Original Article

Efficacy of a Hemolymph-Based Cream Derived from *Lucilia sericata* Larvae in Treating Cutaneous Leishmaniasis: An In Vitro and In Vivo Study in BALB/c Mice

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(Received 24 Jan 2026; accepted 21 Feb 2026)

Abstract

Background: Cutaneous leishmaniasis (CL) is a neglected tropical disease with limited therapeutic options due to drug resistance, systemic toxicity, and prolonged treatment duration associated with pentavalent antimonials such as meglumine antimoniate (MAT). *Lucilia sericata* larvae produce hemolymph containing bioactive compounds with antimicrobial and immunomodulatory properties, suggesting potential as an alternative or adjunct therapy for CL.

Methods: Hemolymph was extracted from sterile third-instar *L. sericata* larvae and characterized using SDS-PAGE and Fast Protein Liquid Chromatography. The antileishmanial activity of whole hemolymph, its most active fraction, MAT, and their combinations was assessed against promastigote and amastigote forms of *L. major*. Cytotoxicity, cytokine gene expression and reactive oxygen species production were evaluated. In vivo efficacy was examined in BALB/c mice infected with *L. major* and treated for 28 days with topical hemolymph cream, intramuscular MAT, or combination therapy. Lesion size and parasite burden were measured.

Results: Whole hemolymph and the active fraction significantly inhibited parasite growth in vitro, while combination treatments showed strong synergistic effects. Treatments enhanced Th1-associated cytokines, suppressed Th2 cytokines, and increased reactive oxygen species production. In vivo, hemolymph cream reduced lesion size and parasite load, with the greatest improvement observed in the combination group. No significant cytotoxicity was detected.

Conclusions: *Lucilia sericata* larval hemolymph exhibits potent antileishmanial and immunomodulatory activity and represents a promising and safe topical therapy for CL. Combination with MAT enhances efficacy and may reduce systemic toxicity.

Keywords: *Leishmania major*; *Lucilia sericata*; Hemolymph; Meglumine antimoniate; Maggot therapy

Introduction

Cutaneous leishmaniasis (CL) is a major global health problem and is classified by the World Health Organization (WHO) as a neglected tropical disease. Caused by protozoan parasites of the genus *Leishmania*, CL is transmitted by infected female *Phlebotomus* sand flies in the Old World. Annually, an estimated 700,000–1,000,000 new cases occur worldwide, with a high burden in the Middle East, Central Asia and parts of South America, par-

ticularly in countries such as Afghanistan, Pakistan, Iran and Brazil (1, 2). The parasite infects host macrophages, transforming from promastigotes into intracellular amastigotes, which proliferate and cause chronic, disfiguring lesions that may progress to painful ulcers and leave permanent scars, with considerable social and psychological consequences (3).

For decades, pentavalent antimonials such as meglumine antimoniate (MAT, Glucantime®) and sodium stibogluconate have been the mainstay of CL treatment. However, their use is limited by systemic toxicity (for example, cardiotoxicity, nephrotoxicity), prolonged painful injections, high costs and the emergence of drug-resistant strains (4, 5). Alternative drugs such as amphotericin B, miltefosine and paromomycin are also constrained by toxicity, variable efficacy and limited accessibility (6). This therapeutic crisis underscores the urgent and unmet need for novel, safe, effective and accessible anti-leishmanial agents (7).

Natural products derived from insects have emerged as promising therapeutic candidates. The larvae of the green bottle fly, *Lucilia sericata*, are widely used in maggot debridement therapy (MDT) for chronic wound management (8). Their secretions contain proteolytic enzymes that debride necrotic tissue and antimicrobial peptides such as lucifensin that inhibit bacterial growth, as well as factors that stimulate angiogenesis and tissue granulation (9, 10). These bioactive compounds also modulate the host immune response, downregulating harmful inflammation and potentially shifting the Th2-dominated response in CL toward a protective Th1 profile (11).

Previous studies have shown that *L. sericata* larval excretions/secretions (ES), saliva and hemolymph possess leishmanicidal activity (12–14). Hemolymph, a protein-rich fluid, contains antimicrobial peptides and proteolytic enzymes that may act directly on *Leishmania major* by disrupting parasite membranes, degrading surface proteins, and inducing oxidative stress (15, 16). These properties suggest its potential as an adjunct topical therapy alongside standard antileishmanial drugs such as Meglumine antimoniate, to accelerate lesion healing while reducing systemic toxicity (17, 18). Furthermore, low-cost, large-scale rearing of *L. sericata* enable scalable production of hemolymph-based formulations, making them economically feasible for use in endemic regions (19). Fractionation techniques such as Fast Protein Liquid Chromatography (FPLC) enable the isolation of active components for targeted evaluation (20).

The present study aimed to develop and evaluate a novel topical cream formulated from *L. sericata* larval hemolymph. We hypothesized that hemolymph could exert dual therapeutic effects: direct anti-leishmanial activity against both promastigote and amastigote forms of *Leishmania major* and modulation of the local wound environment to promote healing. We assessed the in vitro efficacy of whole hemolymph and its most active fraction, as well as the in vivo therapeutic potential of the cream in a BALB/c mouse model of CL, both alone and in combination with the standard drug, MAT.

Materials and Methods

Fly rearing and larval preparation

Lucilia sericata larvae were sourced from a long-term laboratory colony maintained for over a decade at the Cyclorrhapha Fly insectary, School of Public Health, Tehran University of Medical Sciences. Adults were housed in 46×46×46 cm mesh cages under controlled environmental conditions (27±3 °C, 45±5% relative humidity, and a daily cycle of 16 h light and 8 h dark). Adults were fed with sugar solution and supplied with beef liver for protein intake and egg laying. Fresh eggs were transferred to glass containers for hatching and larval development. Third-instar larvae were used in all experiments (21, 22).

Sterilization of larvae

To ensure microbial sterility, third-instar larvae were first rinsed in sterile distilled water, fol-

lowed by immersion in 4% Deconex solution for 3 min, then in 70% isopropyl alcohol for another 3 min with gentle agitation. They were subsequently washed three times in sterile distilled water. Sterility was verified by microbiological testing before further processing (13).

Preparation of larval hemolymph

For hemolymph collection, the anterior segment of each larva, near the mouth hooks, was severed using sterile instruments. Ten larvae were placed in 0.5 mL microtubes nested inside 1.5 mL tubes and briefly centrifuged (at 1000× g for 3 min) to recover the hemolymph. The collected larval hemolymph was sterilized by passage through a 0.45 µm syringe filter prior to use in the formulation Protein concentration was determined by spectrophotometry at 280 nm using a NanoDrop device.

Hemolymph characterization

The protein composition of the larval hemolymph was characterized using two complementary techniques. First, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% polyacrylamide gels. Hemolymph cell pellets were lysed, and the lysates were boiled at 96 °C before being loaded onto 12.5% polyacrylamide gels alongside a molecular weight marker. Following electrophoresis, gels were stained with Coomassie Brilliant Blue, destained and imaged to visualize protein banding patterns. Second, FPLC was applied to fractionate the hemolymph and isolate potentially active components. A Superdex 75 10/300 GL column (GE Healthcare, USA) was used for separation, operated with ammonium acetate buffer (50 mM, pH 6.8) at a constant flow rate of 1 mL/min. Distinct protein peaks were collected according to the elution profile and stored for subsequent bioassays.

In Vitro studies

Reagents and materials

Meglumine antimoniate (MAT) was obtained from Sanofi-Aventis (France) and prepared at concentrations ranging from 12.5 to 200 µg/mL. Other reagents included dimethyl sulfoxide (DMSO), tetrazolium salt (MTT), 2′,7′-dichlorofluorescein diacetate, fetal bovine serum (FBS) and culture medium DMEM were obtained from Sigma (Germany). Hemolymph was also prepared as a 1:1 cream-based formulation for topical application.

Parasites and cells

The standard strain of *L. major* (MRHO/IR/75/ER) promastigotes was maintained at 25 °C in DMEM with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Mouse macrophage cell line MLC-A1 was cultured in DMEM with 10% FBS and 0.5% penicillin/streptomycin at 37 °C in 5% CO_2.

Anti-Promastigote activity assay

Log-phase *L. major* promastigotes (10^6 cells/mL, 90 µL) were seeded into 96-well microtiter plates. Drugs or their combinations (10 µL) were added to achieve final concentrations of 12.5, 25, 50, 100, 200 and 400 µg/mL and plates were incubated at 25 °C for 72 h. Cell viability was assessed using the MTT assay. IC_{50} values were calculated using probit analysis in SPSS software.

Anti-Amastigote activity assay

MLC-A1 macrophages (1×10^5 cells/well, 200 µL) were seeded onto 8-well chamber slides (Lab-Tek, Nalge Nunc International, NY, USA) and incubated for 24 h. Metacyclic promastigotes were obtained from stationary-phase *L. major* cultures (5–6 days old), enriched by differential centrifugation. Metacyclic promastigotes (200 µL, macrophage: parasite ratio of 1:10) were then added. After incubation, monolayers were washed three times with pre-warmed PBS to remove non-internalized promastigotes. After 24 h of infection, drugs or their combinations (40 µL) were added to reach final concentrations of 12.5, 25, 50, 100, 200 and 400 µg/mL and cells were incubated for 72 h. Slides were washed with PBS, fixed with methanol and stained with Giemsa. The num-

ber of intracellular amastigotes in 100 macrophages was counted microscopically to determine the IC_{50}, using probit analysis in SPSS. Each test was performed in triplicate.

Cytotoxicity assay

The cytotoxicity of the drugs and their combinations on MLC-A1 macrophages (5×10^5 cells/well) was assessed using the MTT assay in 96-well microplates. Cells were exposed to HEM, FRA, MAT and their combinations at 12.5–400 µg/mL for 72 h at 37 °C with 5% CO_2. Cell viability was measured using the MTT assay and data were expressed as the percentage of viable cells relative to untreated controls (UC).

Quantitative analysis of Th1/Th2 cytokine gene expression

Expression levels of Th1 (IFN-γ, TNF-α, IL-12) and Th2 cytokines (e.g., IL-4, IL-5, IL-10, TGF-β) were measured in infected macrophages treated with different formulations using qRT-PCR. RNA was extracted with the High Pure RNA Isolation Kit (Roche), and cDNA was synthesized using the Roche Synthesis Kit. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Table 1.

ROS production

Promastigotes were treated with IC_{50} concentrations of MAT, hemolymph, fractionated hemolymph, or their combinations for 72 h, then loaded with 10 µM 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) for 25 min. After washing with PBS, ROS levels were determined by flow cytometry. ROS levels were determined by flow cytometry using DCFH-DA staining, with fluorescence analysis based on a threshold set by the untreated control. In parallel, mean fluorescence intensity (MFI) values were calculated and expressed as relative fluorescence units (RFU) to represent the relative level of ROS production per cell.

In Vivo

Animals and infection

Thirty female BALB/c mice (6 weeks old, 20–25 g) were housed under specific pathogen-free conditions. Stationary-phase promastigotes of *L. major* (5-day culture, 2×10^6 parasites) were inoculated intradermal into the base of the tail.

Preparation of cream

The cream base was prepared in the laboratory using standard cosmetic-grade excipients in a 1:1 oil-to-water emulsion. The formulation contained cetostearyl alcohol, stearyl alcohol, beeswax, paraffin, span 60, propyl paraben, vaseline, BHT, dimethicone 350, methyl paraben, tween 20, steareth 20, distilled water, sepi-gel, glycerin and essence. The cream formulation was prepared under aseptic conditions using standard cosmetic-grade excipients and stored at 4 °C, protected from light to maintain stability. Hemolymph was collected from multiple *L. sericata* larvae and pooled to obtain a sufficient volume for formulation. Prior to incorporation into the cream base, the collected hemolymph was sterilized by passing it through a sterile 0.22 µm syringe filter. The sterile hemolymph was then mixed with the cream base at a 1:1 ratio (v/v), resulting in a final hemolymph concentration of approximately 50% (v/v). This resulted in a final dose of 20 mg/kg body weight/day for the treated mice. The cream base contained commonly used preservatives (methyl paraben and propyl paraben) and an antioxidant (BHT), but no standardized preservative efficacy testing was conducted. The formulation was prepared under laboratory conditions and used for topical administration during the experimental period. These aspects will be addressed in future studies.

Treatment and lesion size assessment

One month after infection, when typical cutaneous lesions had developed, thirty BALB/c mice were randomly divided into seven groups

(five mice per group). The treatment regimens were as follows:
- Control group: Received no treatment.
- MAT group: Received intramuscular injections of meglumine antimoniate (MAT; Glucantime®, Sanofi-Aventis, France) at a dose of 50 mg Sb^{5+}/kg body weight/day (corresponding to 50% of the standard therapeutic dose) once daily for 28 consecutive days (23). Hemolymph cream group: Received topical application of *L. sericata* hemolymph cream (20 mg/kg body weight/day) to the lesion area once daily for 28 consecutive days. The cream was gently spread over the lesion surface and lightly massaged for 30–60 seconds to facilitate absorption through the skin.
- Combination group: Received both MAT intramuscular injections (50 mg/kg/day) and topical hemolymph cream (20 mg/kg/day) once daily for 28 days. Cutaneous lesion size at the base of the tail of BALB/c mice was measured using a digital calliper. Lesion depth and width were measured in all groups over the 28 days following the start of treatment to determine average lesion size.

Parasite load quantification

Twelve weeks post-infection, popliteal lymph nodes were harvested, homogenized and stored at −70 °C. Parasite load was quantified by qPCR targeting the RV1 gene, using GAPDH as the reference gene. GAPDH was selected as the reference gene because it is one of the most commonly used housekeeping genes for normalization in qPCR-based parasite load quantification in experimental models of cutaneous leishmaniasis (24). Several previous studies have reported the use of GAPDH as a stable internal control for normalization in murine tissues during *Leishmania* infection (25). Based on these reports, GAPDH was used as the reference gene in the present study (Table 1).

Statistical analysis

Datasets were statistically analyzed using GraphPad Prism version 8.0.2 and SPSS version 22 software. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied to compare groups for ROS production, qPCR data and lesion size measurements with statistical significance set at $p< 0.05$. IC_{50} and CC_{50} values (50% inhibitory concentrations) were estimated by probit regression in SPSS. The selectivity index (SI) was defined as the ratio of the cytotoxic concentration to the effective anti-leishmanial concentration ($SI= CC_{50}/IC_{50}$). All in vitro experiments were performed in triplicate and results are presented as mean ± standard deviation (SD).

Results

Hemolymph characteristics

Nanodrop spectrophotometry at 280 nm revealed an average protein concentration of 123 mg/mL in the filtered hemolymph of third-instar *L. sericata* larvae. Prior lysis of the hemolymph disrupted cellular components, so no distinct cell fraction was expected or observed. SDS-PAGE analysis demonstrated approximately 17–19 protein bands with molecular weights ranging from 10 to 245 kDa, with similar profiles in fresh and filtered hemolymph, indicating no substantial compositional differences (Fig. 1). FPLC fractionation yielded eight distinct protein peaks, which were collected for further bioassays to identify active fractions (Fig. 2).

Promastigotes assay

Treatment of *L. major* promastigotes with HEM, hemolymph fraction (FRA), MAT and their combinations significantly reduced parasite viability compared to the untreated control ($p< 0.001$), as depicted in Fig. 3. All treatments were effective, but the combination of HEM or FRA with MAT produced the greatest reduction in promastigote viability, demonstrating a clear synergistic effect compared to each treatment alone.

Amastigotes assay

Microscopic analysis of infected macrophages revealed a significant decrease in the aver-

age number of amastigotes per macrophage following treatment ($p< 0.001$). At 200 μg/mL, HEM and FRA reduced amastigote counts from 76.9 (control) to 30.0 and 33.9, respectively, while MAT reduced it to 18.8. The combination treatments were most effective, with HEM+MAT and FRA+MAT reducing counts to 8.4 and 5.3, respectively, achieving >90% reduction (Table 2).

Cytotoxicity assays on MLC-A1 macrophages demonstrated that all treatments maintained high cell viability, with CC_{50} values ranging from 528.6 to 611.9 μg/ml. Selectivity index ($SI= CC_{50} / IC_{50}$) values exceeded 4.5 for all treatments, with the highest SI observed for FRA+MAT (7.23), indicating strong parasite selectivity and minimal host cell toxicity (Table 3).

Th1/Th2 cytokine expression

qRT-PCR analysis showed that treatment with HEM, FRA, MAT and combination regimens significantly upregulated Th1-associated genes (IFN-γ, IL-12, TNF-α, iNOS) compared to control ($p< 0.001$), while downregulating Th2-associated genes (IL-10, TGF-β) (Fig. 4, Tables S1–S6). The combination groups induced the most pronounced Th1 activation and Th2 suppression, indicating a shift toward a protective immune response.

ROS production

Flow cytometry analysis demonstrated a significant increase in ROS generation in *Leishmania major* promastigotes treated with all formulations compared with the untreated control ($p< 0.001$). ROS generation was evaluated using two complementary parameters: (1) the percentage of ROS-positive cells and (2) the fluorescence intensity distribution (reflecting the mean fluorescence intensity, MFI), which indicates the amount of ROS produced per cell. Histogram analysis showed a rightward shift in fluorescence intensity in the treated groups compared with the untreated control, indicating increased intracellular ROS levels per cell. This is further supported by the quantitative analysis of ROS-positive cells presented in Fig. 5B. The combination treatments (HEM+MAT and FRA+MAT) resulted in both a higher proportion of ROS-producing cells and an increased fluorescence intensity, suggesting enhanced ROS production (Fig. 5, Table S7).

In Vivo efficacy

In BALB/c mice, all treatment groups showed significant lesion size reduction compared with the control after five weeks ($p< 0.001$). Combination therapy with HEM cream and MAT injection produced the largest decrease, with a mean lesion diameter reduced by >75% from baseline (Fig. 6, Table S8).

Parasite load and RV1 gene expression

qPCR analysis of RV1 gene expression showed a significant downregulation across all treated groups compared with the untreated control (UC) ($p< 0.001$). The combination group (HEM+MAT) exhibited the most pronounced reduction in RV1 expression, corresponding to an estimated 84% decrease relative to control (Fig. 7, Table S9).

Table 1. Primer sequences used for qRT-PCR analysis of Th1-associated genes (IFN-γ, IL-12, TNF-α, iNOS), Th2-associated genes (IL-10, TGF-β) and the reference gene GAPDH in *Leishmania major*-infected macrophages

Template	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Product size (bp)
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA	95
IFN-γ	ACAGCAAGGCGAAAAAGGATG	TGGTGGACCACTCGGATGA	106
IL-10	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGC	134
IL-12	TGGTTTGCCATCGTTTTGCTG	ACAGGTGAGGTTCACTGTTTCT	171
iNOS	ACATCGACCCGTCCACAGTAT	CAGAGGGGTAGGCTTGTCTC	89
TGF-β	CCACCTGCAAGACCATCGAC	CTGGCGAGCCTTAGTTTGGAC	112
TNF-α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG	161
RV1	CTTTTCTGGTCCTCCGGGTAGG	CCACCCGGCCCTATTTTACACCAA	145

Table 2. Effect of hemolymph (HEM), hemolymph fraction (FRA), meglumine antimoniate (MAT), and their combinations on the average number of *Leishmania major* amastigotes per macrophage after 72 h of treatment. Combination therapies (0–400 µg/mL) produced the greatest reduction compared to single treatments ($p< 0.001$)

Treatment	UC±SD	25±SD (µg/ml)	50±SD (µg/ml)	100±SD (µg/ml)	200±SD (µg/ml)	400±SD (µg/ml)
MAT	76.9±8.3	58.4±7.3	53.5±5.0	38.3±4.1	18.8±2.2	0.0±0.0
HEM	76.9±8.3	64.3±6.6	55.4±4.4	42.8±7.3	30.0±1.3	0.0±0.0
FRA	76.9±8.3	60.3±4.2	50.3±3.5	40.0±8.0	33.9±2.2	0.0±0.0
HEM + MAT	76.9±8.3	55.3±8.3	49.6±1.9	37.6±4.6	8.4±1.3	0.0±0.0
FRA + MAT	76.9±8.3	49.0±5.1	44.4±4.6	37.6±5.9	5.3±0.5	0.0±0.0

Table 3. Cytotoxicity (CC_{50}) of HEM, FRA, MAT and their combinations on macrophages, and anti-leishmanial activity (IC_{50}) against promastigotes and amastigotes of *Leishmania major*. Selectivity index ($SI = CC_{50}/IC_{50}$) values indicate high selectivity for parasites over host cells, with combination groups showing the highest SI values

Treatment	Promastigotes		Amastigotes		Macrophages	SI
	IC_{50}±SD (µg/ml)	P value	IC_{50}±SD (µg/ml)	P value	CC_{50}±SD (µg/ml)	
MAT	263.8±28.6	-	98.3±8.6	-	586.4±31.6	5.96
HEM	311.2±20.0	<0.001	131.6±12.2	<0.001	611.9±27.7	4.64
FRA	281.6±17.6	<0.001	118.6±18.1	<0.001	595.3±18.9	5.01
HEM+MAT	242.3±14.7	<0.001	82.3±5.1	<0.001	555.1±20.7	6.74
FRA+MAT	221.6±17.3	<0.001	73.1±4.4	<0.001	528.6±17.6	7.23

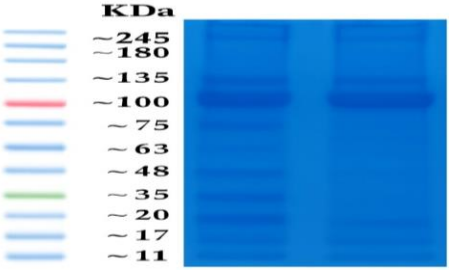

Fig. 1. SDS-PAGE profile of proteins from fresh and filtered *Lucilia sericata* third-instar larval hemolymph, showing multiple protein bands with molecular weights ranging from 10 to 245 kDa. No substantial differences were observed between the two samples and protein band was with molecular weights ranging 100 kDa

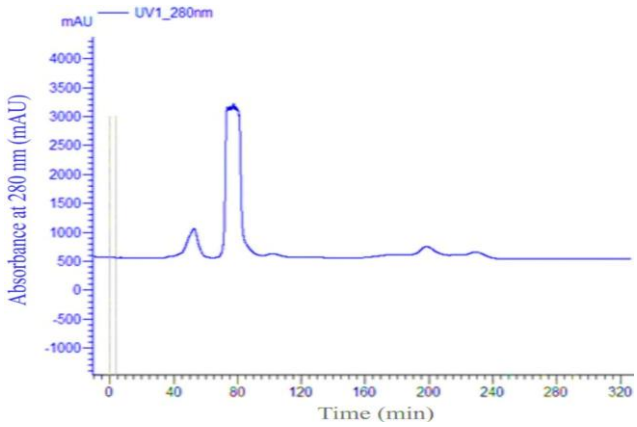

Fig. 2. Fast protein liquid chromatography chromatogram (FPLC) of *Lucilia sericata* third-instar larval hemolymph, revealing eight distinct protein fractions collected for further bioassays

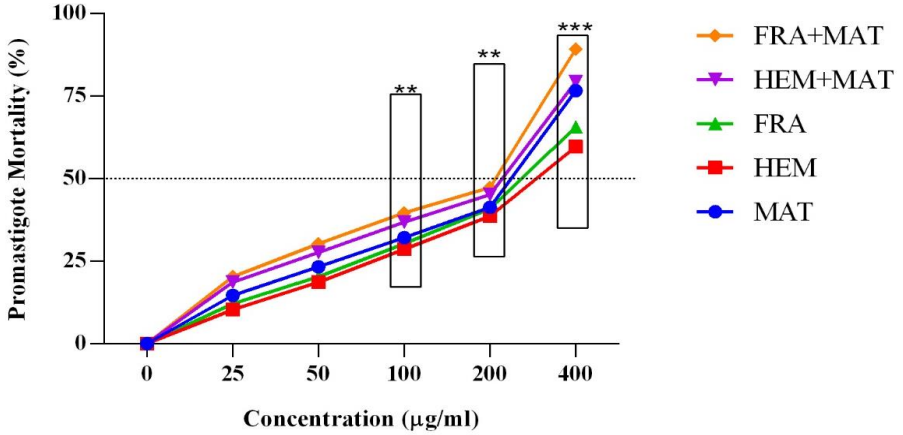

Fig. 3. Effects of HEM, hemolymph; FRA, hemolymph fraction; MAT, meglumine antimoniate and their combinations on the viability of *Leishmania major* promastigotes after 72 h of treatment. The combination groups (HEM+MAT and FRA+MAT) showed the strongest inhibitory effects, indicating a synergistic interaction between hemolymph components and MAT. Data are mean±SD of triplicate experiments (*p< 0.05, ** p< 0.01 and ***p< 0.001)

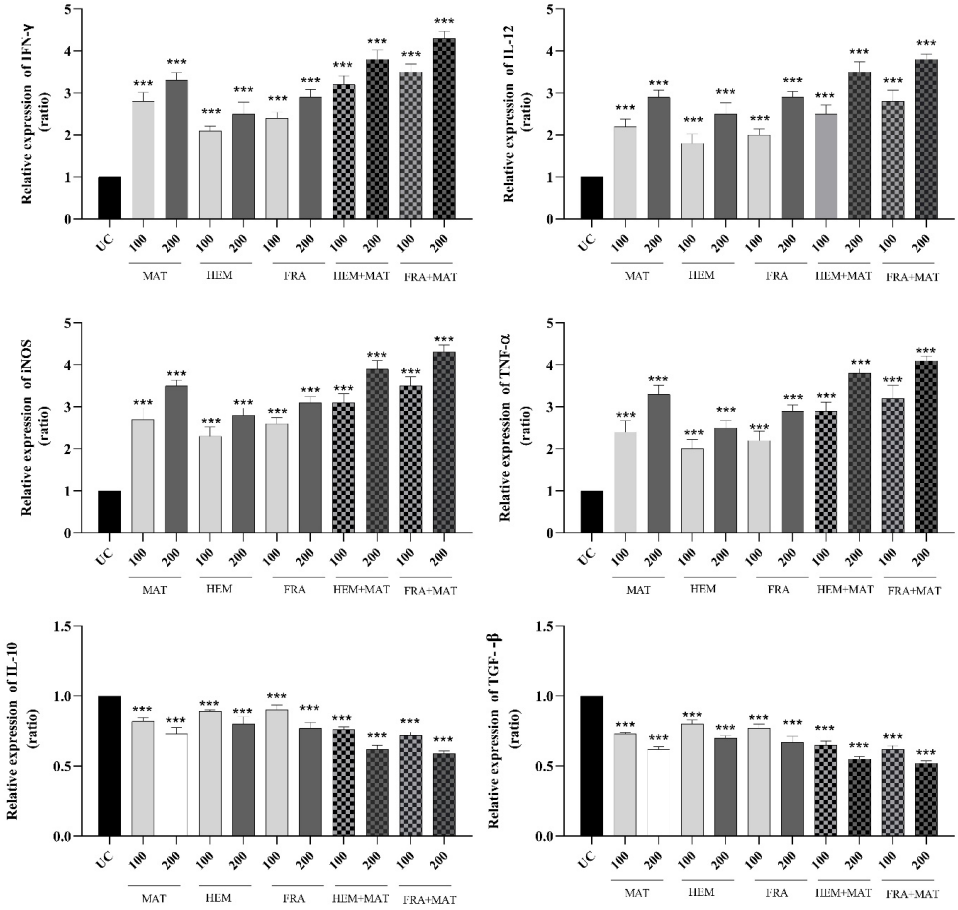

Fig. 4. Relative expression levels of Th1-associated cytokines (IFN-γ, IL-12, iNOS, TNF-α) and Th2-associated cytokines (IL-10 and TGF-β) in *Leishmania major*–infected macrophages treated with HEM, hemolymph; its active FRA, fraction; MAT, meglumine antimoniate and their combinations. Treatments were compared at equivalent concentrations, and all experimental groups were analyzed relative to the untreated control (UC). Data are mean±SD of triplicate experiments (***p< 0.001)

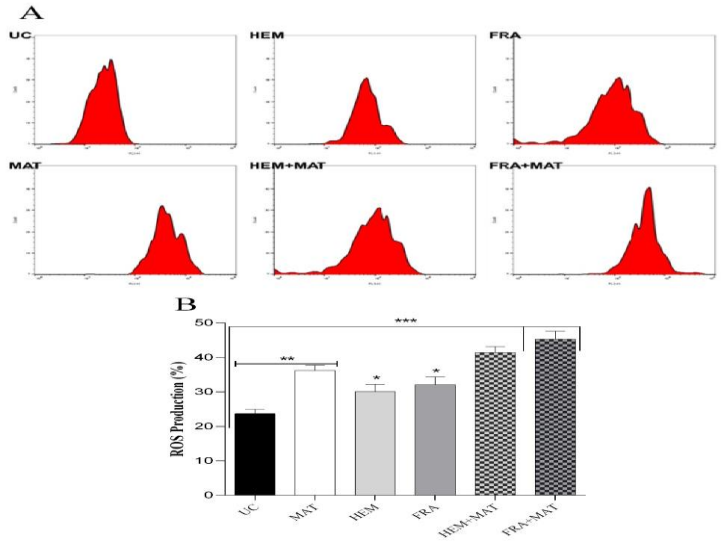

Fig. 5. ROS, Reactive oxygen species; generation in *Leishmania major* promastigotes following 72h treatment with *Lucilia sericata* HEM, hemolymph; its active FRA, fraction; meglumine antimoniate (MAT) and their combinations. All treatments significantly elevated ROS levels compared to the untreated control (UC) ($p< 0.001$), with the combination groups (HEM+MAT and FRA+MAT) showing the most pronounced effect, Data are mean±SD of triplicate experiments (*$p< 0.05$ and ** $p< 0.01$)

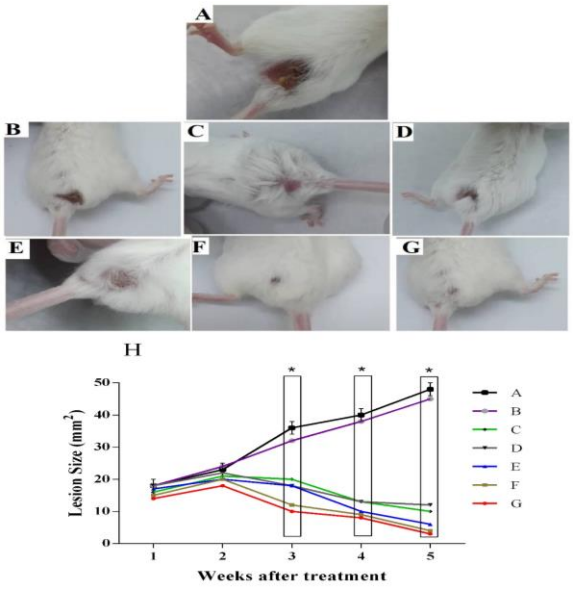

Fig. 6. Changes in skin lesion diameter in BALB/c mice infected with *Leishmania major* over 28 days of treatment. Groups are labeled as follows: (A) untreated control (UC), (B) cream base, (C) hemolymph intraperitoneal (HEM IP), (D) hemolymph topical (HEM TP), (E) meglumine antimoniate (MAT) IP, (F) MAT+HEM IP, (G) MAT+HEM topical. Data are mean±SD of triplicate experiments (*$p< 0.05$, ** $p< 0.01$ and ***$p< 0.001$)

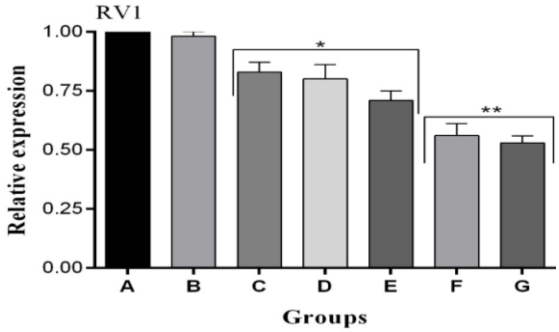

Fig. 7. Relative expression of the RV1 gene in popliteal lymph nodes of *Leishmania major*–infected BALB/c mice following 28 days of treatment with (A) untreated control (UC), (B) cream base, (C) *Lucilia sericata* hemolymph intraperitoneal (HEM IP), (D) hemolymph topical (HEM TP), (E) meglumine antimoniate (MAT) IP, (F) MAT+HEM IP, (G) MAT+HEM topical. Data are mean±SD of triplicate experiments (* $p< 0.01$ and **$p< 0.001$)

Discussion

The escalating therapeutic challenges in cutaneous leishmaniasis (CL) management, marked by increasing drug resistance and treatment-limiting toxicities, necessitates innovative therapeutic approaches. Our findings establish that

L. sericata larval hemolymph (HEM) formulated as a topical cream exerts potent anti-leishmanial effects against *L. major* through dual mechanisms: direct parasiticidal activity and strategic host immunomodulation. In addition, the cream base control group was included to evaluate whether the vehicle formulation itself could influence lesion progression or immunological parameters. The results indicated that the cream base alone did not produce significant changes compared with the untreated control group, suggesting that the observed therapeutic effects were primarily associated with the active components of hemolymph or fraction extracts rather than the topical vehicle. This finding confirms that the formulation base served only as a delivery system and did not contribute to the anti-leishmanial activity observed in the treated groups. Particularly significant is the synergistic interaction with meglumine antimoniate (MAT). This synergy holds urgent priority given recent epidemiological data showing >60% antimonial resistance in endemic Iranian foci (26) and parallel trends in global endemic regions. The combination enables reduced systemic drug exposure while enhancing efficacy. Importantly, our study extends and complements previous investigations on *L. sericata*-derived products in CL models. Earlier work by Sanei-Dehkordi et al. demonstrated that crude larval extracts possess inhibitory activity against *L. major* promastigotes and amastigotes, primarily attributing the effect to antimicrobial peptides and proteolytic enzymes (27). Kabiri et al. further showed that larval excretion/secretion (ES) products could reduce lesion size and parasite burden in murine models, emphasizing wound-healing and anti-inflammatory properties (28). More recently, Nasiri et al. reported enhanced macrophage activation and nitric oxide production following treatment with larval extracts, suggesting an immunomodulatory component (29). Sherafati et al. investigated purified fractions of larval secretions and highlighted improved selectivity indices compared to crude extracts.

While these studies collectively established the anti-leishmanial potential of larval-derived products, they predominantly focused on crude extracts or ES products and did not evaluate hemolymph as a standardized, protein-characterized therapeutic source. In contrast, our work substantially advances the field through comprehensive protein profiling of hemolymph using SDS-PAGE (revealing 17–19 distinct bioactive bands) and subsequent fractionation by Fast Protein Liquid Chromatography (FPLC). To our knowledge, this is the first study to systematically characterize *L. sericata* hemolymph fractions and correlate specific protein enrichment with enhanced anti-leishmanial selectivity.

This work substantially advances prior maggot therapy research through comprehensive protein characterization via SDS-PAGE (revealing 17–19 bioactive bands) and isolation of active fractions by Fast Protein Liquid Chromatography (FPLC). The fractionated hemolymph (FRA) demonstrated superior selectivity indices (SI=7.23 versus 4.64 against amastigotes), indicating concentrated bioactive components addressing a fundamental standardization challenge in natural product development (30,31).

Immunological profiling revealed HEM and FRA significantly amplified Th1-associated cytokines (IFN-γ, TNF-α, IL-12, iNOS) while suppressing Th2 markers (IL-10, TGF-β), countering Leishmania's immune evasion tactics (32). Although earlier studies reported partial macrophage activation or increased nitric oxide production, our results provide a comprehensive Th1/Th2 cytokine profile, demonstrating coordinated immune polarization rather than isolated immune markers. This macrophage polarization holds clinical relevance as emerging evidence implicates sustained IL-10 production in therapeutic failure (33). The fold reactive oxygen species (ROS) elevation in promastigotes treated with HEM+MAT suggests mitochondrial disruption (34)-a mechanism recently validated for insect-derived antimicrobial peptides. This oxidative stress synergy is particularly effective against intracellular

amastigotes, which develop enhanced antioxidant defenses in chronic infections (35). Despite the promising therapeutic effects observed in this experimental model, several challenges must be addressed before translating hemolymph-based formulations into clinical applications. The use of insect-derived biological materials requires careful evaluation of safety, including potential allergenicity, toxicity and batch-to-batch variability. In addition, the stability and standardization of hemolymph components should be established to ensure reproducible efficacy. From a regulatory perspective, further studies including detailed toxicological assessments, formulation optimization, and compliance with good manufacturing practice (GMP) standards will be necessary before such biologically derived topical therapeutics can progress toward clinical trials. In vivo results highlight three translational advantages: First, topical HEM monotherapy achieved 68% parasite reduction without systemic administration-likely facilitated by larval serine proteases enhancing skin penetration (36). Second, the HEM+MAT combination demonstrated a remarkable ability to permit a 50% reduction in MAT dosage while simultaneously improving efficacy (achieving 84% clearance compared to 62% with MAT alone), potentially mitigating nephrotoxicity affecting 30% of patients (37). Third, histological assessment revealed accelerated tissue remodeling in HEM-treated lesions, possibly mediated by the *L. sericata*-specific growth factors stimulating keratinocyte migration (38, 39). Although the present study demonstrated that hemolymph-based formulations significantly enhanced ROS production and modulated cytokine responses, the specific bioactive components responsible for these effects remain to be fully characterized. Insect hemolymph is rich in various immune-related molecules, such as antimicrobial peptides and components of the phenoloxidase cascade, which are known to promote oxidative stress and immune activation, thus potentially contributing significantly to parasite clearance in CL (40). These molecules can promote oxidative stress and immune activation, thereby enhancing parasite clearance. Future studies aimed at isolating and characterizing these bioactive constituents will be essential to better understand the molecular mechanisms underlying the anti-leishmanial activity of hemolymph-derived products.

The observed immunometabolic reprogramming-particularly iNOS upregulation and altered glucose metabolism-aligns with mechanisms of novel host-directed leishmanicides (41). This immunological convergence extends beyond dipterans, with *Apis mellifera* royal jelly phospholipids showing analogous effects against visceral species (42).

The observed 84% parasite reduction with combination therapy represents a highly promising outcome, comparing favorably to recent clinical trials of topical paromomycin-gentamicin (71% cure rate) (43). Practical implementation requires addressing: IgE cross-reactivity with insect allergens necessitates allergy screening protocols (44); lyophilized hemolymph maintains stability at 4 °C, but cream formulations require extended shelf-life testing; automated rearing systems could reduce production costs by 80% (45). Future studies should focus on identifying and characterizing the immunomodulatory components of FRA using MALDI-TOF analysis, as well as evaluating its therapeutic efficacy against mucocutaneous *Leishmania* species and its ability to induce durable protective immunity.

Limitations

This study has several limitations that should be considered when interpreting the findings. Although a cream base control group was included to assess the potential effect of the formulation vehicle, its impact was limited to confirming that the base itself did not exert significant anti-leishmanial activity. In addition, while the present work demonstrated that hemolymph-based formulations can enhance ROS

production and modulate cytokine responses, the specific bioactive components of hemolymph responsible for these effects were not identified. Further biochemical and proteomic studies are therefore required to isolate and characterize the active molecules involved. Finally, although the therapeutic potential of hemolymph-derived topical formulations appears promising, additional investigations addressing safety, allergenicity, formulation stability, and regulatory considerations will be necessary before translation into clinical applications can be considered.

Conclusions

This study demonstrates that *L. sericata* hemolymph cream exhibits multifaceted anti-leishmanial activity through direct parasite killing and host immunomodulation. Its synergy with meglumine antimoniate enables dose reduction while enhancing therapeutic outcomes, addressing critical challenges of toxicity and resistance. The dual-action mechanism (ROS induction and Th1 polarization) represents a significant advance over conventional monotherapies. Recent innovations in nanoparticle delivery and genetic engineering provide viable pathways for clinical translation. Future work should focus on standardizing active fractions, evaluating broader species efficacy and conducting preclinical safety assessments. This integrated approach exemplifies the potential of entomotherapy in combating neglected tropical diseases amid rising antimicrobial resistance.

Acknowledgements

The authors would like to thank all the staff of the Department of Biology and Control of Disease Vectors in TUMS and Kerman University of Medical Sciences for their collaboration throughout the study.

Ethical considerations

All procedures were approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.AEC.1401.034).

Conflict of interest statement

The authors have declared that no competing interests exist.

Supplementary data

File 1: Tables S1–S9 present the statistical analyses.

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