Full length article

Anti *Leishmania* activity of *Lucilia sericata* and *Calliphora vicina* maggots in laboratory models

Alireza Sanei-Dehkordi a, b, Ali Khamesipour c, *, Kamran Akbarzadeh d, Amir Ahmad Akhavan d, Akram Mir Amin Mohammadi c, Younes Mohammadi e, Yavar Rassi d, Mohammad Ali Oshaghi d, Zahra Alebrahim f, Seyed Ebrahim Eskandari c, Javad Rafinejad d, **

a Department of Medical Entomology and Vector Control, Faculty of Health, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
b Social Determinants in Health Promotion Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
c Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran
d Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
e Department of Biostatistics & Epidemiology, School of Public Health, Hamadan University of Medical Sciences, Hamadan, Iran
f Department of Pediatrics, Faculty of Medicine, Hormozgan University of Medical Sciences, Bandar Abbas, Iran

**Corresponding author.

E-mail addresses: ali.khamesipour@gmail.com (A. Khamesipour), jrafinejad@yahoo.com (J. Rafinejad).

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Abstract

Use of sterile fly larvae (maggots) of blow flies for the treatment of many different types of skin and soft tissue wounds is called Maggot debridement therapy (MDT). The larvae of blow flies secrete a broad spectrum of compounds with diverse mechanisms of action in the gut and salivary glands called excretion/secretion (ES) products which showed to have antimicrobial activities against Gram negative and positive bacteria. Cutaneous leishmaniasis (CL) which is the common form of leishmaniasis is difficult to treat.

In this study, the effect of ES from 2nd and 3rd stages of *L. sericata* and *C. vicina* larvae on *in vitro* *Leishmania major* amastigote growth in macrophage was evaluated. The effect of ES on *Leishmania* growth was estimated by assessing the rate of macrophage infection and the number of amastigotes per infected macrophages. In addition, the anti *Leishmania* activities of larval and ES of *L. sericata* and *C. vicina* on the skin lesion induced by *L. major* infection was evaluated in susceptible BALB/c mice.

Highlights

- Anti *Leishmania* activity of *Lucilia sericata* and *Calliphora vicina* maggots on *Leishmania major* were investigated.
- *L. sericata* excretion/secretion (ES) was significantly more effective than *C. vicina* ES against *L. major*.
- Pre-treatment with ES derived from *L. sericata* may have some protective effects on the development of *L. major* lesion.
- ES of *L. sericata* maggots can be considered as a candidate for the treatment of cutaneous leishmaniasis.
1. Introduction

Leishmaniasis is a vector-borne disease caused by various species of intracellular Leishmania parasites which reside and proliferate solely in mammalian macrophage lineage (Bogdan and Röllinghoff, 1999). Leishmaniasis reported from 98 countries on four continents. According to World Health Organization (WHO) estimation, 10th of the world population are currently at risk with 12 million prevalence and 1–1.5 million incidence rate for cutaneous form (CL) and 0.5 million for visceral form of the disease (Alvar et al., 2012; WHO 2010a,b). Leishmaniasis is one of the 15 most Neglected Tropical Disease (NTD) (WHO 2010a,b). Clinical manifestations of the disease classified in 4 major forms including cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). Cutaneous leishmaniasis is a self-healing skin disease and the most common form of the disease. More than 90% of the CL occurs in seven countries including Iran. Zoonotic CL (ZCL) caused by Leishmania major and Anthroponotic CL (ACL) induced by Leishmania tropica are endemic in different parts of Iran. Among 17 out of 31 provinces of Iran, about 90% of reported leishmaniasis belongs to ZCL with a large geographical distribution (Akhavan, 2011; Khamesipour, 2014).

Maggot debridement therapy (MDT) is the medical term of using sterile fly larvae (maggots) for the treatment of chronic wounds. The use of maggot-therapy in medicine has been practiced sporadically for centuries and revived in recent years (Fleischmann et al., 2004). The most commonly used species of flies are blowflies, Calliphoridae family, including Lucilia sericata and Calliphora vicina. However, L. sericata which is commonly called green bottle fly is used mostly in MDT (Sherman et al., 2013). To our knowledge through search in internet only one study investigated. In vivo the effect of larvae and ES of L. sericata and C. vicina on the size of skin lesion induced by L. major infection in BALB/c were monitored.

2. Materials and methods

2.1. Rearing of flies

The female adult L. sericata and C. vicina are collected and identified (James, 1947; Whitworth, 2006) and then each female was placed in a plastic cup with a piece of fresh cow meat (10 g) for egg laying. Deposited eggs were transferred into a bigger cylindrical jar. When the larval growth was completed, 3rd instar larvae and larvae in post-feeding stage were transferred into another plastic container with dry wood chips for pupation. The pupae were transferred into big cages (40 × 40 × 40 cm) and incubated for 5 days for emerging of adults. The adults were placed at least 20 pairs in each cage. The colonies were maintained at insectary condition at 25 ± 1 °C, 16:8 light and dark period and 60 ± 5% of relative humidity. A cotton pad which was soaked in a sugar solution and a piece of chicken liver was placed in each cage for feeding of adult flies. A piece of fresh cow meat was placed in each cage as a substrate for egg laying. The rearing process continued till the fourth generation (F4) and to the end of the study period.

2.1.1. Sterile maggots preparation

Eggs were collected from the cages and washed using sterile distilled water and subsequently the surface of the eggs was sterilized using 1% sodium sulfite in normal saline containing 2.5% formaldehyde. The eggs were then transferred into the blood agar plates and incubated at 25–27 °C. The larvae were removed from the blood agar plates at 48 h after the hatching and washed with sterile distilled water and filtered through a sieve.

2.2. Collection of the ES

In this study, sterile second and third-instar larvae of L. sericata and C. vicina were separately transferred into sterile 200 ml-conical flasks in a density of 100 larvae in 750 μl of Phosphate Buffered Saline (PBS). The larvae were incubated in dark at room temperature (25 ± 2 °C) for 5 h. The resultant liquids in the flask were collected and centrifuged at 13,000 × g for 7 min to remove particulate materials. Subsequently, ES was sterilized using millipore bacterial filters (0.22 μm) and then were transferred immediately into sterile tubes, about 20 ml of ES was prepared for each species, then was aliquoted, 1 ml per cryovial and were stored at −75 °C until use.
2.3. Cell culture

Murine macrophage cells (line J774A- ECACC number 91051511) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin and streptomycin (200 μg/ml).

2.4. Cytotoxicity tests

Mouse macrophages (line J774A) were cultured and treated with different concentrations of ES (5%, 10%, 20% and 40%) for 72 h. As a control, J774A cell line was treated with PBS. The experiment was done in triplicate. The viability of treated J774A was checked using tripian blue. The cytotoxic concentration for 50% (CC50) and 90% (CC90) of cells were calculated using Probit analysis (Finney, 1947).

2.5. In vitro Leishmanicidal activity assays

For each amastigote assay 200 μl of J774 A.1 murine macrophage cell lines (10^6 cells/ml) were attached to the 8-chamber slide (Lab-Tek, Nalge Nunc International NY, USA), and incubated at 37 °C with 5% CO2 for 24 h. Then the promastigotes of L. major harvested at stationary phase at a ratio of 10:1 were added to the adherent macrophages and incubated for an additional 12 h. Non phagocytosed parasites were removed by washing, and the infected cells were treated with different concentrations of ES (0.625, 1.25, 2.5 and 5%) and incubated for another additional 12 h. The cells were washed three times and cultured for additional 72 h. Every experiment was performed in triplicate. Negative and positive controls were included; macrophage without amastigote and macrophage with amastigotes without treatment, respectively.

Then the slides were dried, fixed, stained using Giemsa and examined under a light microscope. The effect of ES on Leishmania growth was studied by checking two criteria; first, the mean number of infected macrophages in 100 counted macrophages and the second, the mean number of amastigotes per 100 counted macrophages.

2.6. Animals and parasites

All experimental procedures involving animals were approved by the Ethics Committee of Tehran University of Medical Sciences.

BALB/c mice were purchased from Pasteur Institute, Tehran, Iran and maintained in the animal house of the Center for Research and Training in Skin Disease and Leprosy, Tehran, Iran.

Female BALB/c mice, 6–8 weeks old were subcutaneously (SC) inoculated with 2 × 10^6 L. major (MHROM/IR/75/ER) promastigotes, harvested at stationary phase in 50 μl into the hind left footpad.

Upon lesion development, the mice were randomly divided into 10 groups (10 mice per group) and were treated according to Fig. 1. Briefly, preimmunization was done by 3 subcutaneous injections of larval secretions in the hind left footpad at the same site of L. major inoculation on days 11, 9 and 4 before L. major inoculation and 4 injections at one-day interval after L. major inoculation (G1, G2).

Groups G3 and G4 were received ES mixed with L. major and inoculated SC and then were treated with ES, 4 times at one day intervals. At day 42 after L. major inoculation, two groups (G5, G6) were treated with free larval, as free larval were placed on footpad lesion 5 times at 48 h interval, two groups (G7, G8) were treated with larval secretions 6 times at days 2, 6, 9, 12, 21, one group (G9) was treated with standard treatment meglumine antimoniate (Glucantime®) 200 mg/kg IP daily for 30 days, one group (G10), only were inoculated with L. major and kept as control. Two perpendicular diameters of the footpad lesions were measured weekly for 15 weeks by using a vernier caliper and compared with the thickness of the uninfected footpad.

2.7. Statistical analysis

SPSS ver.18 program was used to analyze the data, independent t-test and one-way ANOVA were used to compare the data. Probit analysis was used to compare the macrophage infectivity data. The method of Finney was used to determine IC50 [Inhibitory concentration (50%)] values for the respective species (Finney, 1947). A repeated measure analysis of variance (ANOVA) was used to analyze the data, p < 0.05 was considered significant.

3. Results

The results of cytotoxicity tests of ES was obtained for both species of the flies which showed that the concentration of 40% was highly toxic to J774A cell line with cell viability <20%, while the concentration of 5% showed no toxic effect on the cells with cell viability >98% (Fig. 2). The CC50 and CC90 values of ES from L. sericata and C. vicina larvae on J774A cell line were 16.32% and 40.11%, 18.7% and 48.01, respectively.

The effect of ES of L. sericata and C. vicina on in vitro susceptibility of L. major is presented in Table 1. In comparisons with control group it has been proved that some concentrations of ES, 1.25%, 2.5% and 5% from L. sericata and 2.5% and 5% from C. vicina, significantly inhibited the mean number of infected macrophages and the mean number of amastigotes per each macrophage (1.25%, 2.5% and 5% from L. sericata and 5% from C. vicina) (p < 0.05) (Fig. 3). It has also revealed that the dosage of 5% in both flies was the most effective on reducing the number of infected macrophages. The IC50 values infection rate of L. major amastigotes were 0.84% and 1.36% for ES of L. sericata and C. vicina, respectively (Fig. 4, Table 2).

Experimental results of measuring the lesion development after parasite inoculation in study groups at 15 consecutive weeks showed that G4 group showed the largest mean lesion size and the G1 group showed the smallest mean lesion size (Fig. 5).

The results of lesion development in BALB/c mice in study groups were compared with the control group (G10) (Fig. 6). The mean size of lesions in seven groups (G1, G2, G3, G4, G5, G6, and G8) were smaller than the control group (G10), on the contrary, the mean size of lesions in two groups (G2, G4) were more than the control group (G10). Significant difference was observed between the groups 1, 2, 3, 7, 8, 9 compared with the control group (G10) (p values < 0.02). There was no statistical difference between groups G4, G5 and G6 compared with the control group (p values > 0.24).

On the other hand, the mean size of lesion in the group received Glucantime® (G9) compared with the other groups showed that the mean size of lesions in the groups of eight groups (G2, G3, G4, G5, G6, G7, G8, and G10) were larger than the group G9, but this difference was not significant in the two groups (G3, G7). The mean size of the lesions in G1 was smaller than the G9 but this difference was also not significant (Fig. 7).

4. Discussion

Cutaneous leishmaniasis is the most common form of leishmaniasis. The pentavalent antimonials (meglumine antimoniate and sodium stibogluconate) remain the first line treatment for CL; however, the toxicity, high cost, multiple injections, long-term treatment, emerging resistance justify to search for new therapeutic strategies (Croft et al., 2006; Hadighi et al., 2007; Tuon et al., 2008). Different medications such as ketoconazole (Momeni et al., 2003; Singh et al., 1995), Paromomycin, dapsone (Osorio et al.,
allopurinol (Velez et al., 1997), mefloquine (Hendrickx et al., 1998), paromomycin (Bavarsad et al., 2012; Jaafari et al., 2009; Mohebali et al., 2004; Shazad et al., 2005), gentamicin (Tolouei et al., 2011), verapamil (Shokri et al., 2012) have been used to treat CL with limited efficacy and as such justify search for novel new medications.

Sterile maggots of blowflies apply to various chronic wounds. The maggots’ proteolytic enzymes including collagenase, trypsin-like and chymotripsin-like enzymes, take part in the breakdown of macromolecules on the wound surface (Beasley and Hirst, 2004). Previous study showed that compounds such as lysozymes present in the gut of L. sericata and showed antibacterial activities (Valachova et al., 2014). Moreover, various antimicrobial peptides such as Lucimycin (Poppel et al., 2014), Lucifensin (Cerovsky et al., 2011, Cerovsky et al., 2010) and Lucifensin II (Shazely et al., 2013) have been identified in maggot ES. The maggots’ ES have been studied for antibacterial activities against a variety of Gram negative and positive bacteria (Daeschlein et al., 2006; Huberman et al., 2007; Jaklic et al., 2008; Kerridge et al., 2005).

In this study, in vivo and in vitro effect of larval and their ES of the L. sericata and C. vicina on growth of L. major under in vivo and in vitro conditions were evaluated.

### Table 1

The effect of various concentrations of ES from Lucilia sericata and Calliphora vicina on in vitro growth of Leishmania major in J774A cell line.

<table>
<thead>
<tr>
<th>Concentrations (%)</th>
<th>L. sericata</th>
<th>C. vicina</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of infected macrophage ±SD</td>
<td>Number of amastigote ±SD</td>
</tr>
<tr>
<td>0 (Control)</td>
<td>56.33 ± 4.11</td>
<td>68.33 ± 5.56</td>
</tr>
<tr>
<td>0.625</td>
<td>51.00 ± 2.83</td>
<td>62.67 ± 3.40</td>
</tr>
<tr>
<td>1.25</td>
<td>47.33 ± 3.21*</td>
<td>58.00 ± 3.61*</td>
</tr>
<tr>
<td>2.5</td>
<td>37.67 ± 2.52*</td>
<td>46.33 ± 3.21*</td>
</tr>
<tr>
<td>5</td>
<td>21.67 ± 4.04*</td>
<td>33.67 ± 4.51*</td>
</tr>
</tbody>
</table>

*A significant difference compared with control group was observed (P value < 0.05).*

The results showed that the concentration of 5% ES from both flies’ species can reduce the number of infected macrophages about...
2.6 and 1.5 fold for *L. sericata* and *C. vicina* respectively. The inhibition growth of amastigotes in macrophages was 2.03 and 1.36-fold reduction when treated with ES of *L. sericata* and *C. vicina* respectively, which seems that the ES were competent enough to reduce the number of infected macrophages and the mean number of amastigotes per macrophage.

The findings also indicated that some concentrations of ES of *L. sericata* were significantly more effective than those of *C. vicina* on inhibiting the number of infected macrophages and the number of amastigotes infected macrophage ([Fig. 3](#fig3)). The results showed that the *L. sericata* ES was more effective on inhibition of growth of *Leishmania* in infected macrophages than *C. vicina* ES.

Regression analysis showed a concentration dependent significant correlation of the ES exposure with infected macrophages. The Chi-Square ($\chi^2$) values for inhibitory tests showed heterogeneity and non-heterogeneity in *L. sericata* ES and *C. vicina* ES, respectively.

Significant differences have been shown between the study groups; the mean lesion size during 15 weeks (F: 10.68, p: 0.0001 after Greenhouse-Geisser correction). In general, the results showed that the effect of *L. sericata* ES on decreasing the size of the lesion development after *Leishmania major* inoculation in BALB/c mice in study groups.

![Fig. 3. Comparative effectiveness of ES from *Lucilia sericata* and *Calliphora vicina* on the average number of *Leishmania major* amastigotes per infected macrophage by examining 100 macrophages.](#fig3)

![Fig. 4. Probit regression line of ES from *Lucilia sericata* and *Calliphora vicina* on the infection rate of *Leishmania major* amastigotes per infected macrophage by examining 100 macrophages.](#fig4)

![Fig. 5. The mean size of lesion development after *Leishmania major* inoculation in BALB/c mice in study groups.](#fig5)

![Fig. 6. The difference between mean size (cm) of lesion induced by *Leishmania major* inoculation in BALB/c mice in the control group (G10) compared with the other study groups [Y-axis represents the control group (G10)].](#fig6)

### Table 2

Probit regression line parameters of ES from *Lucilia sericata* and *Calliphora vicina* on the infection rate of *Leishmania major* amastigotes per infected macrophage by examining 100 macrophages.

<table>
<thead>
<tr>
<th>Excretion/secretion (ES) products</th>
<th>A</th>
<th>Slope ± SE</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (%)</th>
<th>95% C.I.</th>
<th>$\chi^2$ (df)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. sericata</em></td>
<td>-0.066</td>
<td>-0.67 ± 0.199</td>
<td>0.84</td>
<td>0.84</td>
<td>6.34 (2)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><em>C. vicina</em></td>
<td>0.072</td>
<td>-0.54 ± 0.109</td>
<td>1.36</td>
<td>1.36</td>
<td>0.15 (2)</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

A = y-intercept, B = the slope of the line, SE = Standard error, IC50, 95% C.I. = Inhibitory concentration (%) causing 50% Inhibitory and its 95% confidence interval, $\chi^2$ = heterogeneity about the regression line.
lemon is more effective than use of its free larval. In vivo results suggested that pre-treatment with ES from L. sericata may have some protective effects on the development of L. major lesion and later on the size of the lesion was smaller.

To our knowledge, there is no data on the effect of maggot ES on Leishmania major infection. Current results are in parallel with a similar study which was performed by Polat et al. (2012), in inhibitory effect on L. tropica promastigotes in vitro and on amastigote forms in vivo conditions, the results of this study support our findings that the L. sericata had effects on Leishmania parasite (Polat et al., 2012). There is another report about the therapeutic effects of the L. sericata maggot on the skin lesion caused by L. amazonensis on Mesocricetus auratus, results have shown that the size of lesion decreased 80%–100% after using of maggot (Arrivillaga et al. 2008). However, the therapeutic effects on reducing the size of lesion were significantly higher than our finding.

Maggot-based products that purify maggot-derived substances with conventional technology have recently been developed and may bring further benefits. It seems necessary to screen maggot’s compounds as pharmaceutical products and as candidate for the treatment of some skin ulcerative diseases.

In this study, it was demonstrated that ES of L. sericata has a significant effect on L. major growth in vivo and in vitro conditions. It seems that maggots’ ES of L. sericata might be considered as a candidate for the treatment of cutaneous leishmaniasis. However further research is necessary to clarify the mechanisms of parasite growth inhibition by maggot ES and identify the effective substances in ES.

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