Efficacy of cryotherapy plus topical *Juniperus excelsa* M. Bieb cream versus cryotherapy plus placebo in the treatment of Old World cutaneous leishmaniasis: A triple-blind randomized controlled clinical trial

Mohammad Mahdi Parvizi¹,²,³, Farhad Handjani²,⁴*, Mahmoodreza Moein⁵,⁶, Gholamreza Hatam⁷, Majid Nimrouzi¹,³, Jafar Hassanzadeh⁸, Nasrin Hamidizadeh², Hamid Reza Khorrami⁹, Mohammad Mehdi Zarshenas⁵,¹⁰

¹ Research Center for Traditional Medicine and History of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, ² Molecular Dermatology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, ³ Department of Traditional Persian Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, ⁴ Department of dermatology, Shiraz University of Medical Sciences, Shiraz, Iran, ⁵ Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, ⁶ Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran, ⁷ Basic Sciences in Infectious Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, ⁸ Research Center for Health Sciences, Institute of Health, Department of Epidemiology, Shiraz University of Medical Sciences, Shiraz, Iran, ⁹ Department of Parasitology and Mycology, Shiraz University of Medical Sciences, Shiraz, Iran, ¹⁰ Department of Phytopharmaceuticals (Traditional Pharmacy), School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

*hanjanif@yahoo.com*

Abstract

**Background**

Cutaneous leishmaniasis is one of the highly prevalent endemic diseases in the Middle East and North Africa. Many treatment modalities have been recommended for this condition but success rates remain limited. Herbal remedies have also been used for treatment but evidence-based clinical trials with these products are sparse. In-vitro and in-vivo studies have shown the anti-leishmanial and curative effects of extract of fruits and leaves of *Juniperus excelsa* (*J. excelsa*). The aim of this study was to determine the efficacy of topical *J. excelsa* M. Bieb extract as an adjuvant to cryotherapy for the treatment of human CL.

**Materials and methods**

This study was designed as a two-arm triple-blind randomized placebo-controlled clinical trial using a parallel design. Seventy-two patients with clinical diagnosis of CL confirmed by leishmania smears were allocated to receive either a topical formulation of leaf of *J. excelsa* extract (group A) or placebo (group B) for 3 months. Both groups received cryotherapy as baseline standard treatment. Patients were evaluated before and weekly after the intervention was initiated until complete cure.
Results

Overall, 82% of patients in group A, experienced complete cure and 9% of them had partial cure. On the other hand, 34% in group B reported complete cure, while 14% of them had partial cure at the end of treatment protocol with a significant difference between the two groups (P<0.001). The mean duration to healing of the lesions in patients who received *J. excelsa* extract was statistically significantly shorter than the placebo group (p = 0.04). No significant side effect was seen in the *J. excelsa* extract group except for mild to moderate local irritation after a few weeks in a few numbers of patients.

Conclusion

The results of this study showed that topical *J. excelsa* extract can be used as an adjuvant treatment modality in addition to cryotherapy for accelerating the time to cure in addition to increasing the complete cure rate in CL.

Trial registration

ClinicalTrials.gov IRCT2015082523753N1

Author summary

Many people are afflicted in the world by cutaneous leishmaniasis (CL). The pathogen in this disease is an intracellular parasite. In 2007, The World Health Organization, defined leishmaniasis as a neglected disease. Many treatment modalities have been recommended for cutaneous leishmaniasis, but success rates remain limited. Experimental studies have shown the anti-leishmanial and curative effects of extract of fruits and leaves of *Juniperus excelsa* (*J. excelsa*), but there are no documentation in this regard in humans. This is the first randomized controlled clinical trial which evaluated the efficacy of the leaf extract of *J. excelsa* M. Bieb on human CL. According to the results of this present study, topical *J. excelsa* M. Bieb hydroalcoholic extract could be a good choice for treatment of CL in conjunction with cryotherapy. Using this extract along with cryotherapy can decrease the duration of CL treatment and also increase the success rate of CL treatment without any significant adverse effect.

Introduction

Leishmaniasis refers to a set of diseases caused by intracellular protozoan of the genus *Leishmania* [1]. The pathogen in cutaneous leishmaniasis (CL) is an intracellular parasite. Rodents and canines are the common reservoir of the parasite. Unfortunately, humans are the causal host [1, 2].

Evidence suggests that CL is one of the oldest diseases throughout human history [3]. The concept of this disease goes back to 650 BC [4]. In medieval manuscripts, CL was described as early as the 10th century by Avicenna as “Balkh sore” [5].

In 2007, the World Health Organization, designated leishmaniasis as a neglected disease, since more than 2 million people were afflicted in the world per year [6]. Ecological epidemiology of this disease is very diverse and dispersed in the world [7]. CL is endemic in 90 countries,
mostly located in tropical, subtropical and southern Europe [1, 2]. Old World CL refers to this disease in the Middle East and North Africa [8]. Although, CL has been reported from 20 of 31 provinces in Iran, Fars province and Shiraz (the capital), in southwestern Iran, is one of main endemic areas for this disease in Iran [9, 10].

Many cases of cutaneous leishmaniasis are self-limiting and usually heal within a year [11, 12]. However, not all patients who have received treatment, eventually report the recovery and eradication of infection [13]. In the initial steps of treatment, it is very necessary to prevent chronicity of the lesions, which can cause malformed lesions on body surfaces [11, 12]. Reducing the severity of the infected wound and the patient’s mental and emotional concerns [14, 15], and diminishing the reservoir and transmission of leishmaniasis are the most common reasons for treatment of this disease [16]. In some cases, CL ulcers can turn into large and disfiguring scars [17].

Several systemic and topical remedies are recommended for the treatment of CL. In the past few decades, antimonials, including meglumine antimoniate, have been the first line for the treatment of leishmaniasis. However, it is still accompanied by many complications [18]. Some studies have demonstrated that the efficacy of parenteral antimonials for the treatment of CL in Iran is 60–80 percent [19]. Intralesional injection of meglumine antimoniate, is also used for the treatment of CL and the cure rate reports of this route in Iran is 55–75% [20, 21]. However, since systemic antimonials have many side effects, clinicians face poor patient compliance [22–24].

Cryotherapy is a common treatment for CL, especially when the lesions are: non-complicated, less lymphocutaneous, less than three months duration, small lesions less than four in number, and for those who cannot receive systemic treatment [21, 25, 26]. Generally, cryotherapy with liquid nitrogen includes a cycle of freeze-thaw-freeze resulting in intracellular ice to form, which destroys the cell leading to localized ischemic necrosis [27]. This procedure decreases the local tissue temperature and metabolism which may result in cryonecrosis and can destroy the amastigotes and activate an immune response produced by the liberation of antigenic substances [28, 29].

Several studies have demonstrated that combination of cryotherapy with meglumine antimoniate increases the cure rate of leishmaniasis [21]. Besides, the side effects and limitation for using antimonials for treatment of CL, patient compliance has also led to its limited use [30]. However, use of adjuvant drugs could be useful for improving wound healing and increasing the rate of healing [31].

*Juniperus excelsa* (Cupressaceae) is divided into two main subspecies including *excelsa* M. Bieb and *polycarpus*, the first is called Persian Juniper [32, 33]. *Juniperus excelsa* M. Bieb (*J. excelsa*) is one of the most common genera in the related family [34]. It is called “Urs” or “Abhol” in famous textbooks of traditional Persian medicine, such as Avicenna’s (980–1037 AD) [35], Canon of Medicine or the “Storehouse of Medicaments” by Aghili Alavi Shirazi (1670–1747 AD) [36]. Rhazes (865–925 AD) [37], who is known as a pioneer in the field of dermatology [38], mentioned its different medicinal uses, including its application as a remedy for treatment of old wounds and infected wounds [39]. Fruits, leaves, wood and extracts of parts of these plants have been used for medical, sanitary and cosmetic purposes [40, 41]. *J. excelsa* essential oil has several compounds with proved anti-inflammatory, anti-toxicity and wound healing effects, which have been reported in several studies [42, 43]. In addition, there is some evidence that shows that this plant has anti-leishmaniasis activities in in-vitro and in-vivo studies [44]. Therefore, the aim of this study was to assess the efficacy of *J. excelsa* 5% hydroalcoholic extract cream in conjunction with cryotherapy versus cryotherapy plus placebo in search of an adjuvant treatment for CL.
Materials and methods

Design of the study and ethics statements

This study was designed as a triple blinded randomized controlled clinical trial. The Ethics Committee of Shiraz University of Medical Sciences approved the protocol (IR.SUMS.REC.1394.91) on August 23, 2015. This clinical trial was then registered at Iranian Registry of Clinical Trials (IRCT) website by IRCT2015082523753N1 code (http://en.search.irct.ir/view/25321).

All patients were adult (above 18 years old) and were aware of the trial plan. Written informed consent was obtained from all participants prior to enrollment and patients were free to withdraw from the project at any time. All patient data were also anonymized.

Sample size and study population

This study was performed at the Molecular Dermatology Research Center and Shohadaye-Enghelab Health Center, Shiraz University of Medical Sciences, Shiraz, Iran from September 2015 to September 2016.

Patients 18 to 70 years old with a maximum number of four lesions, ulcer size with maximum diameter of five centimetres, duration of lesions no more than four months, and patients receiving no anti-leishmania treatment remedies during the past four months were eligible for inclusion. Pregnant and lactating women, patients with regional adenopathy and those who had face involvement were excluded from the trial.

It was calculated that a minimum sample size of 25 patients per group was required to demonstrate a difference of 35% (80% versus 46%) in complete cure rate between the groups by accepting a two-tailed alpha error of 0.05 and a beta error of 0.20. To allow for incomplete follow up, with estimated lost-to-follow-up, it was decided to randomize 72 patients.

A total of 72 patients with positive skin smear for CL were enrolled. Skin scraping was taken from the margin of the active lesion, smeared on a glass slide, fixed with methanol and then, Giemsa stained and microscopically inspected for amastigotes of leishmania.

Randomization and blinding

Random allocation software Ink (Version 1.0, May 2004) was used to create a randomization table by a block size of four. Therefore, the patients were allocated to A and B groups according to the randomization table, respectively. This study was a triple blind trial. The herbal extract and placebo were delivered to the patient in a similar laminate tube marked as “A” or “B”. A dermatologist assessed the lesions of both groups, blindly. Also, the person who performed the statistical analysis was blinded and was only aware of the groups by the allocations “A” or “B”.

Data collection

Demographic data including sex and age of the participant, location and number of the ulcers, size of the lesions, and duration of lesions were recorded for all patients. Visits of the patients were done weekly and treatment outcomes and changes in size of ulcers were recorded.

Interventions and follow up

All patients received cryotherapy as a standard and baseline treatment for CL. Cryotherapy was done by liquid nitrogen (−195°C), once weekly for each lesion with a margin of about 1–2 millimetres with a cotton swap. The freezing time was up to 20 seconds in each visit. Based on the improvement or worsening of lesions, the assessor dermatologist decided if the patient needed further cryotherapy sessions or not.
At the time of the first session of cryotherapy, patients in group A received 5% hydroalcoholic extract of leaves of *J. excelsa* as a topical cream, in addition to cryotherapy, three times daily. Patients in group B received placebo cream three times daily (although a small number of patients reported twice daily applications), in addition to cryotherapy. Following applying the cream each time, the patients were recommended to wash the site of the lesion with water and soap to prevent possible secondary infection. None of the patients were prescribed either topical or oral antibiotics at the first week of intervention. Follow-ups for each patient were continued for three months (12 weeks). For compliance issues, each patient was asked to come back weekly with the container that was given to him/her on the previous week and the investigator could assess the approximate amount of topical treatment that was used.

At any time, after eight-weeks of starting therapy, if there was evidence of deterioration of the lesion, the patient was treated with other available remedies due to ethical reasons. In this condition, the case was considered as failure to treatment. Although, a long-term follow up of the patients was not included in the initial plan of our study, we decided to follow up these patients either by personal visits or by telephone for up to six months after completing the study to check for possible recurrence of the lesions.

**Drug and placebo preparation**

Leaf of *J. excelsa* was used to prepare the cream. Leaves of the plant was collected from Geno Biosphere Reserve, Bandar Abbas, Hormozgan province, south of Iran. Authentication of the plant was performed by a botanist at School of Pharmacy, Shiraz University of Medical Sciences with a specified voucher number (NO: PM 852).

The hydroalcoholic extract of the leaf was yielded using percolator apparatus. The obtained extract was then concentrated and dried. Both *J. excelsa* extract (JE) and placebo were made in cream (oil in water) form.

The amount and type of ingredients in both the JE and placebo creams were the same and included beeswax, thickeners agent, petrolatum, paraffin and ethanol (as solvent for dissolving the dry extract ≈ 5% of aqueous phase in cream), except for 5% of the extract which was added to the main cream. To create a similarity in the appearance of the placebo and JE cream, a standard green color powder was used in the placebo cream to make it similar to the JE cream. All steps in the preparation of the cream were performed under sterile air inside a laminar airflow cabinet. All equipments associated with the production of creams, including glass supplies, laboratory equipment, digital scales, Ben Murray, and homogenizer were previously cleaned by ethanol 70%. The JE and placebo creams were distributed to patients in similar 50g white laminated tubes.

**Phytochemical assessment**

**Volatile oil extraction.** In this study, both employed extract of *J. excelsa* and finished product (*J. excelsa* 5% cream) were analyzed in regard of the volatile constituents. To this, the extract and prepared cream were individually subjected to hydrodistillation for 3 hours using a Clevenger-type apparatus. Respective essential oil samples were dried and kept in 4°C for further steps.

**Analysis of the essential oil.** Initially GC/FID oil analysis was carried out to find a proper analytical condition. This process was performed on a gas chromatograph Agilent technologies model 7890A (USA) apparatus attached to HP-5 column (25 m × 0.32 mm, 0.52μm film thickness) and connected to a Agilent technologies (USA) flame ionization detector (FID). Nitrogen gas was employed as carrier gas with a flow rate of 1 ml/min (split ratio was 1:30). The injector temperature was 250°C, and detector temperature was 280°C, while column
temperature was linearly programmed from 60 to 250˚C (at rate of 5˚C/min) and held for 10 min at 250˚C. Solutions of anhydrous and diluted essential oil samples from cream and extract were consecutively injected. The above method was considered for GC/MS analysis. The process was carried out via using Agilent technologies model 7890A gas chromatograph (USA) connected to a mass detector (Agilent technologies model 5975C- USA). The GC was equipped with a HP-5MS capillary column (phenyl methyl siloxane, 30 m × 0.25 mm i.d., Agilent technologies). Helium was employed as carrier gas with the same flow rate as for GC/FID. The mass spectrometer was acquired in EI mode (70 eV) in a mass range of 30–600 m/z. The interface temperature was 280˚C. Identification and analysis of components was based on the comparison of their mass spectra with Willey (nl7) and Adams libraries spectra as well as with those cited in literature.

**Determination of total phenol and flavonoid content (nonvolatile constituents) in prepared cream.** Total flavonoid content in *J. excelsa* cream was determined using an Aluminium chloride colorimetric assay (A modified Dowd method). A solution of 5 ml Aluminium trichloride (2%) (Sigma Aldrich- USA) in methanol (Merck- Germany) was mixed with same volume of different concentrations of quercetin (as control). Absorption was read at 415 nm (PG instrument T90 spectrophotometer- Germany) after 10 minutes against a blank sample (5 ml extract solutions with 5 ml methanol in the absence of AlCl₃). Using a preparing quercetin (Sigma-Aldrich) standard curve (concentrations ≈ 0–80 mg/L), total flavonoid content of samples were determined. The mean of three readings for each sample was considered and expressed as mg of quercetin equivalents (QE)/g of dry plant leaves [45].

Total phenol content in finished product was carried out using Folin–Ciocalteu method. To this, 0.5 ml aliquots of 0.024, 0.075, 0.105 and 0.3 mg/ml methanol Gallic acid (Merck- Germany) solutions were mixed with 2.5 ml Folin Ciocalteu reagent (Sigma Aldrich- USA) and 2 ml (75 g/l) sodium carbonate. Absorption (765 nm) was read after 30 min at 20˚C, and calibration curve was drawn. Approximately, 0.5 ml cream (10 g/L) was mixed with the above reagents. Absorption was read (after 1 hour) for the determination of JE cream phenolics. All determinations were done in triplicate. Total content of phenolic compounds in the methanol extracts in Gallic acid equivalents (GAE) was calculated via the following formula:

\[
C = c \times \frac{V}{m}
\]

Where: “C” is the total content of phenolic compounds, mg/g extract, in GAE; “c” is Gallic acid concentration established from the calibration curve, mg/ml; “v” is the extract volume, ml; and “m” is the methanol extract weight, mg [46].

**Evaluation of the lesions**

Evaluation of the lesions was carried out weekly (the interval between each visit was 7± 2 days). The improving rate was determined by measuring the lesion size at baseline and also weekly by a scaled ruler (in millimeter) up to the time of cure or the end of the study. The size of the lesions were measured in two perpendicular directions by the dermatologist. The area of the lesion was calculated with ImageJ® software 1.44p (An open platform for scientific image analysis, Wayne Rashband, National Institute of Health, Bethesda, Maryland, USA). Clinical response to treatment was defined based on the following criteria:

1. Complete cure (decrease in lesion size with re-epithelization > 90%)
2. Partial cure (decrease in lesion size with re-epithelization between 50–90%)
3. No improvement (decrease in lesion size and re-epithelization < 50%).
Preparation of the smear and diagnosis of leishmaniasis

Cytological smears were prepared by scraping of the skin lesions with a scalpel. Multiple smears were made on slides and were both air dried and alcohol fixed and then stained by the Wright method.[47, 48] Review of cytological smears was conducted by a single expert laboratory personnel. Microscopic examination showed the amastigote forms of *Leishmania* in magnification, ×200.

DNA extraction and nested PCR

Leishmania species identification was determined using nested PCR method amplifying the kinetoplastid DNA from Giemsa-stained smears of CL lesions. DNA was extracted using phenol–chloroform–isoamyl alcohol as previously described [49]. AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea), was performed to extract genomic DNA from each clinical sample, according to the manufacturer’s instructions. Special primers related to variable regions of kDNA were used in PCR analysis as previously described [49, 50].

External primers CSB2XF and CSB1XR in the first round of PCR and internal primers 13Z and LiR in the second round of PCR were applied. The PCR products were analyzed by 1.5% agarose gel electrophoresis. We used PCR products on the promastigote cultures of the reference strains of *L. infantum*, *L. major* and *L. tropica* as positive controls. Nested PCR analysis resulted in a fragment of 680 bp [49, 51].

Statistical analysis

Statistical analysis was conducted using the SPSS version 22 (IBM Corporation, Armonk, NY). In addition, R Statistical Software (version 3.3.1; R Foundation for Statistical Computing, Vienna, Austria) was carried out to drawing a plot for comparing the changing size of the lesions in both groups over time. Normally distributed quantitative variables were demonstrated as mean ± standard deviation (SD). The normality distribution of the quantitative variables was investigated using the Kolmogorov-Smirnov test. Chi-square was used for assessing the relationship between categorical variables and groups. Generalized Estimating Equation model (GEE) was used for modeling the response (size of the lesion) with time and to assess the effect of the JE on it. Finally, multinomial logistic regression was used for estimating the odd ratio of JE effectiveness. The p-value of less than 0.05 was considered as significant.

Results

Patient enrolment

From a total number of 368 visited patients, 72 were enrolled in the study, based on our inclusion criteria. Overall, 10 out of 72 patients who were enrolled in the study, left the study. Therefore, 62 patients including 33 in group A (21 males and 12 females) and 29 in group B (16 males and 13 female) completed the study protocol and were analyzed at the end of the study. Because a few patients used the topical cream two times daily for a few days due to forgetfulness or busy job schedule, we used intention to treat method for analyses of the data. Detailed information about the study CONSORT flow chart is shown in Fig 1. All remaining patients were in-line with the protocol of the study.

Demographic characteristics

The gender distribution showed a male majority in both groups, however, the gender difference between the two groups was not statistically significant.
The mean ± SD of age was 38.91 ± 13.49 years in group A and 42.10 ± 14.54 years in group B, presenting no statistically significant differences between both groups. There were no statistically significant differences for the other baseline demographic data between the two groups as shown in Table 1.

The majority of patients had only one lesion in both groups (18 in group A vs. 17 in group B). Also, most patients had the lesion in the upper extremity (19 in group A vs. 16 in group B).

Number of lesions, duration of lesions, baseline vertical diameter size, baseline horizontal diameter size and the baseline area of lesions showed no statistically significant differences between both groups (Table 1).

Leishmania species identification

An example of a nested PCR analysis is shown in Fig 2. All patients in both groups were infected with *L. major* except one in group B, who was infected by *L. infantum*. Also five and four patients in group A and B, respectively, had negative results in PCR for leishmaniasis.

Response to the treatment

Overall, 27 out of 33 (82%) patients in group A, experienced a complete cure and three of them (9%) had partial cure. On the other hand, 10 out of 29 patients (34%) in group B reported...
complete cure, while four of them (14%) had partial cure. Three patients in group A and 15 patients in group B were designated as treatment failures. The details are shown in Table 2.

The mean duration between the starting point of treatment to the time of complete cure in group A was significantly shorter than group B. Also, the average number of cryotherapy sessions in patients with complete cure in group A was 3.85 ± 2.03 as compared to 6.54 ± 3.35 sessions in group B, as shown in Table 2.

The overall area of the lesions in group A and B were 100.89 ± 14.58 mm² and 217.62 ± 31.73 mm², respectively. The GEE analysis revealed that the area of lesions of CL decreased during the 12 week treatment plan in patients in both JE and placebo groups, showing that the effect of time was statistically significant (coefficient parameter estimation: 21.36 ± 3.17; p < 0.001).

Moreover, our findings indicated that the time interval to healing in the JE group was shorter than in the placebo group and this difference was statistically significant (coefficient parameter estimation: 21.36 ± 3.17; p < 0.001).

Table 1. Demographic characteristics of CL patients in both groups (Group A and Group B).

| Variables                             | Cryotherapy plus JE (Group A) | Cryotherapy plus placebo (Group B) | P-value |
|---------------------------------------|-------------------------------|-----------------------------------|---------|
| Sex                                   |                               |                                   |         |
| Male                                  | 21 (67%)                      | 16 (55%)                          | 0.49    |
| Female                                | 12 (36%)                      | 13 (45%)                          |         |
| Age (year, mean± SD)                  | 38.91±13.49                   | 42.10±14.54                       | 0.437   |
| Marriage status                       |                               |                                   |         |
| Single                                | 9 (27%)                       | 12 (41%)                          | 0.24    |
| Married                               | 24 (73%)                      | 17 (57%)                          |         |
| Educational status                    |                               |                                   | 0.53    |
| Under-Diploma                         | 21 (64%)                      | 17 (59%)                          |         |
| Diploma                               | 5 (15%)                       | 7 (24%)                           |         |
| Associate Degree                      | 3 (9%)                        | 2 (7%)                            |         |
| Bachelor Degree                       | 4 (12%)                       | 3 (10%)                           |         |
| Location of the lesions               |                               |                                   | 0.61    |
| Upper extremity                       | 19 (58%)                      | 16 (55%)                          |         |
| Lower extremity                       | 11 (33%)                      | 8 (28%)                           |         |
| Both upper and lower extremities      | 3 (9%)                        | 5 (17%)                           |         |
| Number of the lesions                 |                               |                                   | 0.65    |
| 1                                     | 18 (55%)                      | 17 (57%)                          |         |
| 2                                     | 8 (24%)                       | 5 (17%)                           |         |
| 3                                     | 5 (15%)                       | 3 (10%)                           |         |
| 4                                     | 2 (6%)                        | 4 (14%)                           |         |
| PCR characterization of microscopic positive samples | |                                   | 0.85    |
| Leishmania major                      | 28 (85%)                      | 24 (83%)                          |         |
| Leishmania infantum                   | 0 (0%)                        | 1 (3%)                            |         |
| Leishmania tropica                    | 0 (0%)                        | 0 (0%)                            |         |
| Negative result of PCR                | 5 (15%)                       | 4 (14%)                           |         |
| Duration between time of lesion occurrence and the time of first visit for treatment (month, mean± SD) | 1.56±0.74                     | 1.44±0.83                         | 0.34    |
| Vertical diameter of lesion on first visit (mm, mean± SD) | 21.38±7.87                   | 20.41±7.95                        | 0.65    |
| Horizontal diameter of lesion on first visit (mm, mean± SD) | 16.66±5.63                   | 17.52±7.05                        | 0.6     |
| Area of the lesion (mm², mean± SD)    | 306.26±226.60                 | 336.95±303.32                     | 0.94    |

JE: Juniperus excelsa M. Bieb extract, mm: millimeter; mm²: square millimeter; SD: standard deviation; PCR: Polymerase chain reaction

https://doi.org/10.1371/journal.pntd.0005957.t001
estimation: 116.96±35.35; p<0.001). Detailed information about estimation of this parameter by GEE is shown Fig 3. In addition, multinomial logistic regression revealed that the rate of complete cure in patients who received the JE was 13.5 times in comparison with those who received placebo (OR = 13.50, 95% CI 3.210–56.770, P-value< 0.001). Fig 4 shows complete cure for the patient who received JE.

In long term follow-up using personal visits and telephone contacts, all patients had complete cure after six months and none of them showed or reported any evidence of disease relapse.

Safety profile

Five out of 33 patients in group A, experienced local irritation including redness and itching after about 5 weeks of JE application (delayed local reaction to the herbal extract), but none of
the reactions were severe. Only one patient who returned on the sixth week of treatment with moderate erythema, redness and itching in her arm stopped using the medication although she had achieved complete cure at that time. One of the patients with irritation due to the JE

Table 2. Outcome of the treatment in both groups (Group A and Group B).

| Variable                                      | Cryotherapy plus JE (Group A) | Cryotherapy plus placebo (Group B) | P-value |
|-----------------------------------------------|-------------------------------|-----------------------------------|---------|
| Result of treatment N (%)                     |                               |                                   | <0.001* |
| Complete cure                                 | 27 (82%)                      | 10 (34%)                          |         |
| Partial cure                                  | 3 (9%)                        | 4 (14%)                           |         |
| Failure to treatment                          | 3 (9%)                        | 15 (52%)                          |         |
| Duration to cure (mean ± SD of weeks)         | 6.48±2.96                     | 8.72±3.34                         | 0.04*   |
| Drug reaction N (%)                           |                               |                                   | 0.055   |
| No                                            | 28 (85%)                      | 29 (100%)                         |         |
| Yes                                           | 5 (15%)                       | 0 (0%)                            |         |
| Number of cryotherapy sessions in patients with complete cure (mean ± SD) | 3.85±2.03                     | 6.54±3.35                         | 0.026*  |

*Significant at 5%, JE: Juniperus excelsa M. Bieb extract, SD: standard deviation

https://doi.org/10.1371/journal.pntd.0005957.t002

Fig 3. Changes in the size of CL lesions in both groups during three months. The numbers on the left side of the chart indicate the changes of length of the ulcers (mm) and the numbers on right side of the chart indicate the area changes (mm²) in the duration of treatment (12 weeks).

https://doi.org/10.1371/journal.pntd.0005957.g003
failed treatment. The other three patients continued using the JE cream as they experienced mild degrees of redness and itching.

### Phytochemical content

Considering that the JE has been evaluated for anti-leishmanial activity in a human study, it was introduced into the semi-solid cream product. Therefore, in order to achieve some therapeutic responses in terms of repeatability, some of the chemical compounds and metabolites in final product containing 5% of the JE was evaluated. These metabolites were volatile compounds and phenolic compounds as well as total flavonoids in the JE cream product.

GC/MS analysis of the plant extract volatile constituents revealed a total of 28 constituents (Table 3). Major compounds were sesquiterpenes (≈ 75% of total identification). On the other hand, most fractions of metabolites in volatile profile extracted from prepared cream were hydrocarbons (38.63%), phenols (34.74%) and sesquiterpenes (21.29%) (Table 3).
Table 3. Volatile constituents of both the plant methanol extract and prepared JE cream.

| No. | Component        | Area % (Extract) | Area % (Cream) | KIC | KIR |
|-----|------------------|------------------|----------------|-----|-----|
| 1   | α-Pinene         | -                | 0.24           | 935 | 939 |
| 2   | Limonene         | -                | 0.21           | 1030| 1029|
| 3   | Linalool         | 1.02             | 0.34           | 1100| 1096|
| 4   | trans-Pinocarveol| -                | 0.27           | 1142| 1139|
| 5   | Borneol          | 0.36             | 0.38           | 1168| 1168|
| 6   | α-Terpinenol     | 0.31             | -              | 1193| 1188|
| 7   | n-Dodecanol      | -                | 0.45           | 1200| 1200|
| 8   | n-Decanol        | -                | 0.19           | 1272| 1269|
| 9   | Thymol           | 0.71             | -              | 1291| 1290|
| 10  | n-Tridecane      | -                | 0.53           | 1300| 1300|
| 11  | Carvacrol        | 1.55             | -              | 1301| 1290|
| 12  | α-Cubebene       | 0.5              | -              | 1353| 1348|
| 13  | Hexyl hexanoate  | -                | 0.3            | 1387| 1383|
| 14  | n-Tetradecane    | -                | 0.83           | 1400| 1400|
| 15  | β-Funenebren     | -                | 1.37           | 1419| 1414|
| 16  | trans-Caryophyllene | -     | 0.32           | 1425| 1419|
| 17  | α-Humulene       | -                | 0.37           | 1459| 1454|
| 18  | n-Dodecanol      | 2.74             | -              | 1477| 1472|
| 19  | γ-Muurolene      | 0.53             | 0.22           | 1478| 1479|
| 20  | α-Amorphene      | 0.71             | -              | 1481| 1484|
| 21  | Germacrene D     | 0.53             | -              | 1497| 1485|
| 22  | n-pentadecane    | -                | 1.63           | 1503| 1500|
| 23  | α-Muurolene      | 1.72             | -              | 1505| 1500|
| 24  | γ-Cadinene       | 1.51             | -              | 1519| 1513|
| 25  | Butylated hydroxytoluene | - | **34.74** | 1524| 1515|
| 26  | Methyl dodecanate| 1.45             | -              | 1525| 1525|
| 27  | Δ-Cadinene       | 4.42             | 0.65           | 1528| 1523|
| 28  | Elemol           | **13.36**        | 0.7            | 1555| 1549|
| 29  | Germacrene B     | -                | 0.45           | 1564| 1561|
| 30  | Dodecanoic acid  | 1.78             | -              | 1570| 1566|
| 31  | Spathulenol      | 0.6              | 0.73           | 1582| 1578|
| 32  | Caryophyllene oxide | 0.83     | -              | 1588| 1583|
| 33  | Ethyl dodecanate | 0.42             | -              | 1593| 1595|
| 34  | n-Hexadecane     | -                | 1.37           | 1600| 1600|
| 35  | β-Oploopenone    | 1.23             | -              | 1613| 1607|
| 36  | Cedrol           | -                | **13.66**      | 1613| 1619|
| 37  | α-Cadinol        | 7.9              | 1.17           | 1636| 1640|
| 38  | β-Eudesmol       | 7.51             | 0.79           | 1647| 1650|
| 39  | α-Eudesmol       | **30.02**        | 0.46           | 1660| 1653|
| 40  | n-Heptadecane    | 1.16             | 0.87           | 1698| 1700|
| 41  | Methyl tetradecanoate | 0.91 | -              | 1723| 1723|
| 42  | n-Octadecane     | -                | 0.64           | 1797| 1800|
| 43  | n-Hexadecanol    | -                | **19.99**      | 1886| 1875|
| 44  | n-Nonadecan      | -                | 0.24           | 1898| 1900|
| 45  | Methyl hexadecanoate | 0.84     | -              | 1923| 1921|
| 46  | Hexadecanoic acid| 1.26             | -              | 1958| 1960|
| 47  | n-Eicosane       | 0.69             | 0.19           | 1996| 2000|

(Continued)
Total phenolic compounds and total flavonoids in the prepared JE cream were 1.85±0.014 mg Gallic acid equivalent/g and 0.31±0.006 mg quercetin equivalent/g, respectively. Accordingly, the amount of phenolic compounds and total flavonoids in the tube containing 50 g of prepared JE cream were 93.03±1.13 mg and 16.31±0.05 mg, respectively.

### Discussion

Few studies have shown the in-vitro leishmania promastigocidal effect of *J. excelsa* M. Bieb [44, 52], and also its efficacy in the treatment of CL in an animal model [53]. Therefore, the current randomized controlled clinical trial has evaluated the efficacy of the leaf extract of this native plant on human CL for the first time.

Nowadays, there is an increase in patient interest for complementary and alternative medicine (CAM). Therefore, there is an essential need for evidence-based assessment of different CAM modalities, including herbal medicines [54–56]. According to medical and pharmaceutical manuscripts in traditional Persian medicine, CL appears as a dry or wet wound caused by insect (sand-fly) bites [5, 57]. This disease was described as *Rish-e-Balkhi* (Balkh Wound) or *Balkhi'ye* in traditional Persian medicine. The medieval descriptions and signs and symptoms of this wound is very close to what is called cutaneous leishmaniasis in current medicine [58, 59]. *J. excelsa* which is known as *Abhol* or *Urs* in *Makhzan al-adviyah* (the Storehouse of Medicament authored by Aghili Shirazi in 18th century A.D.), was administered for the treatment of severe infected wounds called *Qūrūh-e-khabisa* (non-healing wounds) [60]. Abu Mansour Heravi, the author of "*al Abnieh an-Haghayegh al-Advieh*” (10th century A.D.) [61, 62], prescribed *Abhol* for the treatment of infected wounds and also fresh wounds as well as *Rish-e-bal-khi* [63].

Effectiveness of *J. excelsa* in the healing process of CL is related to the presence of various classes of metabolites in the extracted sample. Some of these are mentioned here: *J. excelsa* is a plant rich in phenols, terpenoids and flavonoid components [64]. Several studies have represented the remarkable antileishmanial activities of phenols, flavonoids or terpenoids in various extracts [65, 66]. The underlying mechanism of phenolic compounds and flavonoids is related to the release of lacyate dehydrogenase by macrophages which results in the antileishmanial activity of *J. excelsa* [67, 68]. The antioxidant and anti-inflammatory activity of JE can lead to acceleration in healing of chronic ulcers like CL. Antioxidants have an important role in suppression of the oxidative processes in the early phase of wound healing. However, antioxidative mechanisms are based on gradual detoxification of free radicals and oxidative agents and on gradual return of cells to the state of redox homeostasis [69, 70].
Tendency to chronicity of CL, is the most important reason for the treatment of this disease [12]. There are several recommended remedies in the treatment of CL including: cryotherapy and thermotherapy as physical methods [26, 71], topical and systemic medications and herbal remedies and natural products [72, 73].

Antimonials, are the most common reagents used to treat CL since 50 years ago in both intra-lesional and systemic form. Many patients refuse to use these medicaments due to possible pain and or other adverse effects. Also some physicians dislike to prescribe antimonials due to some important systemic and local side effects or absolute or relative contraindications [74, 75]. On the other hand, there is some evidence revealing the presence of some resistant strains of CL to meglumine antimoniate in recent years [76, 77]. Therefore, finding alternative remedies with less complications are necessary to manage this disease.

Cryotherapy, is the most local standard therapeutic method for the treatment of CL with variable efficacy [29]. Using this method is accompanied with a painful sensation and has several mild to serious complications. On the other hand, the wound may become susceptible to infection due to the induced necrosis and secondary ulceration of the tissue [78, 79]. Several studies have demonstrated that combination therapy in the treatment of CL can improve the treatment duration [80] and accordingly, the combination of cryotherapy and intra-lesional meglumine antimoniate, can increase the efficacy up to 89% [20].

We conducted the first triple blind randomized clinical trial to compare the efficacy of combination therapy of a topical cream containing J. excelsa leaf hydroalcoholic extract in association with cryotherapy versus cryotherapy and placebo in CL patients. Overall, 82% of patients in the treatment group (group A) had complete cure, and 9% of them showed partial cure. In comparison with patients in the placebo group (group B), the result of our study confirmed that topical 5% cream of JE applied three times daily for around three months was more effective compared to cryotherapy plus placebo. These results are in-line with the wound healing effect of J. excelsa in traditional Persian medicine sources, as Makhzan al adviyah [60, 81]. Outcomes of our study is close to the effectiveness of combination therapy of meglumine antimoniate and cryotherapy in Salmanpour’s study [20].

Several investigations have demonstrated the therapeutic effects of J. excelsa in various pharmacological modalities. One study has revealed the anti-inflammatory effect of the plant’s subtypes [82]. Other studies demonstrated anti-parasitic, anti-fungal and anti-microbial effect of J. excelsa essential oil or hydroalcoholic extract, which is in relation with the presence of potent fractions or metabolites in the essential oil or extracts of this plant [81, 83]. Anti-tumor and cytotoxic activity of J. excelsa leaves and fruit essential oil have also been shown in other studies [84]. Persian Juniper has been shown to have an effect on cell-cycle phases of MCF-7 breast cancer cell line [85]. Satisfactory antileishmanial activity of Juniperus species have also been demonstrated in a few in-vitro and in-vivo studies [44, 53, 86]. In addition, antioxidant and radical scavenging activities of high content of terpenoids in essential oil and considerable phenolic content in extracts of J. excelsa have been indicated useful for wound healing [87–89]. Mirzavand et al reported that the extract of the leaf of J. excelsa has more inhibitory effects on Leishmania amastigote in comparison with meglumine antimoniate in an animal model. In that study, the reduction of diameter of the lesions became statistically significant in the sixth week of treatment in the treatment group with J. excelsa as compared to that of the control group. However, there were no statically significant differences in reduction size of mice lesions in the J. excelsa group in comparison with the intra-lesional meglumine antimoniate group [53].

There are also several plants which have been examined for antileishmanial activity. Zerehsaz et al, showed the therapeutic effect of Z-HE (a traditional medicine) in healing of CL[90]. Another study demonstrated the anti-leishmanial effect of Peganum harmala L [91]. Chan-
Bacb et al showed that Annona senegalensis Pers possessed several components with antileishmanial activity against related promastigotes [92]. Additional in-vitro and in-vivo studies revealed the leishmanicidal activity and cytotoxicity of extracts of Ricinus communis L. and Azadirachta indica A.Juss., especially in combination therapy [93].

In our study, five patients experienced local irritation after five to nine weeks of the JE administration in group A. However, this difference was not statistically significant as compared to that of group B. Overall, no acute reaction was observed. To our knowledge, this is the first report of any side effects related to the use JE in the medical literature.

The result of GEE model in our study demonstrated that combination of cryotherapy with a cream containing J. excelsa hydroalcoholic extract can decrease the duration of CL treatment and also result in a decrease in the number of cryotherapy sessions. Therefore, it seems that a cream containing JE in combination with cryotherapy in the treatment of CL can be a good alternative to meglumine antimoniate.

In our study, except for one authentication of leishmania species as L. infantum, all cases with positive PCR, were identified as L. major. Predominancy of the L. major subtype in Iran has been reported in previous studies [10, 94, 95]. The L. infantum case was in group B, which received cryotherapy plus placebo. Therefore, we did not evaluate the effect of the JE, according to the species of CL, in this study. It is interesting to note that the patient with L.infantum was labeled as failure to treatment at the 12th-week of follow-up.

The PCR method, did not identify the strain of Leishmania amastigote in a few of the patients who had positive smears for Leishmania amastigote. This finding may be due to technical errors in sampling of the tissue and/or processing of the PCR. On the other hand, it is worth mentioning that the sensitivity of PCR for detection of leishmaniasis is not perfect [96]. Four patients out of 33 in group A and one out of 29 patients in group B (a total of 5 patients with PCR-undetected strains of Leishmania), had complete cure in this study. The other 4 patients (one patient in group A and 3 patients in group B), with PCR-undetected strains of Leishmania, failed treatment.

Limitations of the study
Firstly, our project is a combination study. Both groups received cryotherapy as baseline therapy. As cryotherapy was done 1–2 millimeters around the lesions, the size of the lesions were affected with this procedure, and it can be a confounding factor in both groups. Secondly, dose assessment was not performed in our study. In other words, we did not evaluate different concentrations of the JE in the cream base. Also, we excluded the patients who had CL lesions on the face due to ethical considerations. Therefore, we could not evaluate the effect of this herbal extract on facial ulcers and subsequently, hypersensitivity or side effects on the facial skin. Moreover, children under 18 years of age were excluded from this study due to ethical issues. In this regard, we could not evaluate this product in children. We expect that the response in children will be acceptable due to a thinner skin and easier penetration, in comparison with adults. Furthermore, we could not evaluate the serum level of active constituents of JE in order to estimate the dermal absorption of this extract.

Conclusion
The result of this study demonstrated a good efficacy of JE in a 5% cream base, for the treatment of CL. Since JE, is a cheap, easy available and safe product, it can be considered as an alternative adjuvant treatment modality in CL. Overall, we suggest further studies in order to focus on mono-therapy with this preparation (prescribing only the cream containing JE without any concomitant modalities), in the treatment of CL.
Supporting information

S1 Consort Checklist.

S1 Fig. CONSORT chart of the clinical trial of therapeutic effect of Juniperus excelsa M. Bieb extract cream on cutaneous leishmaniasis.

S2 Fig. Electrophoresis of PCR products of DNA extracted from positive smears. The 15 lanes are shown in this figure and consist of ladder lanes (1 and 15); weakly positive (lane 2); positive control of L. infantum (lane 3); positive control of L. major (lane 14); Patients samples (lanes 4–13).

S3 Fig. Changes in the size of CL lesions in both groups during three months. The numbers on the left side of the chart indicate the changes of length of the ulcers (mm) and the numbers on right side of the chart indicate the area changes (mm²) in the duration of treatment (12 weeks).

S4 Fig. CL patient who was cured in group A. (A)Before treatment, (B)after one week, (C) after two weeks, (D)after three weeks, (E) after five weeks.

S1 Table. Demographic characteristics of CL patients in both groups (Group A and Group B).

S2 Table. Outcome of the treatment in both groups (Group A and Group B).

S3 Table. Volatile constituents of both the plant methanol extract and prepared JE cream. The significance of bold is to present the most abundant constituents. Compounds have been identified by combination of both mass spectra and retention indices. RI represents the retention indices which were calculated against C8-C24 n-alkanes in the mentioned column. Compounds have been sorted with respect to retention indices on HP-5 MS capillary column.

Acknowledgments

This article was extracted from the thesis written by Dr. Mohammad Mahdi Parvizi as partial fulfillment of the requirements for obtaining his PhD degree in the field of Traditional Persian Medicine at Shiraz University of Medical Sciences, Shiraz, Iran. Hereby, the authors wish to thank the Molecular Dermatology Research Center, Shohadaye-Enghelab Health Center, Department of Parasitology and Mycology, and Medicinal Plants Processing Research Center at Shiraz University of Medical Sciences for their invaluable assistance in helping the authors with this project. The authors are also grateful to Mr. Jamshid Jamali and Mr. Peyman Jafari at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for their helps in statistical analysis.

Author Contributions

Conceptualization: Mohammad Mahdi Parvizi, Farhad Handjani, Mahmoodreza Moein, Gholamreza Hatam, Majid Nimrouzi, Jafar Hassanzadeh, Nasrin Hamidizadeh, Mohammad Mehdi Zarshenas.
Data curation: Mohammad Mahdi Parvizi, Farhad Handjani, Hamid Reza Khorrami, Mohammad Mehdi Zarshenas.

Formal analysis: Mohammad Mahdi Parvizi, Jafar Hassanzadeh.

Funding acquisition: Farhad Handjani.

Investigation: Mohammad Mahdi Parvizi, Nasrin Hamidizadeh, Hamid Reza Khorrami, Mohammad Mehdi Zarshenas.

Methodology: Farhad Handjani, Gholamreza Hatam, Majid Nimrouzi, Jafar Hassanzadeh, Mohammad Mehdi Zarshenas.

Project administration: Farhad Handjani, Gholamreza Hatam, Jafar Hassanzadeh, Nasrin Hamidizadeh, Hamid Reza Khorrami.

Resources: Farhad Handjani.

Software: Mohammad Mahdi Parvizi, Jafar Hassanzadeh, Mohammad Mehdi Zarshenas.

Supervision: Farhad Handjani, Mahmoodreza Moein, Gholamreza Hatam, Majid Nimrouzi, Mohammad Mehdi Zarshenas.

Validation: Farhad Handjani, Jafar Hassanzadeh.

Visualization: Mahmoodreza Moein, Gholamreza Hatam.

Writing – original draft: Mohammad Mahdi Parvizi, Gholamreza Hatam, Mohammad Mehdi Zarshenas.

Writing – review & editing: Farhad Handjani, Gholamreza Hatam, Majid Nimrouzi, Mohammad Mehdi Zarshenas.

References

1. Sundar S, Longo D, Fauci A, Kasper D, Hauser S, Jameson J, et al. Leishmaniasis. Harrison's Principles of Internal Medicine, 18th Edition 18th Edition ed. New York: McGraw-Hill Medical Publishing Division; 2011.

2. Habif T. Clinical Dermatology. 5 ed. USA: Mosby; 2010. 1040 p.

3. Azizi MH, Bahadori M, Dabiri S, Shamsi Meymandi S, Azizi F. A History of Leishmaniasis in Iran from 19th Century Onward. Archives of Iranian medicine. 2016; 19(2):153–62. Epub 2016/02/04. https://doi.org/0161902/AIM.0016 PMID: 26838089.

4. Bray R. Note on the history of cutaneous leishmaniasis in the Mediterranean and Middle East area. Parasitologia. 1986; 29(2–3):175–9 PMID: 3334081.

5. Avicenna H. The Canon in Medicine. Beirut: Institute of Al-A'lam Li Al-Matbooaat; 2005.

6. World Health Organization. Sixtieth World Health Assembly. Control of leishmaniasis. World Health Organization, Geneva, Switzerland [Internet]. 2007. Available from: http://apps.who.int/iris/bitstream/10665/69916/1/WHO_CDS_NTD_IDM_2007.3_eng.pdf.

7. Sharifi F, Sharifi I, Zarean M, Parizi MH, Aflatoonian M, Harandi MF, et al. Spatial distribution and molecular identification of leishmania species from endemic foci of South-eastern iran. Iran J Parasitol. 2012; 7(1):45–52. PMID: 23133471

8. van Thiel PP, Leenstra T, de Vries HJ, van der Sluis A, van Gool T, Krull AC, et al. Cutaneous leishmaniasis (Leishmania major infection) in Dutch troops deployed in northern Afghanistan: epidemiology, clinical aspects, and treatment. Am J Trop Med Hyg. 2010; 83(6):1295–300. https://doi.org/10.4269/ajtmh.2010-0143 PMID: 21118937

9. Oryan A, Shirian S, Tabandeh MR, Hatam GR, Randau G, Daneshbod Y. Genetic diversity of Leishmania major strains isolated from different clinical forms of cutaneous leishmaniasis in southern Iran based on minicircle kDNA. Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2013; 19:226–31. Epub 2013/07/31. https://doi.org/10.1016/j.megid.2013.07.021 PMID: 23892374.
10. Pourmohammadi B, Motazedian M, Hatam G, Kalantari M, Habibi P, Sarkari B. Comparison of three methods for diagnosis of cutaneous leishmaniasis. Iran J Parasitol. 2010; 5(4):1–8. Epub 2010/12/01. PMID: 22347259; PubMed Central PMCID: PMCPMC3279850.

11. Asilian A. Cutaneous Leishmaniasis: Isfahan university of medical science; 1992.

12. Mapar M, Kvoushi H, MA D. Effect of opium in the treatment of localized cutaneous leishmaniasis. Iranian Journal Of Dermatology. 2001; 4(4):23–8.

13. Mendonça MG, de Brito ME, Rodrigues EH, Bandeira V, Jardim ML, Abath FG. Persistence of Leishmania parasites in scars after clinical cure of american cutaneous leishmaniasis: is there a sterile cure? Journal of Infectious Diseases. 2004; 189(6):1018–23. https://doi.org/10.1086/382135 PMID: 14999605

14. Yani k M, Gurel MS, Simsek Z, Kati M. The psychological impact of cutaneous leishmaniasis. Clinical and experimental dermatology. 2004; 29(5):464–7. Epub 2004/09/07. https://doi.org/10.1111/j.1365-2230.2004.01605.x PMID: 15347324.

15. Marsden PD. Mucosal leishmaniasis (“espundia” Escorial, 1911). Transactions of the Royal Society of Tropical Medicine and Hygiene. 1986; 80(6):859–76. Epub 1986/01/01. PMID: 3037735.

16. Gonzalez U, Pinart M, Sinclair D, Firooz A, Enk C, Velez ID, et al. Vector and reservoir control for preventing leishmaniasis. The Cochrane database of systematic reviews. 2015;(8):CD008736. Epub 2015/08/08. https://doi.org/10.1002/14651858.CD008736.pub2 PMID: 26246011; PubMed Central PMCID: PMC4561525.

17. Handler MZ, Patel PA, Kapila R, Al-Qubati Y, Schwartz RA. Cutaneous and mucocutaneous leishmaniasis: Differential diagnosis, diagnosis, histopathology, and management. Journal of the American Academy of Dermatology. 2007; 57(2):335 e1–29. Epub 2007/03/06. https://doi.org/10.1016/j.jaad.2007.01.016 PMID: 17337090.

18. Croft SL, Coombs GH. Leishmaniasis—current chemotherapy and recent advances in the search for novel drugs. Trends in parasitology. 2003; 19(11):502–8. PMID: 14580961.

19. Khatami A, Firooz A, Gorouhi F, Dowlati Y. Treatment of acute Old World cutaneous leishmaniasis: a systematic review of the randomized controlled trials. Journal of the American Academy of Dermatology. 2007; 57(2):335 e1–29. Epub 2007/03/06. https://doi.org/10.1016/j.jaad.2007.01.016 PMID: 17337090.

20. Salamanpour R, Razmavar MR, Abtahi N. Comparison of intralesional meglumine antimoniate, cryotherapy and their combination in the treatment of cutaneous leishmaniasis. International journal of dermatology. 2006; 45(9):1115–6. Epub 2006/09/12. https://doi.org/10.1111/j.1365-4632.2006.02822.x PMID: 16961529.

21. Asilian A, Sadeghinia A, Faghihi G, Momeni A. Comparative study of the efficacy of the combined therapy of intralesional meglumine antimoniate (Gluconite) vs. cryotherapy and intralesional meglumine antimoniate (Gluconite) alone for the treatment of cutaneous leishmaniasis. International journal of dermatology. 2004; 43(4):281–3. Epub 2004/04/20. https://doi.org/10.1111/j.1365-4632.2004.02002.x PMID: 15090013.

22. Aronson NE, Wortmann GW, Johnson SC, Jackson JE, Gasser RA Jr., Magill AJ, et al. Safety and efficacy of intravenous sodium stibogluconate in the treatment of leishmaniasis: recent U.S. military experience. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 1998; 27(6):1457–64. Epub 1998/12/30. PMID: 9868660.

23. Oliveira LF, Schubach AO, Martins MM, Passos SL, Oliveira RV, Marzochi MC, et al. Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. Acta tropicala. 2011; 118 (2):87–96. https://doi.org/10.1016/j.actatropical.2011.02.007 PMID: 21420925.

24. Tallab TM, Bahamdam KA, Mirdad S, Johargi H, Mourad MM, Ibrahim K, et al. Cutaneous leishmaniasis: schedules for intralesional treatment with sodium stibogluconate. International journal of dermatology. 1996; 35(8):594–7. Epub 1996/08/01. PMID: 8854166.

25. Uzun S, Durdu M, Cuiha G, Allahverdiyev AM, Memisoglu HR. Clinical features, epidemiology, and efficacy and safety of intralesional antimony treatment of cutaneous leishmaniasis: recent experience in Turkey. The Journal of parasitology. 2004; 90(4):853–9. Epub 2004/09/11. https://doi.org/10.1645/GE-185R PMID: 15357081.

26. Layegh P, Pezeshkipoor F, Soruri AH, Naviifar P, Moghimian T. Efficacy of cryotherapy versus intralesional meglumine antimoniate (glucantime) for treatment of cutaneous leishmaniasis in children. Am J Trop Med Hyg. 2009; 80(2):172–5. Epub 2009/02/05. PMID: 19190206.

27. al-Majali O, Routh HB, Abuloham O, Bhowmik KR, Muhsen M, Hebeheba H. A 2-year study of liquid nitrogen therapy in cutaneous leishmaniasis. International journal of dermatology. 1997; 36(6):460–2. Epub 1997/06/01. PMID: 9248896.
28. Panagiotopoulu A, Stavropoulos PG, Hasapi V, Papakonstantinou AM, Petridis A, Katsambas A. Treatment of cutaneous leishmaniasis with cryosurgery. International journal of dermatology. 2005; 44(9):749–52. Epub 2005/09/02. https://doi.org/10.1111/j.1365-4632.2005.02628.x PMID: 16135144.

29. Lopez-Carvajal L, Cardona-Arias JA, Zapata-Cardona MI, Sanchez-Giraldo V, Velez ID. Efficacy of cryotherapy for the treatment of cutaneous leishmaniasis: meta-analyses of clinical trials. BMC infectious diseases. 2016; 16:360. Epub 2016/07/28. https://doi.org/10.1186/s12879-016-1663-3 PMID: 27456008; PubMed Central PMCID: PMC4960741.

30. Masmoudi A, Maalej N, Boudaya S, Turki H, Zahaf A. [Adverse effects of intralesional Glucantime in the treatment of cutaneous leishmaniasis]. Medecine et maladies infectieuses. 2006; 36(4):226–8. https://doi.org/10.1016/j.medmal.2005.11.018 PMID: 16600554.

31. Unger A, O’Neal S, Machado PR, Guimaraes LH, Morgan DJ, Schriever A, et al. Association of treatment of American cutaneous leishmaniasis prior to ulcer development with high rate of failure in northeastern Brazil. Am J Trop Med Hyg. 2009; 80(4):574–9. PMID: 19346378; PubMed Central PMCID: PMC3557504.

32. Emami SA, Asili J, Mohagheghi Z, Hassanzadeh MK. Antioxidant activity of leaves and fruits of Iranian conifers. Evidence-based complementary and alternative medicine: eCAM. 2007; 4(3):313–9. https://doi.org/10.1093/ecam/nem011 PMID: 17965761; PubMed Central PMCID: PMC1978238.

33. Sela F, Karapandzova M, Stefkov G, Cvetkovikj I, Kulevanova S. Chemical composition and antimicrobial activity of essential oils of Juniperus excelsa Bieb. (Cupressaceae) grown in R Macedonia. Pharmacognosy Res. 2015; 7(1):74–80. doi: D—NLM: PMC4285633 OTO—NOTN LM. https://doi.org/10.4103/0974-8490.147212 PMID: 25598638.

34. Unlu M, Vardar-Unlu G, Vural N, Donmez E, Cakmak O. COMPOSITION AND ANTIMICROBIAL ACTIVITY OF Juniperus excelsa ESSENTIAL OIL. Chemistry of Natural Compounds. 2008; 44(1):129–31. https://doi.org/10.1007/s10600-008-0040-x

35. Mosavat SH, Ghahramani L, Haghighi ER, Chaijan MR, Hashempur MH, Heydari M. Anorectal Diseases in Avicenna's "Canon of Medicine". Acta Med Hist Adriat. 2015; 2:103–14 PMID: 26959635.

36. Hashempur MH, Ghasemi MS, Daneshfard B, Ghoreishi PS, Lari ZN, Homayouni K, et al. Efficacy of topical chamomile oil for mild and moderate carpal tunnel syndrome: A randomized double-blind placebo-controlled clinical trial. Complement Ther Clin Pract. 2017; 26:61–7. https://doi.org/10.1016/j.ctcp.2016.11.010 PMID: 28107892.

37. Hashempour MM, Hashempour MH, Mosavat SH, Heydari M. Rhazes—His Life and Contributions to the Field of Dermatology. JAMA Dermatology. 2017; 153(1):70–. https://doi.org/10.1001/jamadermatol.2016.0144 PMID: 28114524.

38. Behbehani AM, Rhazes. The original portrayor of smallpox. Jama. 1984; 252(22):3156–9. PMID: 6389914.

39. Razi M. Al-havi. Tehran, Iran: Academy of Medical Sciences Islamic Republic of Iran (Original work published 10th century)[Arabic]; 2005.

40. Loizzo MR, Tundis R, Conforti F, Saab AM, Statti GA, Menichini F. Comparative chemical composition, antioxidant and hypoglycaemic activities of Juniperus oxycedrus ssp. oxycedrus L. berry and wood oils from Lebanon. Food Chemistry. 2007; 105(2):572–8. https://doi.org/10.1016/j.foodchem.2007.04.015.

41. Orhan N, Orhan Ie Fau—Ergun F, Ergun F. Insights into cholinesterase inhibitory and antioxidant activities of five Juniperus species. Food Chem Toxicol. 2011; 49(9):2305–12. https://doi.org/10.1016/j.fct.2011.06.031 PMID: 21708212.

42. Stanley PL, Steiner S, Havens M, Tramposch K. Mouse skin inflammation induced by multiple topical applications of 12-O-tetradecanoylphorbol-13-acetate. Skin Pharmacology. 1991; 4(4):262–71. PMID: 1789987.

43. Tumen I, Sünitar I, Keleş H, Küpeli Akkol E. A therapeutic approach for wound healing by using essential oils of cupressus and juniperus species growing in Turkey. Evidence-based complementary and alternative medicine: 2011; 2012. https://doi.org/10.1155/2012/728281 PMID:PMC3175711; PMID: 21941588.

44. Moein M, Hatam G, Taghavi-Moghadam R, Zareshenas MM. Antileishmanial Activities of Greek Juniper (Juniperus excelsa M.Bieb.) Against Leishmania major Promastigotes. Journal of evidence-based complementary & alternative medicine. 2016. Epub 2016/01/10. https://doi.org/10.1177/2156587216623435 PMID: 26747836.

45. Pourmorad F, Hosseinimehr S, Shahabimajd N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. African journal of biotechnology. 2006; 5(11) https://www.ajol.info/index.php/ajb/article/view/42999.
46. Singleton VL, Orthofer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods in enzymology. 1999; 299:152–78. https://doi.org/10.1007/978-6879(99)9017-1

47. Mohammadpour I, Motazedian MH, Handjani F, Hatam GR. Cutaneous Leishmaniasis of the Eyelids: A Case Series with Molecular Identification and Literature Review. Korean J Parasitol. 2016; 54(6):787–92. https://doi.org/10.3347/kjp.2016.54.6.787 PMID: 28095664

48. Motazedian MH, Parhizkari M, Mehrabani D, Hatam G, Asgari Q. First detection of Leishmania major in Rattus norvegicus from Fara Province, Southern Iran. Vector Borne Zoonotic Dis. 2010; 10(10):969–75. https://doi.org/10.1089/vbz.2008.0214 PMID: 20426685

49. Noyes HA, Rayburn H, Bailey JW, Smith D. A nested-PCR-based schizodeme method for identifying Leishmania kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of Leishmania tropica in Pakistan. Journal of clinical microbiology. 1998; 36(10):2877–81. Epub 1998/09/17. PMID: 9738037; PubMed Central PMCID: PMCPMC105081.

50. Mohammadpour I, Motazedian MH, Handjani F, Hatam GR. Lip leishmaniasis: a case series with molecular identification and literature review. BMC infectious diseases. 2017; 17(1):96. https://doi.org/10.1186/s12879-016-2178-7 PMID: 28122496; PubMed Central PMCID: PMC5264488.

51. Aransay AM, Scoulica E, Tselentis Y. Detection and identification of Leishmania DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplast DNA. Appl Environ Microbiol. 2000; 66(5):1933–8. PMID: 10788363

52. Nabi Sajid, Ahmed Nisar, Javed Khan Muhammad, Bazai Zahoor, Yasinzaí M, Al-Kahraman YMSA. In vitro Antileishmanial, Antitumor Activities and Phytochemical Studies of Methanolic Extract and its Fractions of Juniperus Excelsa Berries. World Applied Sciences Journal. 2012; 19(10):1495–500.

53. Mirzamand AV, Motazedian MH, Hatam G, Asgari Q. First detection of Leishmania major in Rattus norvegicus from Fars Province, Southern Iran. Vector Borne Zoonotic Dis. 2010; 10(10):969–75. https://doi.org/10.1089/vbz.2008.0214 PMID: 20426685

54. Barreto ALS, Alex J, English M, de Souza APF, da Silva EP, Guimarães EF, et al. Chemical diversity and leishmanicidal activity of Manekia obtusa Miq (Piperaceae). Journal of Medicinal Plants Research. 2013; 7(46):3367–74. https://doi.org/10.5897/JMPR2013.5091
66. Naghavi MR, Alaeimoghadam F, Ghafoori H. Artemisia species from Iran as valuable resources for medicinal uses. World Academy of Science, Engineering and Technology, International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering. 2014; 8(11):1194–200. https://waset.org/Publication/artemisia-species-from-iran-as-valuable-resources-for-medicinal-uses/9999694

67. Muzitano MF, Cruz EA, de Almeida AP, Da Silva SA, Kaiser CR, Guette C, et al. Quercitrin: an antileishmanial flavonoid glycoside from Kalanchoe pinnata. Planta medica. 2006; 72(01):81–3. https://doi.org/10.1055/s-2005-873163 PMID: 16450304.

68. Tasdemir D, Kaiser M, Brun R, Yardley V, Schmidt TJ, Tosun F, et al. Antitrypanosomal and antileishmanial activities of flavonoids and their analogues: in vitro, in vivo, structure-activity relationship, and quantitative structure-activity relationship studies. Antimicrobial agents and chemotherapy. 2006; 50 (4):1352–64. https://doi.org/10.1128/AAC.50.4.1352-1364.2006 PMID: 16569852

69. Soneja A, Drews M, Malinski T. Role of nitric oxide, nitrooxidative and oxidative stress in wound healing. Pharmacological Reports. 2005; 57:108. PMID: 16415491

70. Dröge W. Free radicals in the physiological control of cell function. Physiological reviews. 2002; 82 (1):47–95. https://doi.org/10.1152/physrev.00018.2001 PMID: 11773609

71. Lopez L, Robayo M, Vargas M, Velez ID. Thermotherapy. An alternative for the treatment of American cutaneous leishmaniasis. Trials. 2012; 13:58. https://doi.org/10.1186/1745-6215-13-58 PMID: 22594858; PubMed Central PMCID: PMC3441257.

72. Blum J, Lockwood DN, Visser L, Harms G, Bailey MS, Caumes E, et al. Local or systemic treatment for New World cutaneous leishmaniasis? Re-evaluating the evidence for the risk of mucosal leishmaniasis. International health. 2012; 14(3):153–63. https://doi.org/10.1016/j.ijih.2012.06.004 PMID: 24029394.

73. de Vries HJ, Reedijk SH, Schallig HD. Cutaneous leishmaniasis: recent developments in diagnosis and management. American journal of clinical dermatology. 2015; 16(2):99–109. Epub 2015/02/18. https://doi.org/10.1007/s40257-015-0114-z PMID: 25687688; PubMed Central PMCID: PMCPMC4363483.

74. Beheshti M, Ghotti S, Amirizade S. Therapeutic and Adverse Effects of Glucantime Used for Treatment of Cutaneous Leishmaniasis. Shiraz E-Medical Journal. 2007; 8(4):155–61 http://emedicalj.portal.tools/?page=article&article_id=20679.

75. Rodrigues M, Costa R, Souza C, Foss N, Roselino A. Nephrotoxicity attributed to meglumine antimoniate (Glucantime) in the treatment of generalized cutaneous leishmaniasis. Revista do Instituto de Medicina Tropical de Sao Paulo. 1999; 41(1):33–7. PMID: 10436688

76. Emad M, Hayati F, Fallahzadeh MK, Namazi MR. Superior efficacy of oral fluconazole 400 mg daily versus oral flucanazole 200 mg daily in the treatment of cutaneous leishmaniasis major infection: a randomized clinical trial. Journal of the American Academy of Dermatology. 2011; 64(3):606–8. Epub 2011/02/15. https://doi.org/10.1016/j.jaad.2010.04.014 PMID: 21315963.

77. Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. The Lancet Infectious diseases. 2007; 7(9):581–96. Epub 2007/08/24. https://doi.org/10.1016/S1473-3099(07)70209-8 PMID: 17714672.

78. Bailey MS, Lockwood DN. Cutaneous leishmaniasis. Clinics in dermatology. 2007; 25(2):203–11. https://doi.org/10.1016/j.clindermatol.2006.05.008 PMID: 17350500

79. Al-Majali O, Routh HB, Abuloham O, Bhowmik KR, Muhsen M, Hebeheba H. A 2-year study of liquid nitrogen therapy in cutaneous leishmaniasis. International journal of dermatology. 1999; 38(6):460–2. https://doi.org/10.1111/j.1365-4336.1999.tb03221.x PMID: 10436688

80. Chakravarty J, Sundar S. Drug resistance in leishmaniasis. Journal of global infectious diseases. 2010; 2(2):167–76. https://doi.org/10.4103/0974-777X.62887 PMID: 20606973; PubMed Central PMCID: PMC2889657.

81. Moein M, Ghasemi Y, Moein S, Nejati M. Analysis of antimicrobial, antifungal and antioxidant activities of Juniperus excelsa M. B subsp. Polycarpos (K. Koch) Takhtajan essential oil. Pharmacognosy Research. 2010; 2(3):128. https://doi.org/10.4103/0974-8490.65505 PMID: 21808554

82. ORHAN N, Akkol E, Ergun F. Evaluation of anti-inflammatory and antinociceptive effects of some Juniperus species growing in Turkey. Turkish Journal of Biology. 2012; 36(6):719–26 http://journals.tubitak.gov.tr/biology/abstract.htm?id=13126.

83. Asili J, Emami S, Rahimizadeh M, Fazly-Bazaz B, Hassananzadeh M. Chemical and antimicrobial studies of Juniperus excelsa subsp. excelsa and Juniperus excelsa subsp. polycarpos essential oils. Journal of Essential Oil Bearing Plants. 2006; 11(3):292–302. https://doi.org/10.1080/0972006X.2006.10643633

84. Topçu G, Gören AC, Bilgel G, Bilgel M, Çakmak O, Schilling J, et al. Cytotoxic Activity and Essential Oil Composition of Leaves and Berries of Juniperus excelsa. Pharmaceutical biology. 2005; 43(2):125–8. https://doi.org/10.1080/13880200590919429
85. Andalib A, Jafarian-Dehkordi A, Shokouhi-Shourmasti R, Abdolah-Kohpayeh-Esfahani S. The effect of Persian Juniperus excelsa extracts on cell-cycle phases of MCF-7 breast cancer cell line. Journal of Isfahan Medical School. 2016; 33(360):2004–12.

86. Machado M, Santoro G, Sousa MC, Salgueiro L, Cavaleiro C. Activity of essential oils on the growth of Leishmania infantum promastigotes. Flavour and Fragrance Journal. 2010; 25(3):156–60. https://doi.org/10.1002/ffj.1987

87. Moein M, Moein S. Antioxidant activities and phenolic content of Juniperus excelsa extract. Iranian Journal of Pharmaceutical Sciences. 2010; 6(2):133–40. http://www.ijps.ir/?_action=articleInfo&article=2137

88. Reza MM, Soheila M, Farkhondeh M. Study the relationship between antioxidant potential and phenolic contents of Juniperus Excelsa fruit. International Journal of Pharmacy and Pharmaceutical Sciences. 2014; 6(7):192–4. https://innovareacademics.ir/journals/index.php/ijpps/article/view/1718

89. Emami SA, Abedindo BF, Hassanzadeh-Khayyat M. Antioxidant activity of the essential oils of different parts of Juniperus excelsa M, Bieber, subsp. excelsa and J. excelsa M. Bieber. subsp. polycarpos (K. Koch) Takhtajan (Cupressaceae). Iranian Journal of Pharmaceutical Research. 2011; 10(4):799–810. PMID: 24250416

90. Zerehsaz F, Salmanpour R, Handjani F, Ardehali S, Panjehshahin MR, Tabei SZ, et al. A double-blind randomized clinical trial of a topical herbal extract (Z-HE) vs. systemic meglumine antimoniate for the treatment of cutaneous leishmaniasis in Iran. International journal of dermatology. 1999; 38: 610–2. PMID: 10487453

91. Mirzaie M, Nosratabadi SJ, Derakhshanfar A, Sharif I. Antileishmanial activity of Peganum harmala extract on the in vitro growth of Leishmania major promastigotes in comparison to a trivalent antimony drug. Veterinarski arhiv. 2007; 77(4):365.

92. Chan-Bacab MJ, Pena-Rodriguez LM. Plant natural products with leishmanicidal activity. Natural product reports. 2001; 18(6):674–88. Epub 2002/02/01. PMID: 11820764.

93. Jumba BN, Anjili CO, Makwali J, Ingonga J, Nyamao R, Marango S, et al. Evaluation of leishmanicidal activity and cytotoxicity of Ricinus communis and Azadirachta indica extracts from western Kenya: in vitro and in vivo assays. BMC research notes. 2015; 8:650. Epub 2015/11/07. https://doi.org/10.1186/s13104-015-1605-y PMID: 26541197; PubMed Central PMCID: PMCPMC4635543.

94. Shirian S, Oryan A, Hatami GR, Daneshbod Y. Three Leishmania/L. species—L. infantum, L. major, L. tropica—as causative agents of mucosal leishmaniasis in Iran. Pathogens and global health. 2013; 107(5):267–72. Epub 2013/08/07. https://doi.org/10.1179/2047773213Y.0000000098 PMID: 23916336; PubMed Central PMCID: PMCPMC4001456.

95. Razmjou S, Hejazy H, Motazedian MH, Baghaei M, Emamy M, Kalantary M. A new focus of zoonotic cutaneous leishmaniasis in Shiraz, Iran. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2008; 102(7):727–30. Epub 2009/02/19. https://doi.org/10.1016/j.trstmh.2008.12.013 PMID: 19223055.

96. Ribeiro-Romao RP, Saavedra AF, Da-Cruz AM, Pinto EF, Moreira OC. Development of real-time PCR assays for evaluation of immune response and parasite load in golden hamster (Mesocricetus auratus) infected by Leishmania (Viannia) braziliensis. Parasites & vectors. 2016; 9(1):361. Epub 2016/06/29. https://doi.org/10.1186/s13071-016-1647-6 PMID: 27350537; PubMed Central PMCID: PMCPMC4924296.