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 other specific properties. 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 leishmanicidal effect on promastigotes and intracellular amastigotes without cytotoxic effect on macrophages.

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

Abstract

Background: Leishmaniasis is a skin disease caused by Leishmania parasites. 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 other specific properties. 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 leishmanicidal 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 amphoterin B, aromatic diaminides, 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]

Maryam Tahmasebi, Simindokht Soleimanifard, Ali reza Sanei1, Azadeh Karimy2, Seyed Mohammad Abtahi

From the Department of Medical Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, 1Department of Entomology, Zist Eltiam Sepanta Company, Azad University of Khorasgan, Technology Incubator, Center of Medicinal Plant and Traditional Medicine, Isfahan, 2Department of Medical Entomology, School of Health, Hormozgan University of Medical Sciences, Bandar-Abbas, Iran

Address for correspondence:
Dr. Seyed Mohammad Abtahi, Department of Medical Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: abtahi.mohamad@med.mui.ac.ir

Access this article online
Website: www.advbioresearch.net
DOI: 10.4103/abr.abr_56_19
Quick Response Code:
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 × 10⁴ 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% CO₂ 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% CO₂ 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 × 10⁶ 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% CO₂ 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.

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

**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 \( \mu g/dl \) (41.38% viability) and for extract the highest lethality (35.94% viability) was observed at 200 \( \mu g/dl \) [Figures 1 and 2].

Furthermore, the rate of IC50 for the ES was evaluated about 89.91 and for extract about 136.17 \( \mu g/dl \).

**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 \( \mu g/dl \) [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 \( \frac{\text{CC50}}{\text{IC50}} \).[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.

**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 bio-surgery 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 lysoenzymes 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).

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

**Acknowledgments**

The authors would like to acknowledge Isfahan University of Medical Sciences for approval and financial support of the present study.

**Financial support and sponsorship**

This study has been done by financial support of Isfahan University of Medical Sciences as a MS thesis. Nil.

**Conflicts of interest**

There are no conflicts of interest.

**References**

1. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 2012;7:e35671.

2. Ramezani P, Hejazi S, Narimani M, SoleimaniFarid S. In vitro antileishmanial activity and apoptosis induction of *Pleuratus ostreatus* alcoholic extract on *Leishmania major*. Res J Pharmacogn 2017;4:51-8.

3. Santos DO, Coutinho CE, Madeira MF, Bottino CG, Vieira RT, Nascimento SB, et al. Leishmaniasis treatment – A challenge that remains: A review. Parasitol Res 2008;103:1-10.

4. Sherman RA. Maggot versus conservative debridement therapy for the treatment of pressure ulcers. Wound Repair Regen 2002;10:208-14.

5. Sherman RA, Tran JM, Sullivan R. Maggot therapy for venous stasis ulcers. Arch Dermatol 1996;132:254-6.

6. Sherman RA. Maggot therapy for treating diabetic foot ulcers unresponsive to conventional therapy. Diabetes Care 2003;26:446-51.

7. Jukena GN, Menon AG, Bernards AT, Steenvoorde P, Taheri Rastegar A, van Dissel JT. Amputation-sparing treatment of diabetic foot ulcers unresponsive to conventional therapy. Wound Repair Regen 2002;10:208-14.

8. Sherman RA, Tran JM, Sullivan R. Maggot therapy for venous stasis ulcers. Arch Dermatol 1996;132:254-6.

9. Sherman RA. Maggot therapy for treating diabetic foot ulcers unresponsive to conventional therapy. Diabetes Care 2003;26:446-51.

10. Chambers L, Woodrow S, Brown AP, Harris PD, Phillips D, Hall M, et al. Degradation of extracellular matrix components by defined proteinases from the greenbottle larva *Lucilia sericata* used for the clinical debridement of non-healing wounds. Br J Dermatol 2003;148:14-23.

11. Whitaker JS, Twine C, Whitaker MJ, Welck M, Brown CS, Shandall A. Larval therapy from antiquity to the present day: Mechanisms of action, clinical applications and future potential. Postgrad Med J 2007;83:409-13.

12. Grassberger M, Sherman R, Gileva O, Kim C, Muncuoglu KY. Biotherapy – History, Principles and Practice. Vol. 37. Dordrecht, Heidelberg, New York, London: Springer; 2013. p. 38-9.

13. Sanei-Delkordi A, Khamesipour A, Akbarzadeh K, Akhavan AA, Mir Amin Mohammadi A, Mohammadi Y, et al. Anti Leishmania activity of *Lucilia sericata* and *Calliphora vicina* maggots in laboratory models. Exp Parasitol 2016;170:59-65.

14. Kawabata T, Mitsui H, Yokota K, Ishino K, Oguma K, Sano S. Induction of antibacterial activity in larvae of the blowfly *Lucilia sericata* by an infected environment. Med Vet Entomol 2010;24:375-81.

15. Sadeghi S, Seyed N, Etemadzadeh MH, Abediankenari S, Rafati S, Taheri T. In vitro infectivity assessment by drug susceptibility comparison of recombinant *Leishmania major* expressing enhanced green fluorescent protein or EGFP-luciferase fused genes with wild-type parasite. Korean J Parasitol 2015;53:385-94.

16. Oseo B, Mosigisi A, Ogeto T, Mugambi R, Ingonga J, Kananja R, et al. Effects of glucocorticoids in Leishmania major infection. International J Fauna Biological Studies 2015; 2 (3): 16-22.

17. Pororajab F, Ardestani SK, Foroumadi A, Emami S, Kariminia A, Behrouzi-Fardmoghdam M, et al. Selective leishmanicidal effect of 1,3,4-thiadiazole derivatives and possible mechanism of action against *Leishmania* species. Exp Parasitol 2009;121:323-30.

18. Hadighi R, Mohebali M, Boucher P, Hajarian H, Khamesipour A, Ouellette M. Unresponsiveness to glutamcine treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. PLoS Med 2006;3:e162.

19. Adiele LC, Adiele RC, Euye JC. Wound healing effect of methanolic leaf extract of *Napoleona vogelli* (Family: Lecythidaceae) in rats. Asian Pac J Trop Med 2014;7:620-4.

20. George BP, Parimelazhagan T, Kumar YT, Sajeesh T. Antitumor and wound healing properties of *Rubus ellipticus* smith. J Acupunct Meridian Stud 2015;8:134-41.

21. Beasley WD, Hirst G. Making a meal of MRSA-the role of biosurgery in hospital-acquired infection. J Hosp Infect 2004;56:6-9.

22. Pöppel AK, Koch A, Kogel KH, Vogel H, Kollewe C, Wiesner J, et al. Lucifensin, a novel insect defensin of medicinal maggots of the common green bottle fly *Lucilia sericata*. Biol Chem 2014;395:649-56.

23. Čeřovský V, Slaninová J, Fučík V, Monincová L, Bednárová L, Maloň P, et al. Lucifensin, a novel insect defensin of medicinal maggots: Synthesis and structural study. ChemBiochem 2011;12:1352-61.

24. El Shazely B, Veverka F, Fučik V, Voburka Z, Zdárek J, Cervovský V. Lucifensin II, a defensin of medicinal maggots of the blowfly *Lucilia cuprina* (Diptera: Calliphoridae). J Med Entomol 2013;50:571-8.

25. Deschlein G, Muncuoglu KY, Assadain O, Hoffmeister B, Kramer A. In vitro antibacterial activity of *Lucilia sericata* maggot secretions. Skin Pharmacol Physiol 2007;20:225-30.

26. Huberman L, Gollop N, Mumcuoglu KY, Breuer E, Bhusare SR, Shai Y, et al. Antibacterial substances of low molecular weight isolated from the blowfly, *Lucilia sericata*. J Med Entomol 2007;44:127-31.

27. Jaklic D, Lapanje A, Zupancic K, Smrke D, Gunde-Cimerman N. Cervovský V. Lucifensin II, a defensin of medicinal maggots of the blowfly *Lucilia cuprina* (Diptera: Calliphoridae). J Med Entomol 2013;50:571-8.

28. Huberman L, Gollop N, Muncuoglu KY, Breuer E, Bhusare SR, Shai Y, et al. Antibacterial substances of low molecular weight isolated from the blowfly, *Lucilia sericata*. J Med Entomol 2007;44:127-31.

29. Arrivillaga J, Rodríguez J, Oviedo M. Preliminary evaluation of root extract on *Leishmania major* susceptility comparison of recombinant *Leishmania major* expressing enhanced green fluorescent protein or EGFP-luciferase fused genes with wild-type parasite. Korean J Parasitol 2015;53:385-94.
30. Laverde-Paz MJ, Echeverry MC, Patarroyo MA, Bello FJ. Evaluating the anti-Leishmania activity of Lucilia sericata and Sarconesiopsis magellanica blowfly larval excretions/secretions in an in vitro model. Acta Trop 2018;177:44-50.

31. Kabiri M, Dayer MS, Ghaffarifar F. Therapeutic effects of Lucilia sericata larvae on cutaneous leishmaniasis wounds caused by Leishmania major using BALB/c mice as animal model. J Kerman Univ Med Sci 2017;24:389-96.