_{50} for both species ($p = 0.0001$ ($F = 51.59$)).

3.2. L. panamensis promastigote susceptibility to L. sericata and S. magellanica larval ES

L. sericata-derived ES IC_{50} was determined on parasite promastigotes, giving 41.44 μg/mL (log = 1.617), range 38.57 μg/mL–44.52 μg/mL (log = 1.586–1.649), compared to *S. magellanica* ES where IC_{50} was 23.42 μg/mL (log = 1.370), range 22.48 μg/mL–24.39 μg/mL (log = 1.352–1.387) (Fig. 2B). LC_{50} and IC_{50} dose-response curves were then analysed to determine whether there were any statistically significant differences in the analysis for the same fly species, confirming that both ES had a more toxic effect on promastigotes compared to their effect on macrophages. There were statistically significant differences for both *L. sericata* ($F = 35.98$, $p = < 0.0001$) and *S. magellanica* ($F = 49.89$, $p < 0.0001$) (Fig. 2C and D). Selectivity indexes were determined [*L. sericata* (1.751) and *S. magellanica* (1.49)].

3.3. Amastigote susceptibility to L. sericata and S. magellanica larval-ES determined by microscope

Infection percentage at negative control points (no treatment) varied slightly between experiments; this was 30.06% ± 6.26 for infected cells treated with *L. sericata* larval-ES and 34.56% ± 6.40 for infected cells treated with *S. magellanica* larval-ES (Table 2). Intracellular amastigotes from the same infection batch that was treated with larval-ES, were treated with 100 μg/mL glucantime in order to have a positive control for each experiment. Positive control infection percentage for *L. sericata* larval-ES experiments was 10.94% ± 3.63 and for *S. magellanica* larval-ES experiments was 14.83% ± 3.85. This gave a reduction of 67.7% ± 18.05 in infection percentage in the positive controls for the batch of infected cells that were treated with *L. sericata* larval-ES and 54.81% ± 28.33 for cells treated with *S. magellanica* larval-ES (Table 2 and Fig. 3). Larval-ES treatment at different concentrations for both species produced a drop in infection percentage; *L. sericata* larval-*ES* infection percentage decreased 57.90% ± 41.08 and 77.37% ± 13.55 when using 10 μg/mL and 20 μg/mL, respectively, observing less variability in data as the larval-ES dose increased (Table 2 and Fig. 3). *S. magellanica* larval-ES produced a reduction in infection percentage of 58.41% ± 15.48 by using 5 μg/mL (Table 2 and Fig. 3). Minimal *S. magellanica* larval-ES dose thus induced a drop-in infection percentage equivalent to that observed for positive control.

The parasite load in *L. sericata* tests was 2.30 ± 0.72 amastigotes per cell (ama/cell) for the negative control, 1.44 ± 0.15 ama/cell for positive control and 1.85 ± 0.29 ama/cell for treatment with 10 μg/mL larval-ES (Table 2). The *S. magellanica* ES negative control point was 3.00 ± 0.42 ama/cell, for positive control the parasite load was 1.82 ± 0.26 and for treatment with 5 μg/mL ES it was 1.80 ± 0.27 ama/cell (Table 2).

Table 2 shows intracellular amastigote survival index. Positive control became reduced to 15.92 ± 14.43 in *L. sericata*-derived ES assays compared to negative control (74.87 ± 56.53); treatment with 10 μg/mL gave a 32.74 ± 40.58 reduction and treatment with 20 μg/mL 14.84 ± 16.97. Concerning *S. magellanica* ES, the survival index for the no treatment point was 104.96 ± 33.95 (27.34 ± 14.93 for the positive control). This index was 25.34 ± 8.20 for 5 μg/mL concentration and 20.91 ± 3.76 for the treatment with 10 μg/mL.

4. Discussion

The present study ascertained *L. sericata*- and *S. magellanica*-derived larval-ES LC_{50} and IC_{50} for U937 cell-line macrophages to evaluate their anti-*Leishmania* activity concerning *L. panamensis* promastigotes, this being the species causing most cutaneous leishmaniasis cases in Colombia (Corredor et al., 1990; Ovalle et al., 2006; Urbano et al.,

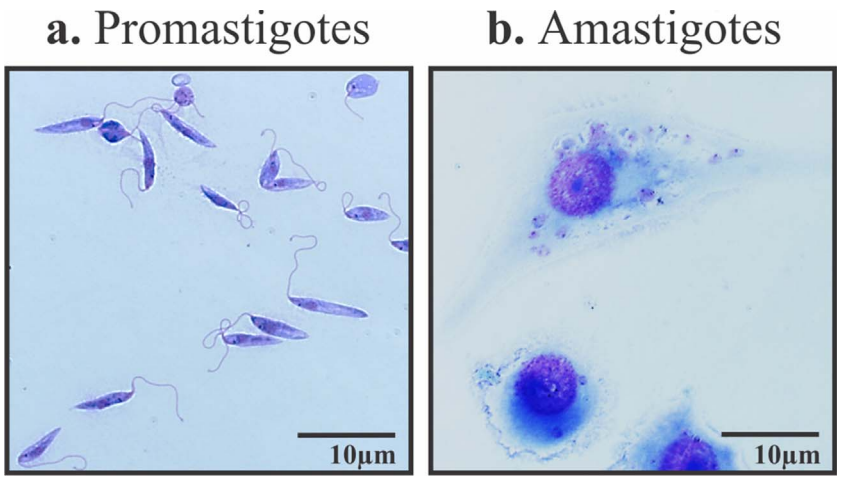

Fig. 1. Different forms of *Leishmania panamensis* stained with Giemsa. A. Free cultured promastigotes. B. Amastigotes in U937 macrophage-derived monocytes. Scale bars are shown in black.

2011).

Larval-ES cytotoxicity for the U937 human histiocytic lymphoma cell line was evaluated. The advantage of using the Alamar Blue technique is that cell lysis is not required for reading absorbance; it is sensitive, fast and relies on the effect of mitochondria, cytochrome reductase and other enzymes occurring in the cytoplasm (Escobar et al., 2012; Rampersad, 2012; Zhi-Jun et al., 1997). A previous study involving *C. vicina*- and *L. sericata*-derived larval-ES cytotoxicity tests on the J774A murine macrophage cell-line showed that both were highly toxic to cells at greater than 40% purity/concentration, thereby making it difficult to compare them due to the ambiguous concentration value (Sanei-Dehkordi et al., 2016). The effect of *S. magellanica*-derived larval-ES on fibroblasts has also been evaluated using the human lung-derived MRC5 cell-line (Pinilla et al., 2015). Viability percentages in that study did not become altered when exposed to concentrations of up to 10 µg/mL *L. sericata* and *S. magellanica* ES; instead, cells exposed to *L. sericata* ES at concentrations close to 20 µg/mL had lower viability percentages, but not cells treated with the same *S. magellanica* ES concentration. Such apparently contradictory results from the present study could be due to differences in the cell types used, variation in the larval-ES components and/or in experimental design and analysis.

The effect of larval ES derived from both blowflies evaluated here highlighted *L. panamensis* promastigote susceptibility to such substances. This agreed with Polat et al. (2012) who observed effective *L. sericata* larval ES action on *Leishmania tropica* promastigotes in spite of using a different evaluation method. However, the quantitative analysis approach attempted in the present study, by using precise larval ES concentrations will facilitate additional *in vitro* and *in vivo* experiments.

Comparing IC_{50} (parasite toxicity) and LC_{50} (host-cell toxicity) dose-response curves revealed that larval-ES from both species had a more toxic effect on promastigotes (statistically significant difference: $p < 0.0001$). A similar pattern has been recorded in another report showing a higher effect on promastigotes than on host cells (de Mello et al., 2014).

The present study found that the lethal effect of *S. magellanica*-derived larval-ES on promastigotes was higher than that for *L. sericata*-derived larval-ES; this agreed with previous findings evaluating these products' toxicity on prokaryote organisms, showing that *S. magellanica* larval-ES had a stronger effect against Gram-positive and Gram-negative bacteria than *L. sericata* larval-ES (Díaz-Roa et al., 2014).

Both species' larval-ES were found to be effective against *L. panamensis* when analysing intracellular parasites; a relevant reduction in infection percentage was observed, being even greater for ES treatment than for positive control (100 µg/mL glucantime). The decrease in infection percentage was greater than 50% with 10 µg/mL *L. sericata* and

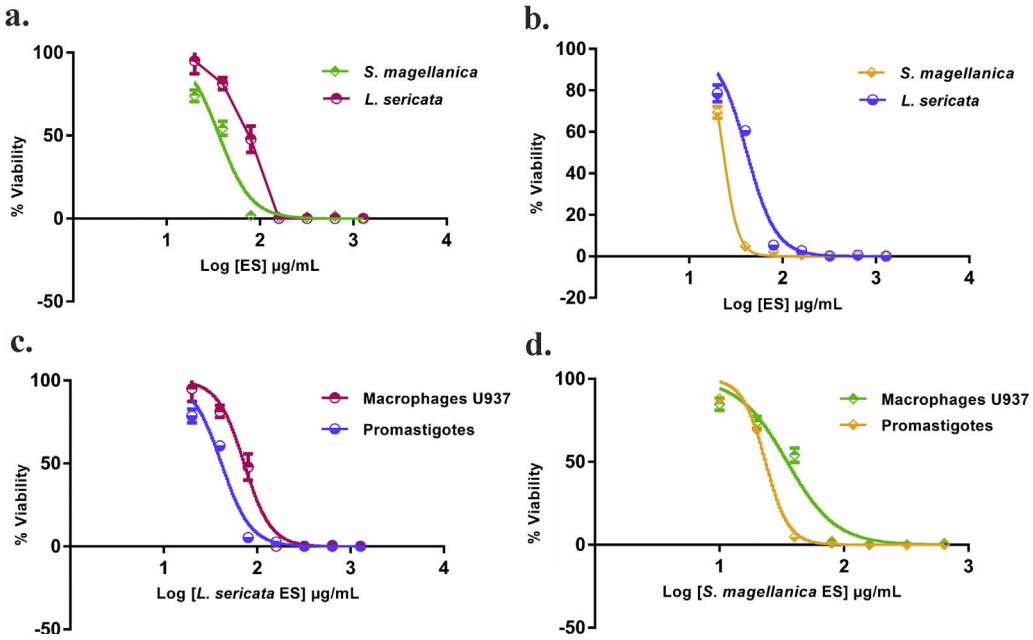

Fig. 2. Dose-response curves regarding the effect of *Lucilia sericata* and *Sarconesiopsis magellanica* larval-ES on U937-derived macrophages (LC_{50}) and *Leishmania panamensis* promastigotes (IC_{50}). A. *Lucilia sericata* LC_{50} *vs Sarconesiopsis magellanica* LC_{50} B. *Lucilia sericata* IC_{50} *vs Sarconesiopsis magellanica* IC_{50} C. LC_{50} *vs* IC_{50} *Lucilia sericata*. D. LC_{50} *vs* IC_{50} *Sarconesiopsis magellanica*.

Table 2
The effect of *Lucilia sericata* and *Sarconesiopsis magellanica* larval-ES on *Leishmania panamensis* amastigotes in *in vitro* conditions.

Treatment (µg/mL)	%I		%DI		PL		SVI	
	L. sericata	*S. magellanica*	*L. sericata*	*S. magellanica*	*L. sericata*	*S. magellanica*	*L. sericata*	*S. magellanica*
No-treatment control	30.06 ± 6.26	34.56 ± 6.40	Not applicable	Not applicable	2.30 ± 0.72	3.00 ± 0.42	74.87 ± 56.53	104.96 ± 33.95
Positive control Glucantime 100	10.94 ± 3.63	14.83 ± 3.85	67.66 ± 18.05	54.81 ± 28.33	1.44 ± 0.15	1.82 ± 0.26	15.92 ± 14.43	27.34 ± 14.93
5		13.94 ± 5.39		58.41 ± 15.48		1.80 ± 0.27		25.34 ± 8.20
10	15.44 ± 0.80	10.06 ± 6.15	57.90 ± 41.08	71.02 ± 1.52	1.85 ± 0.29	2.09 ± 0.55	32.74 ± 40.58	20.91 ± 3.76
20	7.72 ± 1.69	13.17 ± 3.76	77.37 ± 13.55	61.72 ± 2.22	1.58 ± 0.40	2.02 ± 0.49	14.84 ± 16.97	26.65 ± 3.74
40	9.72 ± 4.01	22.50 ± 6.99	70.35 ± 11.95	34.69 ± 2.43	1.66 ± 0.28	2.30 ± 0.54	17.18 ± 15.33	52.21 ± 13.33
60	7.94 ± 2.23	18.58 ± 1.30	75.36 ± 7.9	48.69 ± 31.05	1.41 ± 0.18	2.54 ± 0.23	12.17 ± 10.74	52.49 ± 48.99
70		19.97 ± 4.19		45.04 ± 35.70		2.14 ± 0.28		41.79 ± 30.46
80	7.06 ± 1.64		76.41 ± 0.46		1.74 ± 0.36		13.26 ± 9.75	
100	7.00 ± 0.68		77.61 ± 3.99		2.11 ± 0.31		17.46 ± 17.82	

%I: Infection percentage; %DI: Decreased infection percentage; PL: Parasite load; SVI: Survival index.

with 5 µg/mL *S. magellanica* ES (Fig. 3), implying that amastigotes could be more susceptible to ES than promastigotes, as promastigote IC_{50} was 41.44 µg/mL for *L. sericata* and 23.42 µg/mL for *S. magellanica* (Fig. 2B). This anti-*Leishmania* effect observed for intracellular parasites at low ES concentrations is a promising result for future in *in vivo* tests. *L. sericata* larval-ES LC_{50} (U937 toxicity) was 72.57 µg/mL and *S. magellanica* larval-ES LC_{50} was 41.44 µg/mL (Fig. 2A), suggesting that larval-ES would have low toxic effect on the host cells in doses that are lethal for parasites.

Analysing *L. panamensis* intracellular amastigote susceptibility to the larval-ES suggested that low concentrations were more toxic than high larval-ES concentration on parasite survival, similar behaviour has been reported in earlier work (Sanei-Dehkordi et al., 2016) evaluating *in vitro* susceptibility of *L. major* amastigotes infecting the mouse macrophage cell line J-774. However, it is worth noting that the anti-leishmanial effect, represented as decrease in percentage of infection, was more uniform in *L. sericata* larval-ES from 20 to 100 µg/mL (Table 2 and Fig. 3) compared to *L. magellanica* ES. It was also observed that the maximum reduction in infection percentage occurred between 5 and 20 µg/mL of *S. magellanica*-derived larval ES (Table 2 and Fig. 3). Nevertheless, the standard deviation of the decrease in infection percentage for cells treated with 60–70 µg/mL of *S. magellanica*-derived larval ES showed great variability between experiments [Table 2 (DI = 48.69 and SD ± 31.05 for treatment with 60 µg/mL and DI = 45.04 and SD ± 35.70 for 70 µg/mL), Fig. 3], implying that the toxicity on the host cells at such high concentrations, interferes with the microscopy reading, making data not reproducible.

The *L. sericata* treatment survival index (when analysing infection percentage) behaved more homogeneously at the different concentrations used here. The index values were low compared to those obtained for *S. magellanica* treatment (Table 2). In spite of having a more drastic fall between no treatment control point and ES points, its effect was not maintained as concentrations increased.

The low selectivity indexes for promastigotes obtained at the present study could be explained by the complex mix of active principles in larval-ES, this obstacle can be addressed by a direct evaluation of larval-ES fractions' effect on the parasite. On the other hand, the experimental approach used here did not allow to calculate the selectivity index of the apparently more susceptible amastigotes. In accordance with the results obtained, it is well known that metabolic and phenotype differences between the parasite's two cell stages are so broad and could directly affect any response to the effects caused by the ES (Coombs et al., 1982; Fiebig et al., 2015).

Using microscopy for assessing the effect on intracellular stages limited the present study; this technique may have broad acceptance for this type of study but it is more prone to error, introducing subjectivity regarding readings and variation amongst observers. This could explain why large SDs were observed for values related to reduced infection percentages. A quantitative PCR should thus be used in future studies to minimise bias concerning data recording and facilitate analysis.

Previous evidence regarding antimicrobial peptides (AMPs) in larval-ES (Boman, 1995, 2000; Kerridge et al., 2005) suggested that the effect of these substances has mainly been evaluated on Gram-positive and Gram-negative bacteria. Previous studies have confirmed the presence of AMPs including lucifensin (Čeřovský et al., 2010), lucimycin (Poppel et al., 2014), as well as cecropins, diptericins and proline-rich peptides (Pöppel et al., 2015) in *L. sericata* acting on a wide spectrum of microorganisms. Such findings highlight ES having an anti-*Leishmania* effect, as there are reports of AMPs from other organisms, such as amphibians, mammals, plants and invertebrates, having proven anti-parasite action, triggering several action mechanisms on promastigotes and amastigotes from the following species: *L. donovani*, *L. infantum*, *L.*

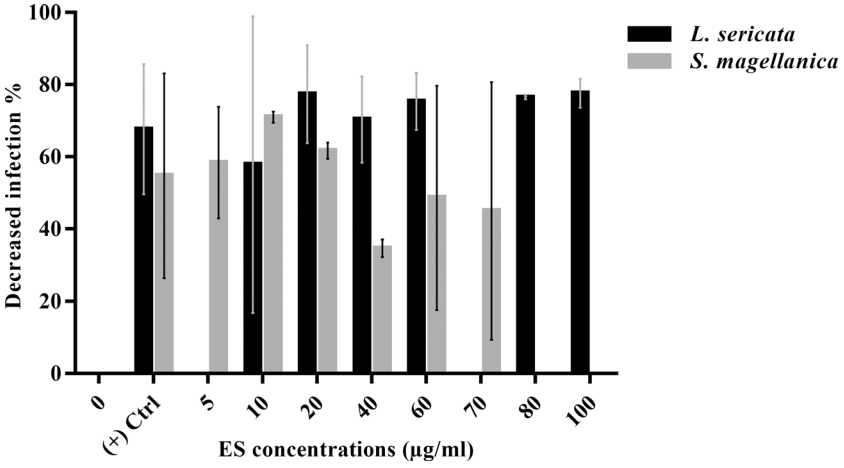

Fig. 3. Evaluating *Leishmania panamensis* amastigote susceptibility to *Lucilia sericata* or *Sarconesiopsis magellanica* larval-ES. Decrease in infection percentage for treatment with *Lucilia sericata* (black bars) or *Sarconesiopsis magellanica* (gray bars) larval-ES.

amazonensis, *L. major*, *L. mexicana* and *L. braziliensis* (Torrent et al., 2012). AMPs may thus have been the main constituents of larval ES producing an anti-leishmanial effect (derived from both flies in the present work).

Other reports (van der Plas et al., 2009a,b) have shown that larval-ES have anti-inflammatory effects which could contribute towards reducing lesion development and leishmaniasis immunopathogenesis, a factor which could have better efficacy regarding action in *in vivo* applications. Some work on *Leishmania* strains in *in vivo* conditions (all having led to promising results concerning treatment for controlling the microorganism's proliferation) has provided evidence of LT and larval-ES effectiveness concerning the *Leishmania* parasite, *i.e.* the sub-genus *L. amazonensis* (Arrivillaga et al., 2008), *L. tropica* (Polat et al., 2012) and *L. major* species (Sanei-Dehkordi et al., 2016) and the *Viannia* sub-genus and *L. panamensis* species (Cruz-Saavedra et al., 2016) concerning cutaneous lesions in BalB/c mice and hamsters. The only reports regarding *in vitro* models have been concerned with *Leishmania* sub-genus species (Polat et al., 2012; Sanei-Dehkordi et al., 2016) determining that 5% concentration *L. sericata* and *C. vicina* larval-ES might reduce the amount of infected macrophages and amastigotes per cell (Sanei-Dehkordi et al., 2016). Work by Polat et al. (2012) used microscope follow-up for evaluating anti-leishmanial *L. sericata* larval ES activity on promastigote stage; the present study confirmed this, even though adapting a more consistent methodological approach. *S. magellanica* larval ES anti-leishmanial effect in *in vitro* conditions has been demonstrated here against the parasite's intracellular form and against *L. panamensis* promastigotes.

5. Conclusions

The direct effect of *L. sericata* and *S. magellanica* larval-ES, simultaneously, on different *L. panamensis* (*Viannia*) stages has been evaluated for the first time by a quantitative approach in this study; their cytotoxicity on the U937 human macrophage cell-line was also assessed. It should be noted that *S. magellanica* larval-ES had equivalent effectiveness at low concentrations when compared to *L. sericata*-derived larval-ES, thereby highlighting a greater effect for *S. magellanica* and its usefulness in future applications. Further studies are required for discerning the larvae ES components involved in anti-*Leishmania* activity.

Conflicts of interest

All authors declare that they have no conflict of interests.

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