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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.