Original Article

A Survey on Inhibitory Effect of Whole-Body Extraction and Secretions of *Lucilia sericata*'s Larvae on *Leishmania major In vitro*

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Abstract

Background: Leishmaniasis is a skin disease caused by *Leishmania* parasite. Despite being self-limiting, must be treated. Available drugs have side effects and drug resistance has also been seen. **Materials and Methods:** Maggot debridement therapy (MDT) is using sterile fly larvae (maggots) of blow flies (*Lucilia sericata*) for the treatment of different types of tissue wounds. Larvae have excreted and secreted substances that have been proved to have antimicrobial effects, in addition to the some other specifications. **Results:** In this study, the anti-leishmanial effects of extracts and secretions of sterile second- and third-instar larvae of *L. sericata* on the growth of *Leishmania major* promastigotes and amastigotes in the J774 macrophages have been evaluated *in vitro*. **Conclusion:** The results showed that extracts and secretions had almost the same leishmaniocidal effect on promastigotes and intracellular amastigotes without cytotoxic effect on macrophages.

Keywords: *Leishmania major, Lucilia sericata larvae, maggot debridement therapy*

Introduction

Leishmaniasis is a parasitic disease caused by different species of *Leishmania* from trypanosomatidae family with cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) forms. According to the reports from 98 countries, there is 12 million prevalence, 1.5 million incidence of cutaneous form (CL), and 0.5 million for visceral form. Three hundred and fifty million people are at risk according to the World Health Organization estimations.[1]

Despite being self-limiting in most cases, CL cause permanent scars on the skin, which even after full healing, can have a lot of social impact on patient's life.[2]

The first-line drugs are pentavalent antimonials compounds (SbV) including meglomine antimonate (glucantime), and sodium stibogluconate (pentostam). Some of the other agents are amphotericin B, aromatic diamidines, and paromomycin (aminosidine). Getting benefit of these treatment methods is limited by renal and cardiac toxicity, relapse, drug resistance, adverse drug reaction, and high costs of treatment.[3]

Hence, the mentioned restrictions in the treatment of leishmaniasis have led the researchers to focus on some ancient treatments. Maggot debridement therapy (MDT) has been known for centuries. It is using fly larvae (maggots) to recover some intractable wounds such as pressure ulcers,[4] venous stasis ulcers,[5] neurovascular ulcers such as diabetic foot wounds,[6] traumatic and postsurgical wounds,[7] osteomyelitis,[8] and burns.[9] They do this by secreting a vast spectrum of compounds with various mechanisms of action in the gut and salivary glands named excretion/secretion (ES). Three main mechanisms have been known in MDT: debridement or cleaning wound healing by the stimulation of wound granulation and subsequently repair and disinfection.[10] Maggot therapy was confirmed by the FDA in 2004, and its compliance is increasing worldwide because of its efficacy, safety, and simplicity.[11]

The main species that have been used by researchers are blowflies, Calliphoridae family, including *Lucilia sericata* and *Calliphora vicina*. However, *L. sericata* (green bottle fly) is the most widely used.[12]

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Some evidence of ES of *L. sericata* larvae effects on leishmaniasis was discovered by Sanei-Dehkordi *et al*.,[13] but the antileishmanial studies are very rare, and hence, the aim of this study was to assess the efficacy of whole-body extraction and secretions of *L. sericata* on the growth rate of *Leishmania major* amastigotes and promastigotes *in vitro*.

Materials and Methods

Collection of the excretion/secretion

Sterile second- and third-instar larvae of *L. sericata* (obtained from the larval breeding center) in a density of 100 larvae in 750 ml of phosphate-buffered saline (PBS) were transferred into 200 ml sterile-conical flasks. After incubation of larvae at room temperature (25°C ± 2°C) and darkness for 5 h, the resultant liquids in the flasks were collected and centrifuged at 13000 g for 7 min to remove particulate materials. Obtained ES was sterilized using Millipore bacterial filters (0.22 mm) and then was aliquoted, 1 ml per sterile cryo-vial and were stored at −70°C.[13]

Collection of the extract

At first, for the purpose of separating foreign matter and obtaining 100 ml of the extract, 160 s and third stages larvae were washed in 110 ml of autoclaved distilled water. They were then centrifuged at 4°C and 2500 g for 10 min. After removing the supernatant, the larvae were placed at −70°C. In order to prepare the extract, the frozen larvae were homogenized with sterile glass rod and 120 ml of PBS was added, the tubes were centrifuged at about 5000 g for 10 min at 4°C. The supernatant was collected as extract and was sterilized using Millipore bacterial filters (0.22 mm).[14]

Antipromastigote assay

The cryopreserved form of *L. major* (MRHO/IR/75/ER) was prepared from Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. Promastigotes were cultured in NovyMacNeal-Nicollem medium with 100 µg/mL streptomycin, 100 IU/mL penicillin, and 100 µg/mL gentamycin and then for mass production were subcultured in RPMI-1640 (Gibco, UK) supplemented with 10% FBS (fetal bovine serum).

The inhibitory effect of *L. sericata* ES and extract against *L. major* promastigotes were determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, 200 µL medium contain Promastigotes (5 × 10^6 promastigote/mL) were seeded into 96-well microtiter plates in the presence of different concentrations of ES (2.3, 4.6, 9.37, 18.75, 37.5, 75, and 150) µg/dl and extract (3.12, 6.25, 12.5, 25, 50, 100, and 200) µg/dl and incubated at 24°C ± 1°C for 24 h. The negative control was complete RPMI 1640 medium with no parasites, and the positive control was complete RPMI 1640 medium with parasites without treating. The medium was discarded, and the cells were incubated with MTT solution (5 mg/mL in PBS) for 4 h and the resulting formazan crystals were solubilized with 100 µl of acidic isopropanol 0.04 N and 50 µl of dimethyl sulfoxide. The absorption was measured using an ELISA reader in 570 nm after incubation for 15 min at room temperature. Results represent the average of three independent experiments.[15]

$$\%\text{viable cells} = \frac{\text{Absorbance of treated promastigotes} - \text{Absorbance of blank(negative control)}}{\text{Absorbance of non treated promastigotes} - \text{Absorbance of blank(negative control)}} \times 100$$

The 50% inhibitory concentration (IC50) of promastigote was calculated by regression analysis.

The J774 cell line culture

The J774 murine macrophage cell line was purchased from the Tehran Pasteur institute and was grown at 37°C and 5% Co_2 in RPMI-1640 supplemented with 20% heat-inactivated fetal calf serum (FCS) and 100 µg/ml streptomycin and 100 IU/ml penicillin in cell culture plates.

Treatment of macrophages and determination of cytotoxicity concentration (CC50)

The cultured macrophage was transferred into 96-well microtiter plates and exposing to mentioned concentrations of the ES and the extract with RPMI1640 and incubated at 37°C and 5% Co_2 for 24 h. The viability of the macrophages was measured by the MTT assay as previously described. This experiment was done in triplicate. The cytotoxic concentration for 50% (CC50) of cells was calculated by regression analysis.

Treatment of infected macrophages and determination of infection rates and multiplication index

J744 cell line was calculated by Neubauer chamber cell counting and 2 × 10^6 cells/well were transferred into a six-well plate with a 22 mm × 22 mm strile coverslip on the bottom with RPMI 1640 medium (2 ml) supplemented with inactivated FCS 20%, penicillin, and streptomycin and was incubated at 37°C, in the presence of 5% CO_2 for 5–6 days.

Macrophages were infected with stationary phase *L. major* promastigotes at a 10:1 parasite/macrophage ratio, incubated at 37°C in 5% CO2 for 4 h, after that free promastigotes removed by washing with PBS. The plates were incubated for an additional 24 h. Subsequently, each culture plate was exposed to different concentrations of the ES and also the extract and the plates were incubated for 24 h. Then, the coverslips were removed and stained with Giemsa. Tow plates without any expose were considered as control.

The number of infected macrophages and the mean number of intracellular amastigotes were determined by studying and counting at least 100 macrophages in duplicate cultures with direct microscopic examination, to obtain multiplication index (MI).[16] Macrophages with grayish cytoplasm and purple-red nucleus were examined. The amastigotes inside were oval-shaped along with kinetoplast

$$MI = \frac{\text{No. of amastigotes in experimental culture per 100 macrophages}}{\text{No. of amastigotes in control culture per 100 macrophages}} \times 100$$

Statistical analysis

The results of the experiment were analyzed using the SPSS ver. 22 (Chicago, Illinois: SPSS Inc.), two-way ANOVA was used to compare the data at a confidence level of $P \leq 0.05$.

Results

In this experimental study, the effect of *L. sericata* extract and ES against the promastigote and amastigote of *L. major* (MRHO/IR/75/ER) and macrophage J774 were examined.

Promastigote assay using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

In the determination of anti-leishmanial effects of ES and extract of *L. sericata* larvae MTT results showed that the percentage of promastigotes proliferation in both extract and ES treated groups was lower than the control group ($P < 0.05$). Increasing the concentration of extracts and ES decreased the viability of living cells. The highest lethal effect of maggot ES was observed at the concentration of 150 µg/dl (41.38% viability) and for extract the highest lethality (35.94% viability) was observed at 200 µg/dl [Figures 1 and 2].

Furthermore, the rate of IC50 for the ES was evaluated about 89.91 and for extract about 136.17 µg/dl.

Macrophage assay and determination of CC50

The cultured macrophages were exposed to mentioned concentrations of ES and extract and their viability was calculated by MTT and the rate of CC50 for the ES was evaluated about 193.08 and for extract was about 281.30 µg/dl [Figures 3 and 4].

Selectivity index (SI) is an indicator that shows the least effective inhibitory effects of a drug on a microorganism in host cells and is the result of $\frac{CC50}{IC50}$.[17] In this study, SI was 2.14 for ES and 2.06 for extract. The statistical examinations did not show any difference between SI of ES and extract.

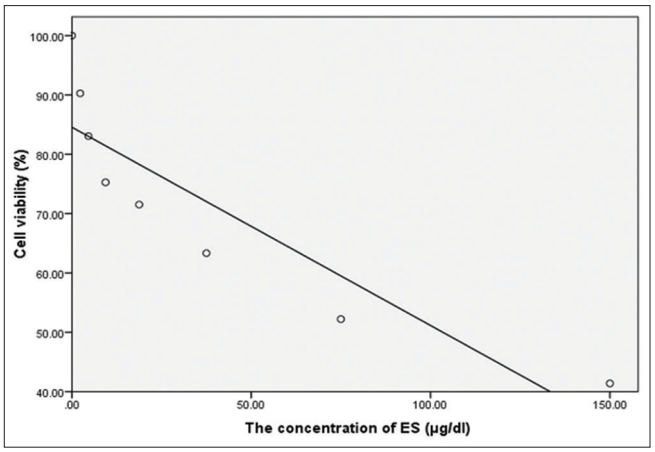

Figure 1: Inhibitory concentration of Lucilia sericata larvae excretion/secretion on promastigot of Leishmania major (MRHO/IR/75/ER)

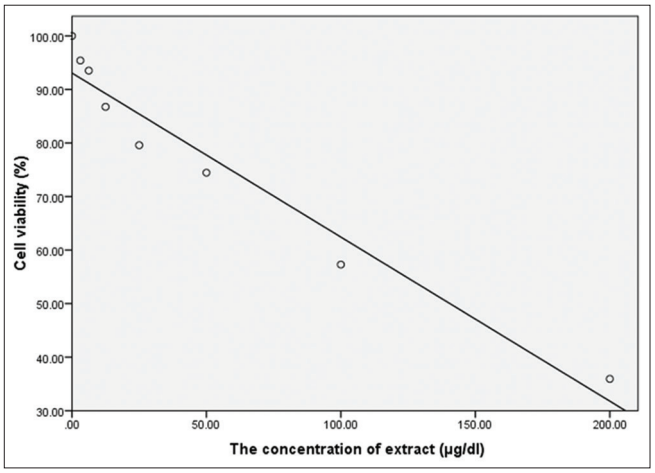

Figure 2: Inhibitory concentration of Lucilia sericata larvae extract on promastigot of Leishmania major (MRHO/IR/75/ER)

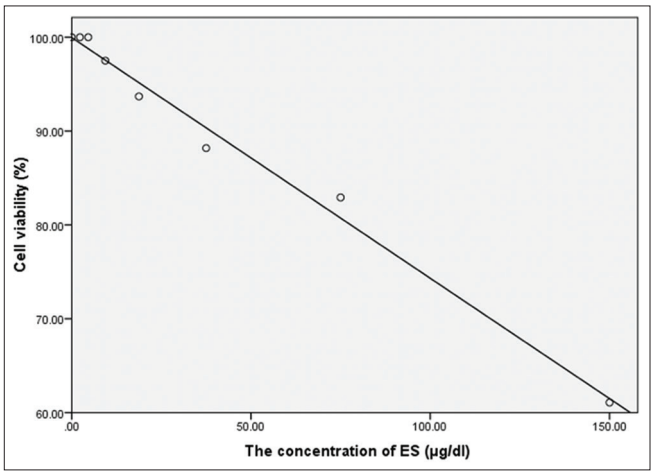

Figure 3: Cytotoxicity concentration of Lucilia sericata larvae excretion/secretion on macrophages

Anti-amastigote effect of the excretion/secretion and extract

In expose to different concentrations of ES and extract the mean number of amastigote multiplication (MI) was

decreased as the concentration of ES and extract were increased, but there was a steady parasite load in the negative control [Figure 5]. This study suggests that ES and extract of *L. sericata* larvae are effective in clearing parasite; although, there is no statistical difference between them.

Discussion

Zoonotic Cutaneous Leishmaniasis is caused by *L. major* and is endemic in some regions of Iran, such as the south, east, and central areas.[18]

Since the early nineteenth century, within the modern medicine progression, although the main focus of wound healing studies has been on the chemical drugs studies in the last decade showed the tendency to use biologic materials, including plant and animal extracts.[19,20] Larval therapy, MDT or biosurgery is the therapeutic use of fly larvae. The practice of larval therapy is increasing worldwide because of its efficacy, safety, and simplicity.[11] Larvae can debride and remove dead and necrotized tissue inside the wound due to its hook and oral fragrances, but this can sometimes be very painful. Furthermore, because of the psychological effects, the presence of alive larvae on the wound is not pleasant for patients.[11] Hence, replacement of complete larvae with larval extract including the same characteristics can be a suitable solution. Maggot's ES and extract have some proteolytic enzymes such as collagenase, trypsin-like and chymotrypsin-like. These enzymes contact with various chronic wounds and take part in the breakdown of macromolecules.[21] In Maggot's ES, various antimicrobial peptides such as Lucimycin,[22] Lucifensin I[23], and Lucifensin II[24] have been identified. Furthermore, in the gut of *L. sericata* some compounds such as lysozymes exist that have antibacterial activities against some Gram-negative and Gram-positive bacteria.[25-27]

This research revealed that the highest lethal effect with secretions was at the concentration of 150 µg/dl (41.38% viability).

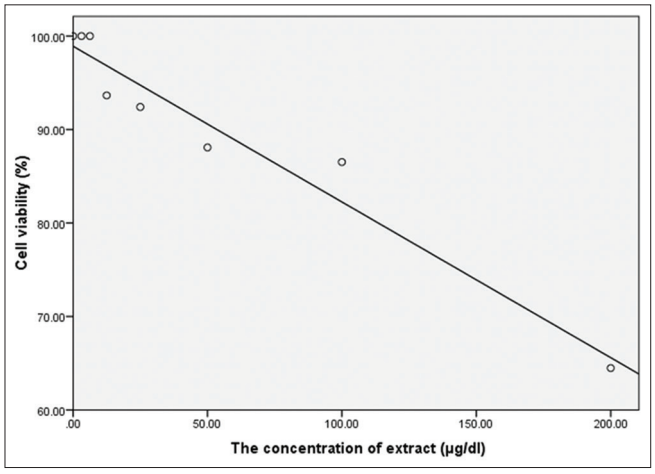

Figure 4: Cytotoxicity concentration of Lucilia sericata larvae extracts macrophages

Results showed that the IC50 of the extract against promastigotes was 136.17 µg/dl and for ES was 89.91 µg/dl and CC50 of the extract against macrophages was 281.30 µg/dl and for ES was 193.08 µg/dl. The two-way analysis statistics showed that extracts and ES had no cytotoxicity effect on macrophages with the IC50 dose on promastigotes.

According to statistical tests, there was no significant difference between the SI (Selective Index) of the extract and the ES. It shows that they had the same effect.

Sanei-Dehkordi detected that the *L. sericata* ES was effective on the inhibition of *L. major*'s growth in infected macrophages and this is in confirmation with our work.[13]

Polat *et al*. evaluated the inhibitory effect of extract of *L. sericata* on *L. tropica* promastigotes and amastigote and the results supported our findings that the *L. sericata* had effects on *Leishmania* parasite.[28] There is another report about the therapeutic effects of the *L. sericata* maggot on the skin lesion caused by *Leishmania amazonensis* in the animal model, results have confirmed 80% decrease in the size of lesion after using maggot.[29] In 2017, Laverde-Paz *et al*. examined the anti-leishmanial activity of the ES larvae, and they showed reduction in the percentage of infected macrophages and intracellular amastigotes.[30] In another report "therapeutic effects of *L. sericata* maggots and larval salivary secretion on cutaneous leishmaniasis caused by *L. major* were examined in BALB/c mice" showing that the use of salivary secretion had significant inhibitory effects on the average number of infected macrophages as well as the number of amastigotes in comparison to the control group.[31]

Conclusion

The leishmanicidal effects of *L. sericata* ES and extract were confirmed by MTT method in promastigotes and by the macrophages-amastigote model in amastigotes inside macrophages. The results proved that the ES and extract reduced the number of alive parasites and also the effect of both are equal. However, further research is necessary

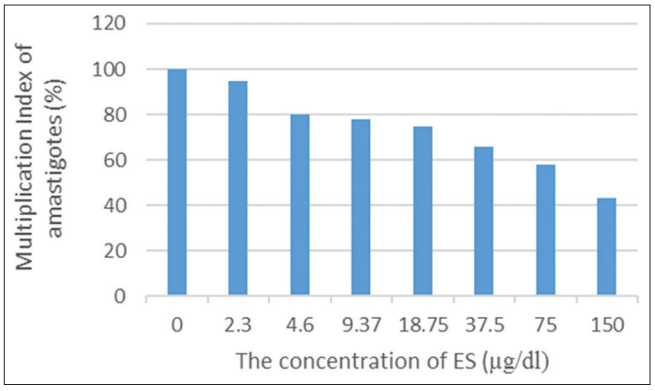

Figure 5: Multiplication index of amastigotes in expose to various concentrations of excretion/secretion of Lucilia sericata larvae

to clarify the mechanisms of parasite growth inhibition by maggot ES and extract.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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