Received: 5 August 2018 | Revised: 23 October 2018 | Accepted: 19 November 2018

DOI: 10.1111/pim.12605

ORIGINAL ARTICLE

WILEY Parasite Immunology

Sambucus ebulus extract stimulates cellular responses in cutaneous leishmaniasis

Maryam Heidari-Kharaji[1] | Vahid Fallah-Omrani[2] | Alireza Badirzadeh[3] | Behnam Mohammadi-Ghalehbin[4] | Mohammad Ali Nilforoushzadeh[1] | Leila Masoori[3] | Hossein Montakhab-Yeganeh[5] | Mehrak Zare[1]

[1]Skin and Stem Cell Research Center, Tehran University of Medical Sciences, Tehran, Iran

[2]Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[3]Department of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

[4]Department of Microbiology and Medical Parasitology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran

[5]Department of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Correspondence
Alireza Badirzadeh, Department of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran.
Email: badirzadeh.ar@iums.ac.ir

Funding information
Shahid Beheshti University of Medical Sciences, Grant/Award Number: 12020

Summary

This is the first study aiming to determine the therapeutic effects of the *Sambucus ebulus* aquatic extract as an antileishmanial herbal drug and evaluate the immune responses in *Leishmania major major* infected BALB/c mice. The antileishmanial activity of *S ebulus* aquatic extract was evaluated using MTT test as well as parasite rescue and transformation assay. Footpad swelling and parasite load of infected mice were measured by several techniques. The immune responses were evaluated by measuring the levels of IFN-γ, IL-4, nitric oxide and arginase. The results indicated that *S. ebulus* can significantly decrease *L. major* promastigotes and amastigotes viability, but it was not toxic to macrophages. The lesion size, parasite burden and the level of ARG decreased in the treated infected mice, while the IFN-γ-to-IL-4 ratio and the level of NO increased significantly. Altogether, the *S. ebulus* extract is an effective compound for killing *Leishmania* parasite without excessive toxicity to the host cells and can cure the CL by switching the host immune responses towards Th1 response. Thus, it may be a perfect therapeutic option for CL treatment.

KEYWORDS

BALB/c, CC_{50}, Cutaneous leishmaniasis, EC_{50}, IC_{50}, *Leishmania major*, *Sambucus ebulus* L.

1 | INTRODUCTION

Leishmania parasites are the causative agents of leishmaniasis, a group of protozoan infection transmitted by a bite of female sand flies to human. Leishmaniasis is the world's most devastating neglected tropical disease (NTD) with a complicated ecology[1-3] whose transmission needs parasites, vectors, reservoirs and human interactions.[4,5] Leishmaniasis is an important endemic disease across the five continents which involve 98 countries, especially the developing countries.[4] Based on World Health Organization (WHO) reports, nearly 12 million people are affected and 350 million individuals are at risk of the disease.[5-7] *Leishmania* infection has been clinically categorized into three major forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL).[1,8] Globally, CL is the most prevalent form of the disease with approximately 1.2 million new cases diagnosed each year, where 90% of cases occur in seven developing countries including Afghanistan, Iran, Algeria, Peru, Brazil, Saudi Arabia and Syria.[9,10]

Although different chemical compounds are available for the treatment of various clinical forms of leishmaniasis, there is no effective treatment against the disease and the current chemical therapies suffer many critical disadvantages.[11] The usual and common treatment of leishmaniasis is the prescription of pentavalent antimony (antimonials) including Pentostam (sodium stibogluconate) or Glucantime (meglumine antimoniate) which have remained the first-line chemical drugs against leishmaniasis globally.[12,13] Another alternative first-line treatment is antifungal drug amphotericin B (AMB) which is used in the case of resistance to the antimony

treatment.[1,14,15] Recently, new chemical therapeutic alternatives have been recommended for the treatment of leishmaniasis including lipid-associated AMB, paromomycin, miltefosine, sitamaquine, azoles and itraconazole.[1,13] All of the mentioned antileishmanial drugs have many shortcomings due to their negative side effects as well as the persistence of *Leishmania* infection because of host immunity and the ability of the parasite to modify the host immune responses.[13,16] Although the recommended antileishmanial drugs such as antimonials can stimulate the host immune responses and resolve the *Leishmania* infection, several documented studies have described the insufficient antileishmanial activity and their different adverse effects which can culminate in treatment failure. Hence, discovering novel compounds with lower toxicity, high immunomodulatory effects and antileishmanial activity is vital.[16]

To date, use of natural product has been one of the main effective choices in the treatment of parasitic diseases including leishmaniasis. Studies have suggested that a natural herbal compound does not have a high probability of causing adverse effects. They are sustainable, economical, available and more importantly have immunomodulatory effects compared with common antileishmanial compounds.[16,17] These herbal extracts can be utilized as alternative natural treatments for cancers and serious infectious diseases such as CL, in which the host immune responses are critical for evolution of the disease.[9,16,18]

In traditional medicine, one of the less studied herbal plants for its antiparasitic characteristics is *Sambucus ebulus* L. (*S ebulus*), known as dwarf elder or elderberry,[19,20] which is ubiquitously present in different parts of Iran.[21] *S ebulus* plant is known as Khamaan in Iranian (Persian) traditional medicine which is prescribed as a natural herbal remedy in several diseases and various painful conditions such as sore throat, arthritis, honey bee bites and haemorrhoid.[19,21] Further, this herbal plant has lately shown to have anti-*Helicobacter pylori* activity, antitumour, antinociceptive, anti-inflammatory, antiangiogenic and anti-oxidative activities.[20-24] Previous studies have revealed that *S ebulus* has different active compounds including glycosides flavonoids, tannins, steroids, colchicine, ebulitins, anthocyanins, caffeic acids and volatile substances.[20,21] The compounds such as flavonoids can trigger the host immune responses.[20]

To the best of our knowledge, this is the first comprehensive study measuring the major efficacy, safety, toxicity and antiparasitic prosperities of *S ebulus* aqueous extract against *L major* in both in vitro and in vivo situations and evaluating the immune responses in mice model of cutaneous leishmaniasis.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Phorbol 12-myristate 13-acetate (PMA) and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Deisenhofen, Germany). Schneider's Drosophila medium, M199 medium, Dulbecco's modified Eagle's medium (DMEM) phenol red-free, RPMI-1640, foetal calf sera (FCS), dimethylsulphoxide (DMSO), gentamicin, L-glutamine, adenosine, HEPES, hemin and amphotericin B (AMB) were purchased from Gibco (Gibco, Life Technologies GmbH, Karlsruhe, Germany) and Sigma (Darmstadt, Germany).

2.2 | Plant material

Sambucus ebulus leaves were collected from forest regions of Amol city (36°23′N 52°20′E) located in the North of Iran (Mazandaran Province) in August 2016. The plant material identification was performed by a botanist in the Shahid Beheshti Medical Herbarium Center and Agricultural Research Center. A voucher specimen (1046A) was deposited at the Herbarium of Shahid Beheshti Medical Herbarium Center. The *S ebulus* material was dried at room temperature and coarsely grounded and powdered using a mixer and then kept in a dark amber-coloured bottle before herbal extraction.

2.3 | Herbal extraction

The aqueous extraction processes of powdered materials of *S ebulus* were applied according to the method as described previously.[25] It was sterilized by filtration via a membrane filter (0.22 μm). The extract utilized freshly to prepare for different concentrations for evaluation of its antileishmanial activity.

2.4 | Cells culture

Human monocyte cell line called THP1 (ATCC® TIB-202 TM) was used in this study. They were cultured in RPMI-1640 supplemented with FCS 10% at a temperature of 37°C in a CO_2 5% incubator. To form differentiated and adherent macrophages, they were counted and treated with 5 μg/mL of PMA. Nonadherent macrophage cells were eliminated by washing twice with RPMI 1640 medium. THP1 cells treated with PMA were utilized for further evaluations.[26,27]

2.5 | Extract cytotoxicity evaluations on macrophages

The cytotoxicity effects of *S ebulus* extract were assessed via cultivating PMA-treated human THP1 macrophages (5×10^6 cells) in the presence of increasing concentrations of the extract (125, 250, 500, 1000, 2000, 4000, 8000, 16 000, 32 000 and 64 000 μg/mL), PBS and AMB as controls in 96-well microtiter culture plates (Orange Scientific, Braine-l'Alleud, Belgium.) at 37°C in CO_2 5% for 48 hours. Cell viabilities were measured by colorimetric MTT assay as described previously elsewhere.[28,29] Absorbance ratios were quantified at the optical density of 570 nm. The viability of macrophages was measured through comparing treated cells and untreated control samples, and finally, CC_{50} was calculated [50% cytotoxic concentration (μg/mL)].

2.6 | Promastigotes extract susceptibility assay

The efficacy of the various increasing concentrations of *S. ebulus* extract (125, 250, 500, 1000, 2000, 4000, 8000, 16 000, 32 000 and

TABLE 1 General safety assessment of *Sambucus ebulus* extract in mice model. G_A with no treatment was used as the control group. Other groups (G_B-G_G) were treated with *S ebulus*. Routes of injection are presented in the table

Groups (G)	Concentrations (mg/kg)	Injection route
G_A	-	-
G_B	100	Intramuscular (IM)
G_C	150	Intramuscular (IM)
G_D	200	Intramuscular (IM)
G_E	100	Intralesional (IL)
G_F	150	Intralesional (IL)
G_G	200	Intralesional (IL)

64 000 μg/mL) on the stationary growth phase of *L major* promastigotes (2×10^6 parasites) was measured at 26°C for 48 hours. PBS and AMB were used as controls. *S ebulus* extract susceptibility was determined by colorimetric MTT assay,[26,30] and the concentration of the extract causing 50% inhibition in promastigotes growth (μg/mL) was computed (IC_{50}).[26]

2.7 | Macrophages infection and EC_{50} determination

Phorbol 12-myristate 13-acetate-treated human THP1 macrophages (5×10^5 cells) were seeded into a glass chamber slide (Thermo Scientific Pierce Chemical Co, Waltham, MA) and in 96-well culture plate in RPMI-1640 medium supplemented with FCS 10%, incubated at 37°C in CO_2 5% for 24 hours. Then, the stationary growth phase promastigotes of *L major* were added into each chamber and well (1:10 macrophages to the parasite) and incubated at 37°C in CO_2 5% for 24 hours allowing the promastigotes to infect the THP1 cells. Thereafter, free promastigotes were removed through washing twice by serum-free RPMI-1640 medium. The infected macrophages were treated with various increasing concentrations of *S ebulus* extract as mentioned previously for 48 hours at 37°C in CO_2 5%.[18,26,31]

2.8 | Parasite rescue and transformation assay

The infected PMA-treated human THP1 macrophages in the 96-well culture plate from the previous step were washed twice with serum-free RPMI-1640 medium to remove medium debris. Next, 20 μL of RPMI-1640 medium containing SDS 0.05% was added to each 96-well for lysis of the infected cells. The plate was shaken on the shaker for about 30 seconds, where Schneider's Drosophila medium supplemented with FCS 10% was added to each well. Then, the plate was incubated for 72 hours at 26°C in order to transform the survived amastigotes of *L major* to promastigote forms. After 72 hours, the survived live amastigote forms of the parasite were transformed into flagellated promastigotes. Finally, the effect of the *S ebulus* extract was measured by colorimetric MTT assay and the effective concentration (EC_{50}: 50%) (μg/mL) was calculated.[27]

2.9 | Ethical statement and mice conditions

This study was approved by the Human and Animal Research Ethics Committee of Shahid Beheshti University of Medical Sciences which is based on the guidelines of the Specific National Ethics for Biochemical Research issued by the Research and Technology Deputy of the Ministry of Health and Medical Education (MOHME) of Iran (issued 2005). In this study, attempts were made to minimize mice suffering within the course of our experiment. Female BALB/c mice (6-8 weeks old) were purchased from Pasteur Institute of Iran (Tehran, Iran). The mice were housed in ventilated plastic cages and kept in a controlled animal care facility (23°C; humidity: 55%-60%) with free access to tap water and enough rodent food.

2.10 | General safety assessment of *S. ebulus* extract in BALB/c mice

The toxicity of the *S ebulus* extract was assessed in susceptible BALB/c mice with the age of 6-8 weeks weighing nearly 20 g. Seven groups (four mice per group) are chosen and nominated as G_A to G_F as presented in Table 1. Group G_A, as the control, received no treatment; groups G_B to G_D received *S ebulus* intramuscularly (IM) at three concentrations of 100, 150 and 200 mg/kg, respectively, and G_E to G_F received *S ebulus* intralesionally (IL) at three concentrations of 100, 150 and 200 mg/kg, respectively (Table 1). After extract treatment, all mice were monitored for 1 week and their vital signs including body weight, sound sensitivity, sleepiness, diarrhoea and hair shedding were monitored.[32,33]

2.11 | Parasite and inoculation of mice

The pathogenic Iranian strain of *L major* (MRHO/IR/75/ER) was used in the current experiment. The parasite was maintained in a virulent state by a continuous passage in BALB/c mice. Isolated lymph nodes (LNs) from infected mice were cultured at 26°C in M199 medium at pH 7.4, supplemented with FCS 5% and gentamicin 50 μg/mL. The promastigotes of the parasite were subcultured and monitored daily. A total of 2×10^6 stationary phase metacyclic promastigotes (4-5 days) isolated by Ficoll 400 was injected subcutaneously (SC) in their right hind footpad.[32] Frozen and thawed (F/T) antigen preparation of *L major* parasite has been described in our previous study.[32,33]

2.12 | Schedule of animal study

For the infected mice, nine groups (n = 15 mice per each group) were used in the following way: G1: infected group with no treatment as control; G2: infected group treated with the standard antileishmanial drug, amphotericin B (AMB) 8 mg/kg; G3: infected group treated with PBS 1X; G4, G5 and G6 infected groups treated with *S ebulus* 100, 150 and 200 mg/kg, respectively, administrated by IM route; and G7, G8 and G9 infected groups treated with *S ebulus* 100, 150 and 200 mg/kg, respectively, administrated by IL route (Table 2). Four weeks post infection, G2 received AMB intraperitoneally (IP)

TABLE 2 Distinct groups of mice for in vivo experiment: the treatment, concentrations and routes of *Sambucus ebulus extract* injection are outlined for each group

Groups (G)	Treated with	Concentrations	Injection route
G1	No treatment	-	-
G2	AMB	8 mg/kg	Intraperitoneal (IP)
G3	PBS	1X	Intramuscular (IM)
G4	*S ebulus*	100 mg/kg	Intramuscular (IM)
G5	*S ebulus*	150 mg/kg	Intramuscular (IM)
G6	*S ebulus*	200 mg/kg	Intramuscular (IM)
G7	*S ebulus*	100 mg/kg	Intralesional (IL)
G8	*S ebulus*	150 mg/kg	Intralesional (IL)
G9	*S ebulus*	200 mg/kg	Intralesional (IL)

on a daily basis, which continued for 14 days. Groups G4, G5, G6, G7, G8 and G9 received different concentrations of *S ebulus* (100, 150 and 200 mg/kg,) by IM and IL routes, respectively, four times a week lasting for 4 weeks.

During the entire infection/treatment course, footpad swelling was monitored weekly and recorded for each mouse separately. The increase in the thickness and width of the infected and uninfected footpads was measured by a metric caliper. After 14 days of AMB treatment and 1 month of *S ebulus* treatment, five mice from each group were sacrificed. Then, their infected lymph nodes (LNs), spleens and footpads were isolated and evaluated for parasite load, cytokine levels and arginase activity.

2.13 | Quantification of parasite burden using limiting dilution assay

Eight weeks after infection, five mice from each group were sacrificed and the motile promastigotes of *Leishmania* in the drained LNs were counted microscopically. Also, the parasite burden was quantified by limiting dilution assay as previously described.[32,34]

2.14 | Quantification of parasite burden using real-time PCR

Eight weeks following infection, quantification of parasite burden (PB) in the isolated LNs was performed using real-time PCR. After LNs homogenization, the total genomic DNA was extracted by GF-1 DNA extraction kit (Vivantis, Canada) according to the manufacturer's instruction. The DNA content was determined by NanoDrop (Nanodrop, ND-1000, USA). A total of 40 ng of extracted genomic DNA was used for real-time PCR. Two sets of primers targeting a region of *Leishmania* kinetoplastid minicircle DNA including RV1 and RV2 primers (forward: 5′-CTTTTCTGGTCCCGCGGGTAGG-3′ and reverse: 5′-CCACCTGGCCTATTTTACACCA-3′) were utilized in this experiment. For quantification of parasite load, 5 μL of genomic DNA, 9 μL of SYBER Green PCR master mix (Qiagen) and 5 pmol of all forward and reverse RV1 and RV2 primers were used in a total volume of 25 μL per PCR. The PCR amplification program was as follows: 95°C for 5 minutes; 40 cycles of 15 seconds at 95°C; 30 seconds at 58°C; and 72°C for 40 seconds. *Leishmania major* genomic DNA was used in eightfold dilutions corresponding to 1×10^7 parasites to draw the standard curve using Applied Biosystem 7500 real-time PCR.[32,35] All of the reactions were done in duplicate.

2.15 | Cytokine production by splenocytes in mice

IL-4 and IFN-γ productions were measured in the supernatant of splenocytes as described elsewhere.[32,33] Briefly, at the end of the treatment (8 weeks after infection), five mice from each group were sacrificed with the spleen of each mouse homogenized using DMEM phenol red-free medium supplemented with inactivated FCS 5%. Then, the cell suspension was treated with ACK lysis buffer (NH_4CL 0.15 mol/L; $KHCO_3$ 1 mmol/L; Na_2EDTA 0.1 mmol/L) at room temperature for 5 minutes to eliminate the erythrocytes. The cells were washed thrice in DMEM phenol red-free by centrifugation at 580 g for 8 minutes. To assess the cell viability, they were counted via trypan blue staining. The cells were seeded at a density of 4×10^6 per well in the presence of the medium alone (no antigen) as a negative control, concanavalin A (Con A) (5 μg/mL) as the positive control and *L major* F/T (10 μg/mL) for all experiments, where the plates were incubated for 5 days at 37°C in CO_2 5% in humidified atmosphere. After 3 days for IL-4 and 5 days for IFN-γ, the production of IL-4 and IFN-γ in supernatants of splenocyte of each mouse in response to no antigen, Con A and F/T was quantified by sandwich ELISA kits (R&D, Minneapolis, MN), following the manufacturer's recommendations. All experiments were done in triplicate.

2.16 | Determination of arginase activity

Four weeks after the treatment, the enzymatic activity of arginase (ARG) was assessed in the footpads of sacrificed mice. It measures the conversion of L-arginine to L-ornithine through the microplate method which has been described elsewhere.[36-38] Briefly, cell suspensions of isolated footpads were homogenate in 500 μL of PBS 1X. The suspensions were centrifuged for 5 minutes at 500 g, and cell-free supernatants were centrifuged at 12 000 g for 10 minutes. A total of 25 μL of cell lysates was solubilized with 25 μL of lysis buffers (Triton 0.1% x-100, $MnCl_2$ 10 mmol/L, and Tris-HCl 50 mmol/L; pH 7.5). Arginase was activated by heating for 7 minutes at 56°C. L-arginine hydrolysis was performed by incubating

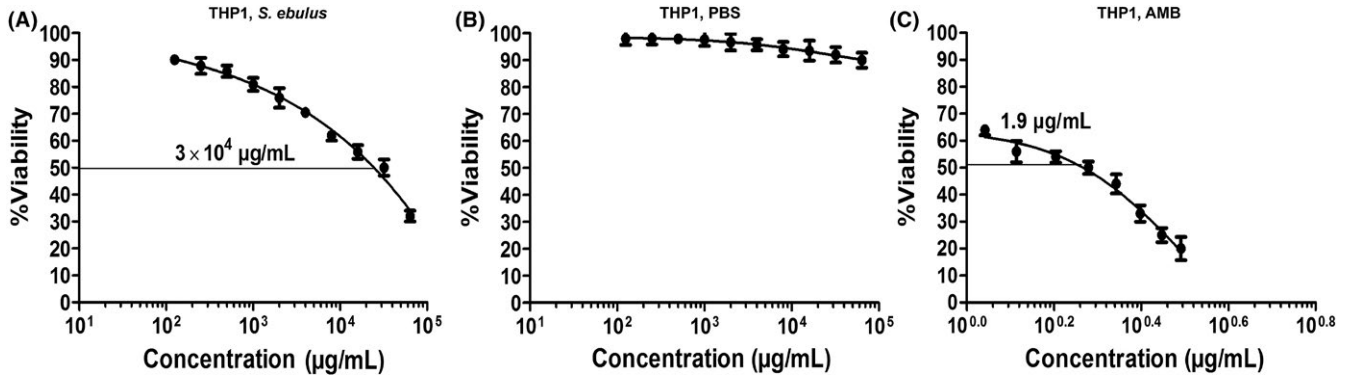

FIGURE 1 Cytotoxicity assay of different concentrations of *Sambucus ebulus* aqueous extract using PMA-treated human monocyte cell line (THP1) after 48 h via MTT assay (A) *S ebulus* extract is only toxic to cells at higher concentrations (30 000 µg/mL); (B) PBS; and (C) amphotericin B (AMB) was used as negative and positive reference controls, respectively. The data are reported here as mean ± SD of three repeated experiments with identical outcomes

the activated lysates with 50 µL of L-arginine (pH 9.7) at 37°C for 60 minutes. The reaction was stopped by the addition of 400 µL of acid solution (H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1:3:7, v/v/v). The urea concentration was measured at 540 nm after addition of 20 µL of α-isonitrosopropiophenone (ISPF, dissolved in 100% ethanol, Sigma) using a spectrophotometer (Power Wave XS, BIOTEK, VT), followed by heating at 100°C for 45 minutes. One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 µmol of urea per minute. The protein content of each sample (25 µL) was measured by BCA Protein Assay Reagent (Thermo Scientific Pierce Chemical Co, Waltham, MA) using serially diluted bovine serum albumin (BSA) as standard.[8,35]

2.17 | Nitric oxide measurement

A total of 100 µL of the stimulated splenocytes supernatant (after 3 days) was obtained from each well and subsequently mixed with an equal volume of Griess reagent [0.1 N (1-naphthyl) ethylenediamine dihydrochloride, sulphanilamide 1% in H_3PO_4 5%], and then incubated 10 minutes at room temperature. The absorbance of the coloured complex was measured at 570 nm. The nitric oxide (NO) concentration of each sample was estimated based on standard curve formula plotted with $NaNO_2$ serial dilution in the culture medium.[32,35]

2.18 | Statistical analysis

CC_{50}, ED_{50} and IC_{50} calculations and statistical analyses were conducted using Prism 6.0 for Windows (GraphPad Prism, San Diego, CA). The differences were analysed by one-way ANOVA and Student's *t* test. The *P*-values less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Cytotoxicity evaluation

As demonstrated in Figure 1, the results of colorimetric MTT assay indicated that *S ebulus* aqueous extract did not exert any toxic effects to the PMA-treated human THP1 macrophages, even at its higher concentrations (125-64 000 µg/mL). The obtained CC_{50} (µg/mL) of *S ebulus* extract on THP1 macrophages and AMB was 30 000 and 1.9 µg/mL, respectively.

3.2 | Effects of extract on *Leishmania* promastigotes and IC_{50} estimation

The antileishmanial effect of *S ebulus* aquatic extract was evaluated against promastigotes of *L major* in order to estimate the IC_{50} values (Figure 2). The results revealed that the extract significantly affected the promastigotes growth, and the IC_{50} of the extract was effective at the concentration of 3500 µg/mL killing 50% of the parasites (Figure 2A). PBS, as a negative control, had no effects on promastigotes of *L major* (Figure 2B). For AMB (tested at 1.1-3.1 µg/mL), as a positive reference control, 50% growth inhibition of *L major* was observed at the concentration of 1.2 µg/mL (Figure 2C).

3.3 | Parasite rescue and transformation assay and EC_{50} estimation

Using the MTT test, the EC_{50} (µg/mL) of *S ebulus* aquatic extract for amastigotes form of *L major* is shown in Figure 3, which demonstrates that for *L major* amastigotes, the EC_{50} of the extract was 6000 µg/mL (Figure 3A). PBS, as the negative control, had no effects on amastigotes of *L major* (Figure 3 B). For AMB (positive control), the EC_{50} of the extract was 2.6 µg/mL (Figure 3C). As the cytotoxicity (CC_{50}) results revealed, the CC_{50} of PMA-treated human THP1 macrophages (Figure 2A) and the EC_{50} of the *S ebulus* aquatic extract were 30 000 and 6000 µg/mL, respectively. This means that *S ebulus* extract was not only toxic to human THP1 cells, but also killed the *L major* amastigotes (Figures 2 and 3).

3.4 | *Sambucus ebulus* extract as a safe compound in BALB/c mice

The results of toxicity assay in BALB/c mice in Figure 4 indicated no evidence of toxicity in any of them after 7-day administration of

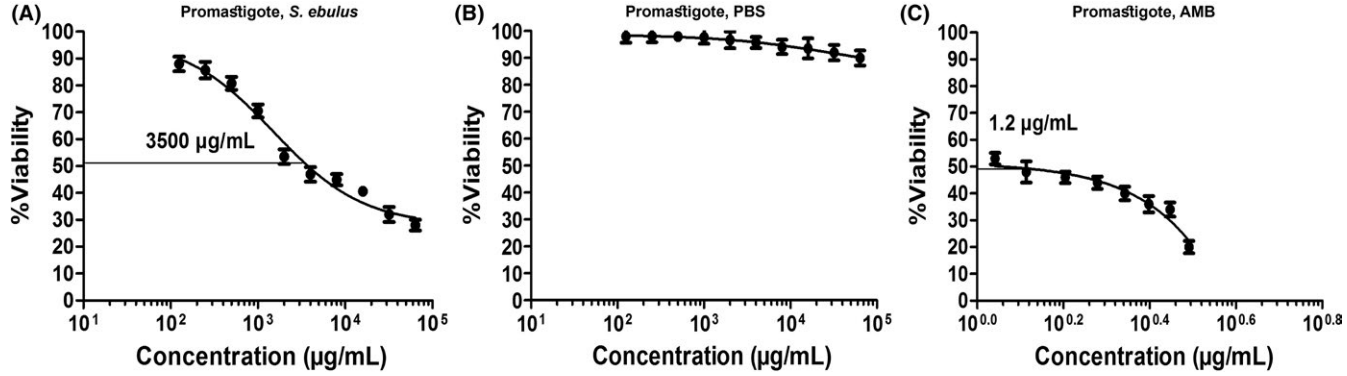

FIGURE 2 The inhibitory effect (IC_{50}) of different concentrations of *Sambucus ebulus* aqueous extract on promastigotes of *Leishmania major* after 48 h using MTT assay; (A) the IC_{50} of *S ebulus* extract killed 50% of the parasites at the concentration of 3500 µg/mL; (B) PBS; and (C) amphotericin B (AMB) was used as negative and positive controls, respectively. The data are reported here as mean ± SD of three repeated experiments with identical outcomes

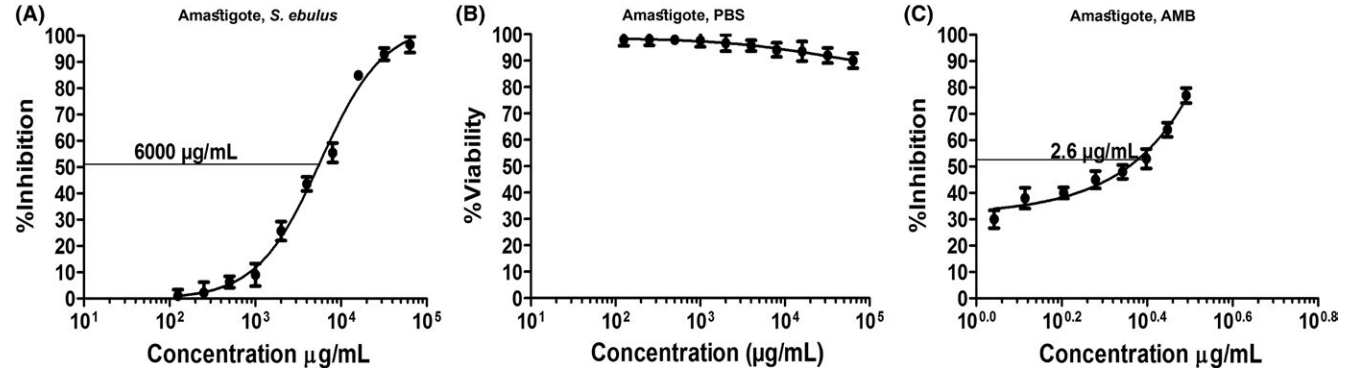

FIGURE 3 Parasite rescue and transformation assay (PRTA) and EC_{50} for *Leishmania major* amastigotes using MTT assay; (A) EC_{50} of *S ebulus* aquatic extract against amastigotes of *L major*; (B) PBS; and (C) amphotericin B (AMB) was used as negative and positive controls, respectively. The data are reported here as mean ± SD of three repeated experiments with identical outcomes

S ebulus extract. There were no considerable changes in the body weight suggesting no toxicity of the different concentrations of the extract. All of the mice across different groups (Table 1) elucidated normal behaviour and body weight as followed daily. There was no difference between treated and untreated groups.

3.5 | Lesion development in *Leishmania major* infected BALB/c mice treated with *Sambucus ebulus* extract

The footpad lesion development across all the infected mice with *L major* was monitored weekly for 10 weeks. Four weeks after the treatment with extract, G2 (AMB, 8 mg/kg), G4 (SE 100 mg/kg, IM), G5 (SE 150 mg/kg, IM), G6 (SE 200 mg/kg, IM), G7 (SE 100 mg/kg, IL), G8 (SE 150 mg/kg, IL) and G9 (SE 200 mg/kg, IL) exhibited significantly smaller lesions compared with G1 (no treatment group) and G3 (PBS group) ($P < 0.05$) (Figure 5). All of the three intralesional (IL) injected groups (G7, G8 and G9) had significantly smaller lesions compared with intramuscularly (IM) injected and control groups as shown in Figure 5 ($P < 0.05$). Note that G2 (AMB 8 mg/kg) as the positive control and G9 (SE 200 mg/kg, IL) among the test groups had the smallest lesion size among all of the groups ($P < 0.05$).

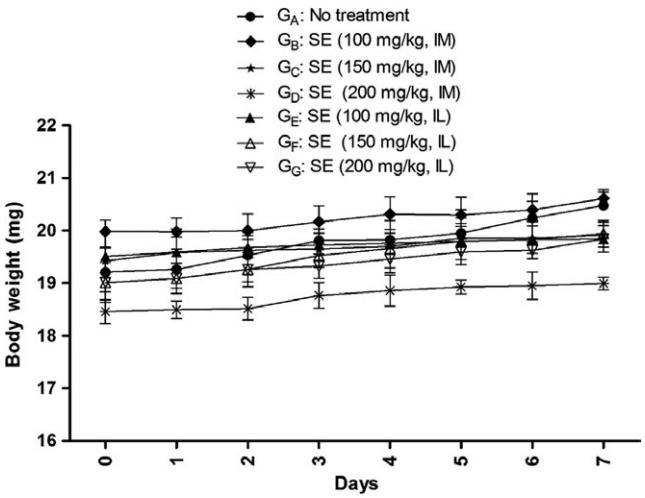

FIGURE 4 Evaluations of toxicity of *Sambucus ebulus* (SE) aquatic extract in BALB/c mice by assessing the vital signs such as body weight (mg); group G_A as the control received no treatment; groups G_B to G_D received different concentrations of SE (100, 150 and 200 mg/k) intramuscularly (IM); and G_E to G_F received them intralesionally (IL) (n = 4, 8 groups)

3.6 | *Sambucus ebulus* extract reduces the parasite burden in the lymph nodes of BALB/c mice

The *Leishmania* parasite burden in the lymph node of BALB/c mice was assessed using conventional limiting dilution assay (microtitration) (Figure 6A) and quantitative real-time PCR (qPCR) (Figure 6B), at 8 weeks after infection in all mice groups. As displayed in Figure 6A,B, in G2 (AMB 8 mg/kg), G4 (SE 100 mg/kg, IM), G5 (SE 150 mg/kg, IM), G6 (SE 200 mg/kg, IM), G7 (SE 100 mg/kg, IL), G8 (SE 150 mg/kg, IL) and G9 (SE 200 mg/kg, IL), the parasite burden decreased significantly in the lymph nodes compared with both control groups including G1 (no treatment group) and G3 (PBS group) ($P < 0.001$). Intralesional-routed (IL) groups including G7 (SE 100 mg/kg, IL), G8 (SE 150 mg/kg, IL) and G9 (SE 200 mg/kg, IL) had the lowest parasite burden compared with intramuscular-routed (IM) groups G4 (SE 100 mg/kg, IM), G5 (SE 150 mg/kg, IM) and G6 (SE 200 mg/kg, IM) ($P < 0.001$). Among all groups treated with *S ebulus* extract, G9 (SE 200 mg/kg, IL) presented the lowest level of parasite load ($P < 0.001$). Importantly, the data showed no significant difference between G9 (SE 200 mg/kg, IL) and G2 (AMB 8 mg/kg). Thus, intralesionally treated (IL) *S ebulus* extract with the concentration of 200 mg/kg and AMB have a similar efficiency with regards to the parasite proliferation and killing (Figure 6).

3.7 | Effects of *Sambucus ebulus* extract on cytokine production in mice

To compare the active immune responses by *S ebulus* extract treated groups and those of positive and negative controls and to find their functions against *L major* challenge in susceptible BALB/c mice, two important cytokine productions including IFN-γ (T-helper 1 (Th1) effector cytokine) and IL-4 (T-helper 2 (Th2 effector cytokine) were evaluated (Figure 7). Concanavalin A (Con A) functioned as a positive control in all performed experiments (data not shown). Significantly higher levels of IFN-γ were found in G2 (AMB 8 mg/kg), G9 (SE 200 mg/kg, IL), G8 (SE 150 mg/kg, IL), G7 (SE 100 mg/kg, IL), G6 (SE 200 mg/kg, IM), G5 (SE 150 mg/kg, IM) and G4 (SE 100 mg/kg, IM), respectively (Figure 7A). The highest level of IFN-γ production was found in G2 (AMB 8 mg/kg) and G9 (SE 200 mg/kg, IL). In contrast, in groups including G1 (no treatment), G3 (PBS), G4 (SE 100 mg/kg, IM), G5 (SE 150 mg/kg, IM), G6 (SE 200 mg/kg, IM), G7 (SE 100 mg/kg, IL), G8 (SE 150 mg/kg, IL) and G9 (SE 200 mg/kg, IL), the level of IL-4 production decreased significantly with elevation of the concentration of *S ebulus* extract ($P < 0.01$) (Figure 7B). Furthermore, we determined the ratio of both cytokines (IFN-γ/IL-4) across all mice groups (Figure 7C). The data indicated that the ratio of IFN-γ /IL-4 was significantly higher in groups G2 (AMB 8 mg/kg), G9 (SE 200 mg/kg, IL), G8 (SE 150 mg/kg, IL), G7 (SE 100 mg/kg, IL), G6 (SE 200 mg/kg, IM), G5 (SE 150 mg/kg, IM) and G4 (SE 100 mg/kg, IM), respectively, compared with the other controls (G1 and G3) ($P < 0.01$) (Figure 7C). The highest level of IL-4 production was found in G1 (no treatment), G3 (PBS) and G4 (SE 100 mg/kg, IM).

3.8 | High NO level and low ARG activity in groups treated with *Sambucus ebulus* extract

The vital amino acid, L-Arginine, is metabolized via iNOS or ARG based upon the important signals from the medium of macrophages. Inside these cells, immune responses through Th1 cytokines (IFN-γ) enhance the iNOS induction and NO production, while the Th2 cytokines (IL-4) promote the generation of ARG enzyme.[9,35] Parasites clearance into macrophage cells and their survival are mediated through NO and ARG, respectively.[9]

As presented in Figure 8A, all of the *S ebulus* extract concentrations including G4 (SE 100 mg/kg, IM), G5 (SE 150 mg/kg, IM), G6 (SE 200 mg/kg, IM), G7 (SE 100 mg/kg, IL), G8 (SE 150 mg/kg, IL), G9 (SE 200 mg/kg, IL) and G2 (AMB 8 mg/kg) significantly raised the level of NO production as an antileishmanial compound compared to the G1 (no treatment) and G3 (PBS), respectively. Intralesionally injected (IL) groups including G9 (SE 200 mg/kg, IL), G8 (SE 150 mg/kg, IL) and G7 (SE 100 mg/kg, IL) had the highest NO production, respectively, compared to intramuscularly injected groups (IM) ($P < 0.05$). Among all groups treated with *S ebulus* extract, G9 (SE 200 mg/kg, IL) had the maximum rise in the NO level. This is in a direct correlation with the increased IFN-γ induction and the active cellular immunity (Th1) through intralesionally injected (IL) groups (G7, G8 and G9) (Figure 8A).

As we expected, ARG level was inversely reduced across all the *S ebulus* treated groups including G4, G5, G6, G7, G8, G9 and G2, respectively, when compared to the control groups ($P < 0.05$) (Figure 8B). As

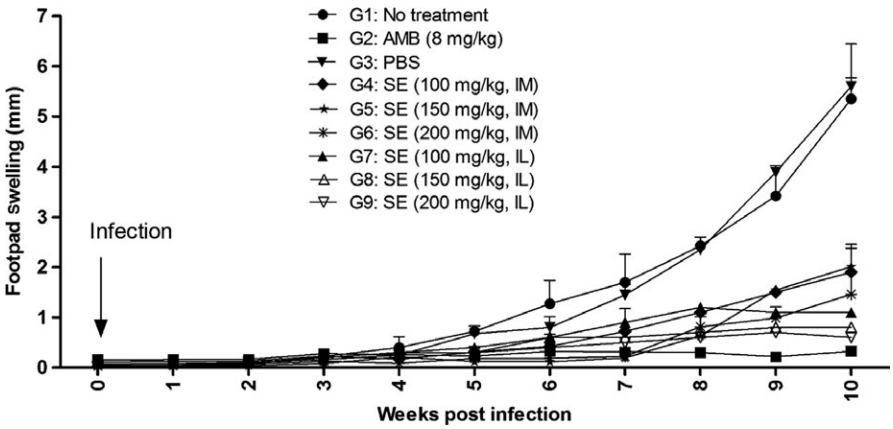

FIGURE 5 Lesion development/ swelling of footpad in *Leishmania major* infected BALB/c mice before and after treatment with *Sambucus ebulus* (SE) aquatic extract. The lesion size was documented by a metric caliper through measuring the increase in footpad thickness/width from 1 to 10 wk after infection

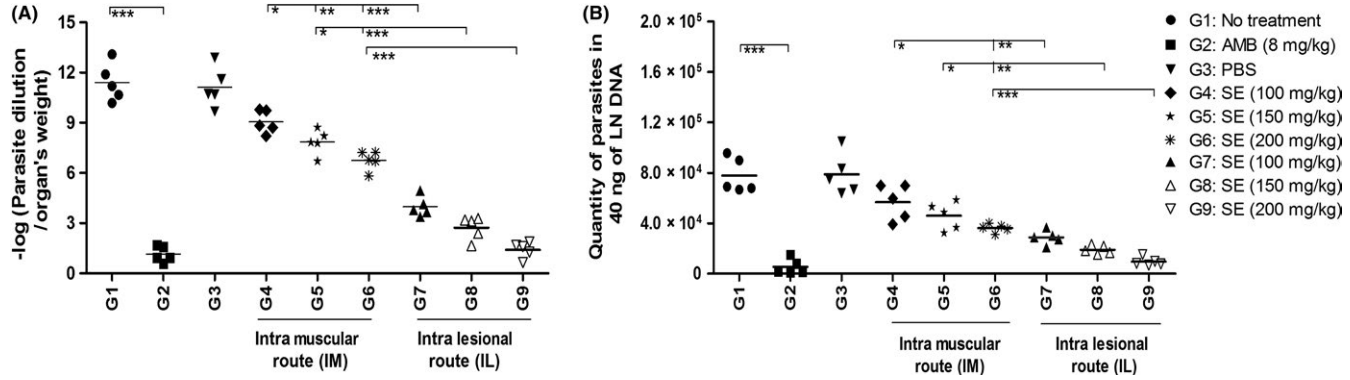

FIGURE 6 Parasite burden in the lymph nodes of distinct mice groups after infection with *Leishmania major* and treatment with *Sambucus ebulus* (SE) aquatic extract at 8 wk postinfection; (A) conventional limiting dilution assay; and (B) quantitative real-time PCR (qPCR) through two administration routes including intralesional (IL) and intramuscular (IM) (*P < 0.05, **P < 0.01, ***P < 0.001)

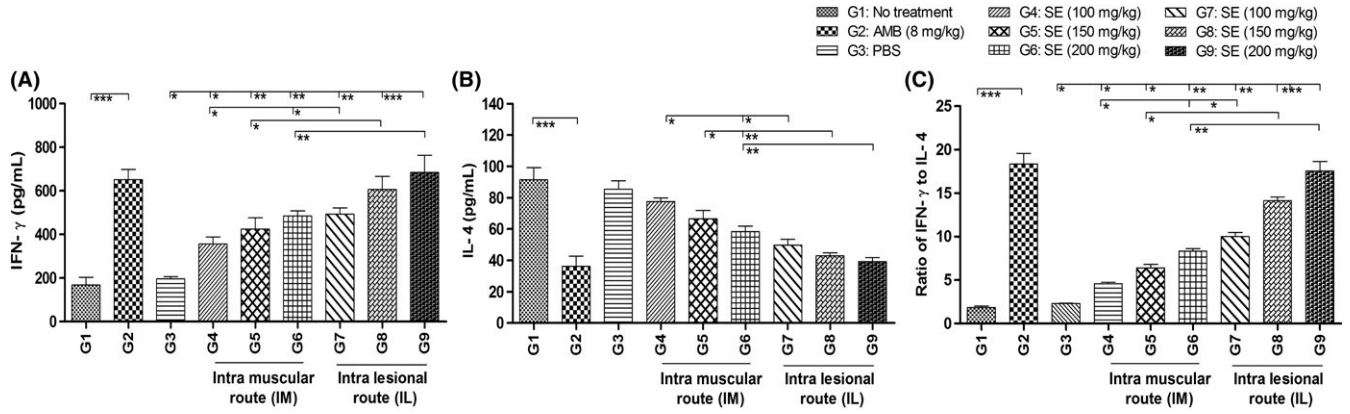

FIGURE 7 Evaluation of cytokine production in the supernatant of frozen and thawed (F/T) antigen-stimulated splenocytes of *Sambucus ebulus* extract treated BALB/c mice groups compared to positive and negative controls; the cytokines including (A) IFN-γ, (B) IL-4 and (C) IFN-γ/IL-4 ratio were evaluated (*P < 0.05, **P < 0.01, ***P < 0.001)

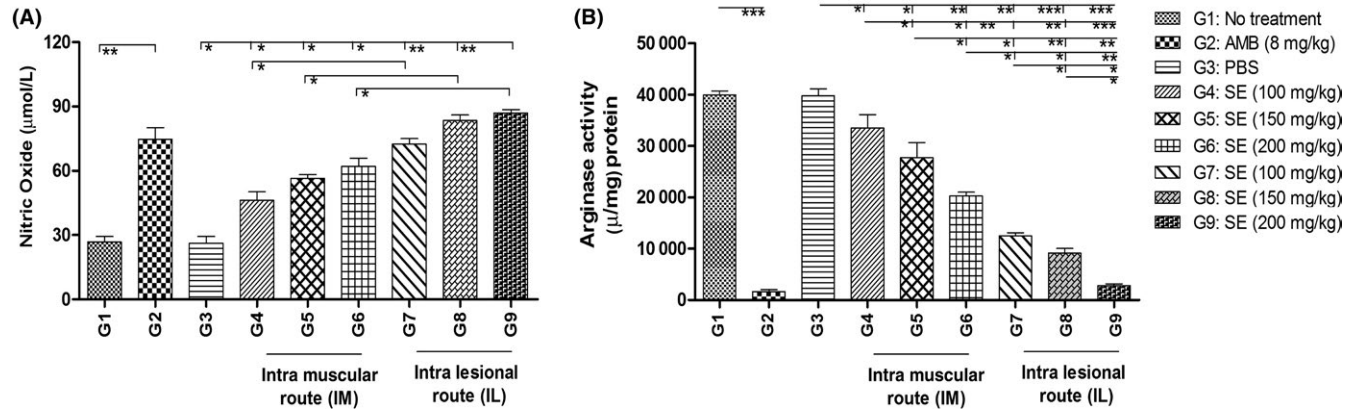

FIGURE 8 Quantification of nitric oxide (NO) production and arginase (ARG) activity in the mice treated with different concentrations of *Sambucus ebulus* extract in the spleen and footpad of BALB/c mice, respectively (n = 5); (A) NO production (μmol/L) was quantified via Griess assay; (B) ARG activity (mU/mL) was measured by microplate method. (*P < 0.05, **P < 0.01, ***P < 0.001)

presented in Figure 8, the data revealed a direct relationship between ARG level and NO production; when NO production was elevated, the ARG level diminished in the treated groups. Our data exhibited that G9 in the treated and G2 in control groups had the highest NO production, while G9 in the treated and G2 in control groups had the lowest ARG activity (Figure 8). According to the increasing production of NO and diminishing ARG activity, the cellular immune responses (Th1) were active through *S ebulus* extract treatment.

4 | DISCUSSION

To date, there has been an urgent increasing need to find novel alternative natural drugs for the treatment of disfiguring cutaneous leishmaniasis capable of providing the best efficiency, high safety, low price and easy drug administration.[13] In addition, due to lack of effective vaccines, the only efficient tool for fighting the disease is antileishmanial drugs.[33] Therefore, in such a scenario, the use of novel herbal drugs as a natural compound such as *S ebulus* is one of the major attractive chemotherapeutic candidates for triggering intense and suitable immune responses in order to kill *Leishmania* inside the host macrophages' cells.

According to WHO, approximately four-fifths of the world's population, mainly in the Middle Eastern and low-income countries, meet their daily primary health care demands via herbal and natural therapies as well as traditional medicines.[39] Nowadays, in developing countries such as Iran, traditional medicine has been utilized as the main source of complementary or alternative medicine in new health care and medicinal products. The use of herbal plants in pharmaceutical research and the drug's development are of uttermost importance.[39] Although the anti-inflammatory, antioxidant, anti-*Helicobacter pylori* and antigiardial activities of *S ebulus* extract have previously been reported,[20,22-24] the antileishmanial activity has not been described yet. Hence, to confirm the claimed traditional administration of *S ebulus* in Iranian traditional medicine (Persian traditional medicine) as a major source for antioxidants,[21] the current work is the first comprehensive study aiming to examine the main therapeutic effects of *S ebulus* aqueous extract as an antiparasitic herbal drug with the plausible immunoregulation of immune cells without excessive toxicity to the host cells and direct antileishmanial activities in in vitro and mice model.

As mentioned earlier, *S ebulus* has been utilized as an important herbal drug in traditional medicine for the treatment of different diseases.[20,40,41] The various medicinal activities of *Sambucus* species have been previously described including anti-*Helicobacter*,[41] antibacterial,[42] antiviral,[43] anti-inflammatory[22] and radical scavenging[44] effects, as well as inhibitory effects of interleukin 1 (IL-1) and tumour necrosis factor-α (TNF-α).[22] Further, it has also shown a rapid wound healing activity.[20,40]

In order to confirm the antileishmanial activity of *S ebulus* extract, cytotoxicity (CC_{50}), inhibitory (IC_{50}) and effective (EC_{50}) concentration assessments were performed. In this work, the results suggested that the extract had a direct and strong action on both forms of *L major* promastigotes and amastigotes without adversely affecting the host macrophages. The best concentrations of the extract for reducing promastigotes and amastigotes growth were 3500 and 6000 μg/mL, respectively, killing 50% of both forms of the parasite. The exact mechanism of action by which *S ebulus* kills the *Leishmania* parasites has remained unclear. Shokrzadeh and colleagues have found that the *S ebulus* has anti-*Helicobacter pylori* activity.[20] There is strong evidence to suggest that the extract may inhibit the urease and growth of *Helicobacter*. The possible explanations for antileishmanial activity of *S ebulus* may involve rapid and intense wound healing activity of the *S ebulus* extract as well as inhibition of parasite urease pathway.[40]

After in vitro assessment of *S ebulus* extract on *L major*, the aquatic extract was used as a safe therapeutic herbal drug in the parasite infected susceptible mice. We used three distinct *S ebulus* extract concentrations (100, 150 and 200 mg/kg) and evaluated the efficiency of *S ebulus* extract in the treatment of infected BALB/c mice with *L major*. In infected BALB/c mice treated with *S ebulus* extract and AMB, the footpad swelling was lower when compared to no treatment and PBS groups. In this study, we utilized two distinct methods for *Leishmania* parasite burden measurement including conventional limiting dilution assay (microtitration) and quantitative real-time PCR (qPCR). The results of both methods revealed a similarly significant difference between parasite burden in *S ebulus* extract and AMB treated groups compared to both control groups (no treatment and PBS groups). Our results suggested that the immune response to the *S ebulus* treated groups was similar to that of the AMB group. AMB can cause elevated NO, IFN-γ and IFN-γ/IL-4 ratios and diminished arginase activity, where our data showed similar results for *S ebulus*. In other experiments, in vitro and in vivo studies have demonstrated that AMB can induce inflammatory cytokine with antileishmanial activity and increase the level of IFN-γ and NO.[45-47]

We observed that in comparison with the control groups (no treatment and PBS groups), test groups especially the intralesionally (IL) injected ones could intensely induce cytokines such as IFN-γ and NO expression as one of the reliable cell-mediated (Th1) immune responses. Different studies have suggested that clearance of *Leishmania* parasite via classically activated macrophage cells (M1) is controlled through the production of superoxide, and they are killed directly by the production of NO.[48,49] The possible explanation for this function can be increase in the direct effects of IFN-γ (as Th1 cytokine) with the production of NO in order to induce *Leishmania* clearance, which contributes to the disease healing.[49,50]

Studies have demonstrated that L-arginine can be catabolized to urea and polyamines via ARG activity and/or NO via iNOS induction in activated macrophages. Th2 cytokines such as IL-4 stimulate ARG activity and polyamine generation, which can help in *Leishmania* proliferation. The current data revealed that the lowest ARG activity and IL-4 production (as Th2 cytokine) were seen across all test groups especially intralesionally (IL) injected samples as compared to both control groups (no treatment and PBS groups). In the infection caused by *Leishmania* parasite, IL-4 induction (as Th2 cytokine) and increased ARG activity with L-arginine metabolism in alternative activated macrophages' cells (M2) play a significant role in establishing the infection and parasite proliferation.[35,51] Both elevated IL-4 and maximum ARG activity are the signs of humoral immunity (Th2), in which the *Leishmania* parasite benefits from[49] and have negative regulatory effects on cytokines in the cell-mediated response (Th1). Our results are in agreement with other studies.[49,52,53]

The ratio of IFN-γ/IL-4 in splenocytes isolated from mice stimulated with *Leishmania* frozen and thawed treated with *S ebulus* extract was significantly higher than that of control groups (no treatment and PBS groups). Both IFN-γ (as a Th1 cytokine) and IL-4 (as a Th2 cytokine) inductions are utilized as main signatures of Th1 and Th2 immune responses, respectively.[49,53] Our results support the

findings of other studies demonstrating the induction of IL-4-driven Th2 immune responses, which have proven to boost nonhealing response in *Leishmania* infection.[53,54]

Notably, in both in vitro and in vivo studies, the antileishmanial activity of *S ebulus* extract was dose-dependent suggesting that with the rise in their concentrations, the effects of the extract were potentiated. This dose-dependent activity is in agreement with other studies.[55] In addition, in the current study, we administered two different treatment routes including intralesional (IL) and intramuscular (IM) for assessing the effects of *S ebulus* extract on mice infected with *L major*. Interestingly, our results indicated significant differences in the parasite load, IFN-γ/IL-4 ratio, NO production and ARG activity. Intralesionally (IL) injected groups (G7, G8 and G9) had significantly smaller lesions compared with intramuscularly (IM) injected and control groups. One possible explanation for the inadequate responses to IM injected therapy of Leishmania infection in mice is that even low concentrations of the extract and AMB can reach the CL infection site. Our findings provide more evidence for the other studies.[56,57]

Altogether, our findings on in vitro assessment of CC_{50}, IC_{50} and EC_{50} as well as in vivo measurement of parasite burden, cytokine production and ARG activity suggest that treatment of *L major* infection with the *S ebulus* aquatic extract as an herbal drug in traditional medicine is effective, safe and capable of efficiently controlling *L major* proliferation in the infected BALB/c mice. To date, no documented reports have investigated the antileishmanial effects of *S ebulus* extract in mice model. Hence, the *S ebulus* extract may be an ideal herbal drug candidate and a novel alternative treatment of CL caused by *L major* which induces Th1 responses. Finally, it can be concluded that the use of *S ebulus* extract can open a novel gate for the treatment of neglected tropical diseases such as leishmaniasis.

ACKNOWLEDGEMENTS

This work was financially supported by Shahid Beheshti University of Medical Sciences with grant number 12020 to Vahid Fallah-Omrani. We declare that we have no conflict of interest in relation to this study.

ORCID

Alireza Badirzadeh https://orcid.org/0000-0001-5315-2559

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How to cite this article: Heidari-Kharaji M, Fallah-Omrani V, Badirzadeh A, et al. *Sambucus ebulus* extract stimulates cellular responses in cutaneous leishmaniasis. *Parasite Immunol*. 2019;41:e12605. https://doi.org/10.1111/pim.12605