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In vitro and in vivo Assessment of Anti-Leishmanial Efficacy of Leaf, Fruit, and Fractions of *Juniperus excelsa* Against Axenic Amastigotes of *Leishmania major* and Topical Formulation in BALB/c Mice

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Abstract

Background: Leishmaniasis is a parasitic disease caused by *Leishmania* protozoa. Iran is an endemic region for leishmaniasis and thus, many natural medicaments, such as *Juniperus excelsa*, are traditionally being used for the treatment of its cutaneous form.
Objectives: The current study aimed at assessing the anti-leishmanial activities of this medicament's leaf and fruit extracts and respective leaf fractions against *Leishmania major* as the causative agent of zoonotic cutaneous leishmaniasis in both in vitro and in vivo models.
Methods: This experimental study was carried out at Shiraz University of Medical Sciences, Shiraz, Iran, during the year 2013. For the generation of axenic amastigotes, promastigotes were mass cultivated and incubated at 33 to 34°C and pH 3.5. The anti-amastigote activity was evaluated using the colorimetric assay. For in vivo study, promastigotes were inoculated in 40 female BALB/c mice tails. Lesions were created after four weeks. Animals were randomly distributed to four groups as test, placebo, Glucantime, and control. Mice were treated for 30 days. Lesion diameters were measured and recorded weekly.
Results: All extracts and fractions possessed potent activities; leaves showed the highest with IC_{50} (SD) (50% inhibitory concentration) 0.97 $\pm$ 3.53 mg/mL, and among fractions, ethyl acetate with IC_{50} 1.95 $\pm$ 5.30 had greater anti-leishmanial activity. In animal models compared with the beginning, the diameter of Cutaneous Leishmaniasis (CL) lesion had a significant difference in the test group ($P < 0.05$ vs. control group) at the end of the protocol. However, no significant differences were seen in placebo and Glucantime groups versus the control group.
Conclusions: Comprehensive and long term therapy assessment with *J. excelsa* is needed to introduce it as a natural anti-leishmanial medicine.

Keywords: Cutaneous, Ethyl Acetate, Inbred BALB C, *Juniperus*, Laboratory, *Leishmania major*, Leishmaniasis, Mice, Meglumine Antimoniate, Natural Products

1. Background

Leishmaniasis is a neglected tropical disease transmitted by arthropods (Female sand fly bites) (1). Causative agents of this infectious disease are protozoan parasites of the genus *Leishmania*. The disease is endemic in 98 countries, including tropical, subtropical, and Mediterranean areas. Having a prevalence of 12 million and 350 million people at risk, leishmaniasis is one of the nine major tropical diseases around the world (2, 3).

Clinical form of leishmaniasis is Cutaneous (CL), Mucocutaneous (MCL), Visceral (VL) Post-Kala-Azar Dermal (PKDL), and diffuse cutaneous leishmaniasis. More than 90% of CL is present in Iran, Afghanistan, Nepal, Syria, Saudi Arabia, and Peru (4). Iran has an exceptional situation in the Middle East, and all clinical forms of CL have been reported during the last decades (5-8). The association of leishmaniasis with immune system deficiency and lack of concerned vaccine encourages manufacturers to release new product lines related to this disorder (9). Chemotherapy is critical to control and manage leishmaniasis. Currently, the first therapeutic line for leishmaniasis is pentavalent antimonial formulations, followed by Amphotericin B and Paromomycin (10). The high cost for low income developing countries and long term treatment as well as toxicity, undesirable effects, relapse, and resistance to these medicaments results in more efforts for new drug

discovery (11, 12). Plant extracts and derived compounds may be an appropriate choice showing achievements in clinical approaches for the management of leishmaniasis (13). *Juniperus excelsa* is a species of Cupressaceae family, including two subspecies: subsp. *excelsa* (Greek juniper) and subsp. *polycarpos* (Persian juniper). Geographically, this plant is distributed in south and east of Europe, Turkey, Iran, Caucasus, central Asia, Iraq, Syria, Lebanon, and Saudi Arabia (14, 15).

Fruits and leaves of Greek Juniper possessed anti-parasitic, anti-fungal, and antibacterial activity (16-18). The plant is also traditionally applied for diseases, such as bronchitis, epistaxis, common cold, hypertension, inflammation, and gout disease (16) A recent study of antibacterial activity of *J. excelsa* against *Mycobacterium* spp. was reported (19). In folk medicine, the efficacy of the herb for diarrhea, abdominal spasm, asthma, fever, gonorrhea, headache, and leucorrhea has also been reported (20). Greek Juniper has been used by Iranian folk practitioners for a long time (21). In recent studies, the anti-leishmanial activity of leaves, fruit, and fractions of *J. excelsa* on *leishmania* promastigotes had a potent effect, moreover, in a clinical trial study, the topical form of *J. excelsa* was effective against leishmaniasis lesions (22, 23). The anti-leishmanial activity of this plant may be regarded as the presence of components, such as alkaloids, monoterpene, diterpene, sesquiterpene, and saponins as well as flavonoids (24, 25).

2. Objectives

To prove folk reports, the current investigation aimed at evaluating the activity of fruit, leaves, and concerned fractions of Greek Juniper on *L. major* amastigotes, using in vitro and in vivo methods.

3. Methods

3.1. Plant Material

Leaves and fruits of Greek Juniper (*Juniperus excelsa* M. Bieb.) were collected from Genu Mountain, north of Bandar Abbas (The center of Hormozgan province south of Iran) during year 2012. The plant was identified by S. Khademian, botanist of Department of Phytopharmaceuticals, School of pharmacy, Shiraz University of Medical Science, Shiraz, Iran. A voucher number was specified for this plant. Preparation of the extracts, fractions, and cream formulation were conducted at the Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

3.2. Preparation of the Extracts and Fractions

Leaves and fruits of *J. excelsa* were dried, powdered, and subjected to ethanol extraction using percolation method at room temperature for 48 hours. The prepared extract was subsequently concentrated and dried via a rotary (Heidolph, Germany) evaporator. For the fractionation, approximately 76 g of leaf extract was dissolved in 90% methanol (V/V) and then transferred to liquid-liquid extractor in addition to 3 $\times$ 800 mL of Petroleum ether (MERK, Germany). The obtained phase was collected and concentrated. The residual of methanol phase was 250 mL and 60 mL of distilled water was added to the obtained 70% methanol; this phase was mixed and extracted with Chloroform (3 $\times$ 250 mL). Subsequently, ethyl acetate (MERK, Germany; 800 mL) and n-butanol (MERK, Germany; 500 mL) was added to the methanol extract in the extractor to prepare respective fractions. All prepared fractions were then concentrated, dried, and kept in a desiccator.

3.3. Cream Formulation

To this, O/W cream formulations containing base and methanol extracts were prepared. Ingredients were cetomacrogol 1000, cetostearyl alcohol, almond oil, butylated hydroxy toluene, and vaseline. Moreover, 0.2 and 0.02% (W/W) methylparaben and propylparaben were added to the formulations. To prepare O/W cream formulation, oil and aqueous phases were heated up to 75 $\pm$ 1°C. Afterwards, the aqueous phase was gently added to the oil phase. Methanol extract, methylparaben, and propylparaben were dissolved in distilled water. Other components were considered as the oil phase. The O/W mixture was then placed under stirring at 500 rpm (20 minutes) to be cooled at room temperature. The concentration of methanol extract for anti-leishmanial assay in the cream formulation was considered as 5% (W/W); placebo was made in basis cream.

3.4. Parasites and Cultivation of Axenic Amastigotes

Characterized strain of *L. major* isolated from a patient referred to Research Laboratory of tissue and blood protozoa at the Department of Parasitology and Mycology at the Faculty of Medicine, Shiraz University of Medical Sciences, was mass cultivated and used. The promastigotes were cultivated in Schneider's Drosophila medium (HIMEDIA, India) supplemented with 15% fetal calf serum (FCS, SIGMA, USA) until a change to stationary phase. The sample was then centrifuged and incubated at 33 to 34°C in the above-mentioned medium. The pH reached 3.5 by adding succinic acid (SIGMA, USA), and culture was maintained for 120 hours for the generation of axenic amastigotes (26).

3.5. In Vitro Anti-Leishmanial Assay Using MTT

Overall, 10 mg of each extract and 40 mg of Glucantime (SIGMA, USA) were dissolved in 1000 μL dimethyl sulfoxide 5% (DMSO, MERK, Germany) and Phosphate-Buffered Saline (PBS), respectively. The extracts and Glucantime were passed through a 0.22-μm syringe filter. Overall, 100 μL of culture medium was added to each vial and by 100 μL of extracts, and Glucantime, separately, serial dilution was performed (5 concentrations of each) ranging from 0.625 to 10 mg/mL and 2.5 to 40 mg/mL for extracts and Glucantime, respectively. For detection, the viability of axenic amastigotes, colorimetric 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT, SIGMA, USA) assay was employed to compare the effectiveness of the extracts and fractions with Glucantime as the gold standard drug. Overall, 100 μL of prepared culture containing 2×10^6 axenic amastigotes were added to each 96 well of flat bottom plates. Then, 10 μL of each dilution of extracts was added to wells and the plate was wrapped with parafilm tape and incubated at 25°C for 48 hours. Control wells contained axenic amastigotes treated by solvent, and blank wells only contained media. Following the incubation, plates were centrifuged and the supernatant was collected. Adequately, 50 μL of MTT was added to each well and incubated in darkness for three hours at 25°C. Then, 100 μL DMSO was added as the stopper. The plate was slowly shaken and the absorbance was determined using an ELISA reader (BioTek, UK) at 570 nm. The percentage of parasite inhibition by extracts, fractions, and Glucantime was calculated by the following formula:

Inhibition% =100 - [(OD test - OD blank/OD control - OD blank)] $\times$ 100

All the in vitro assays were conducted in duplicates, and the means of duplicate wells are presented as the final results. At the 50% inhibitory concentration (IC_{50}), the drug concentration required for inhibition of 50% of amastigotes was calculated, and results are shown as means and standard deviation.

3.6. In Vivo Anti-Leishmanial Assay

This experimental study was conducted during the year 2013 and approved by the Ethical Committee of Shiraz University of Medical Sciences, Shiraz, Iran. The animals were handled and used in accordance with their moral guidelines during the experiment (Thesis number: IR.SUMS.REC.1392-6486). Forty female inbred BALB/c mice that were three to four weeks old were provided by the Experimental Animal Center, Shiraz University of Medical Sciences, and kept under the standard animal protocols for the programmed experiments. Animals were maintained in the animal house of Shiraz University of Medical Sciences, in cages with standard condition in 12-hour light/dark cycle and at a temperature of 25 $\pm$ 1°C; animals were provided food and water ad libitum. Overall, 250000 stationary phase of promastigotes were injected in the base of the tail of animals. After four weeks, the lesions of *L. major* were created. To confirm the presence of leishmaniasis, samples from lesions were smeared and fixed with absolute methanol and stained with Giemsa for observing amastigotes by using the light microscope. The diameter of lesions was measured by caliper, in two diameters (D and d) at right angles to each other, and the size (mm) was determined according to the formula (27):

S = (D + d)/2

Animals were randomly distributed to four groups (group I: infected and untreated control, group II: the reference drug Glucantime administered intramuscularly as 75 mg/kg/day, group III: cream 5% of *J. excelsa* as 1 mg/mm two times daily, topically on lesions, group IV: placebo cream two times daily, topically on lesions) and the treatment was started. Duration of treatment was 30 days, and measuring the diameter of lesions was performed weekly. At the end of the treatment, smears were prepared from lesions, liver, and spleen of each animal.

3.7. Statistical Analysis

Each concentration was repeated in duplicate, and results were presented as mean and standard deviations. The normality of data was checked by the Kolmogorov-Smirnov test, statistical analysis was done by using one-way Analysis of Variance (ANOVA) and Tukey post hoc tests, while the lesion size progression among the groups was evaluated using repeated measures ANOVA. In this study, $P < 0.05$ was considered statistically significant. Values of IC_{50} were calculated using curve expert version 1.3.

4. Results

4.1. In Vitro Anti-Leishmanial Assay

In this study, five different concentrations of extracts and fractions of *J. excelsa* compared with Glucantime as the standard drug were conducted on axenic amastigotes of *L. major*, according to MTT assay; the extracts and fractions had a remarkable inhibitory effect on amastigotes, and results are presented in Table 1. The highest and lowest of anti-leishmanial activity was related to leave extract and n-butanol, respectively. Result of anti-leishmanial activity of Glucantime is presented in Table 2. Moreover, IC_{50} of extracts and fraction were calculated, results are presented in Table 3.

Results of anti-leishmanial activities of all extracts and fractions compared to glucantime as a standard drug is shown in Figure 1.

Table 1. The Inhibition Effect of Various Concentrations Extracts of the Leaves, Fruit and Fractions of Leaves (0.625 to 10 mg/mL) on *L. major* Amastigotes Using MTT Assay[a]

	Concentration				
	10	**5**	**2.5**	**1.25**	**0.625**
Leaves extract	98.00 ± 1.41	93.00 ± 8.48	84.50 ± 13.44	65.00 ±7.07	25.00 ± 7.07
Petroleum ether (fraction)	98.00 ± 2.83	90.00 ± 5.66	57.50 ± 3.54	37.50 ± 3.54	8.50 ± 2.12
Chloroform (fraction)	90.50 ± 6.36	65.00 ± 7.07	26.00 ± 15.56	25.00 ± 7.07	10.00 ± 2.83
Ethyl acetate (fraction)	95.50 ± 0.71	86.00 ± 12.73	55.00 ± 7.07	42.50 ± 3.54	12.50 ± 3.54
n-butanol (fraction)	65.00 ± 7.07	53.50 ± 17.68	46.00 ± 21.21	12.50 ± 9.19	9.00 ± 4.24
Fruit extract	92.00 ± 2.83	27.50 ± 3.54	27.50 ± 3.54	8.50 ± 2.12	4.50 ± 0.71

[a]Values are expressed as mean ± SD mg/mL.

Table 2. The Inhibition Effect of Various Concentrations of Glucantime as Standard Drug (2.5 to 40 mg/mL) on *L. major* Amastigotes Using MTT Assay[a]

	Concentration, mg/mL				
	40	**20**	**10**	**5**	**2.5**
Inhibition effect	45 ± 7.07	42.5 ± 10.6	40	35 ± 7.07	9 ± 1.41

[a]Values are expressed as mean ± SD mg/mL.

Table 3. IC_{50} Related to Extracts of Leaves, Fruit, and Fractions of Leaves of *J. excelsa* on *L. major* Amastigotes[a]

Sample	IC_{50} ± SD, mg/mL[b]
Leaves extract	0.97 ± 3.53
Petroleum ether	1.99 ± 3.53
Chloroform	3.88 ± 11.33
Ethyl acetate	1.95 ± 5.30
N-butanol	4.21 ± 19.44
Fruit extract	5.81 ± 3.182

[a]Values are expressed as mean ± SD.
[b]50% inhibitory concentration.

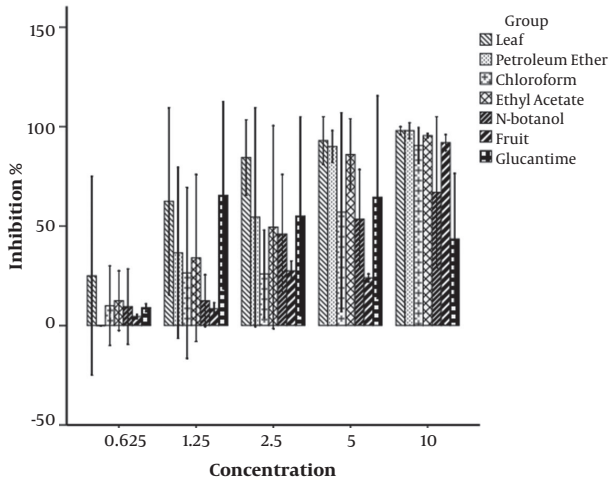

Figure 1. Inhibition effect among extract of leaves, fruit and fractions of leaves compared with Glucantime as the standard drug on axenic amastigotes of *L. major*

4.2. In Vivo Anti-Leishmanial Assay

The in vivo anti-leishmanial activity was assessed among four groups as test, placebo, glucantime, and control for 30 days of intervention. The diameter of the lesions was measured weekly, and their averages were calculated in Table 4. Up to four weeks after treatment, the lesion diameter did not decrease significantly, yet from the 5th to 6th- week post treatment, the mean lesion diameter in the test group was significantly decreased with respect to the control group. As shown in Figure 2, diameter of CL lesion in test, placebo, Glucantime, and control groups from first week to the end of the protocol is highlighted.

5. Discussion

Currently, the main drugs for leishmaniasis are pentavalent antimonial and amphotericin B. Regarding high cost and long treatment periods, side effects, high toxicity to heart, liver, and kidneys, risk of relapse, and resistance of some *Leishmania* species, investigation of natural medicaments is increasing (11, 28). Medicinal herbs for the treatment of parasitic diseases have been studied for a long time (29). In the present study, the efficacy of leaves, fruit, and fractions of *J. excelsa* (0.625 - 10 mg/mL) on *L. major* compared with glucantime (2.5 to 40 mg/mL) were assayed by an in vitro and in vivo model. Based on MTT and IC_{50} results, the effects of herb extracts were more potent with respect to glucantime drug, notwithstanding the con-

Table 4. The Diameter of Leishmaniasis Lesions in Test, Placebo, Glucantime, and Control Groups from the First Week to the End of the Protocol in Female BALB/c Mice Infected with *L. major* (n = 40)[a,b]

	Lesion Size					
	W0	W1	W2	W3	W4	W6
Test	5.150 ± 2.5933	5.800 ± 3.7283	5.900 ± 3.9637	5.800 ± 4.0359	6.000 ± 4.2098	3.900 ± 2.5582
Placebo	4.083 ± 1.6253	5.000 ± 2.5298	5.167 ± 2.7869	5.667 ± 3.5590	5.750 ± 3.4893	4.333 ± 2.2730
Glucantime	4.167 ± 2.0412	3.667 ± 2.0656	3.867 ± 2.2730	4.500 ± 2.9326	4.750 ± 2.6599	4.167 ± 2.7325
Control	4 ± 2.9155	4.450 ± 2.8592	4.533 ± 3.2290	5.083 ± 3.6935	5.117 ± 3.6499	5.417 ± 3.4988

[a] Values are expressed as mean ± SD mg/mL.
[b] $P < 0.05$.

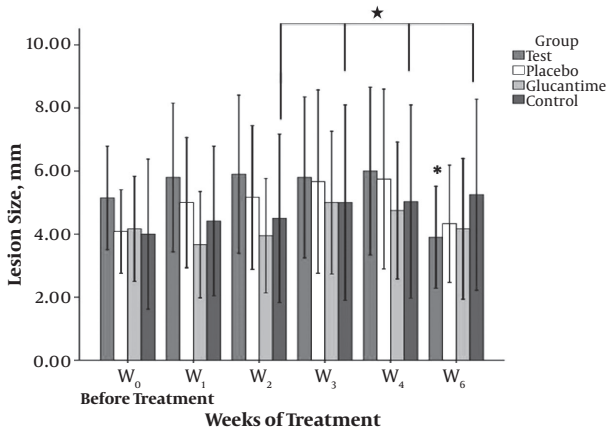

Figure 2. The diameter of leishmaniasis lesions in test, placebo, Glucantime, and control groups from the first week to the end of the protocol in female BALB/c mice infected with *L. major* (n = 40). Test group (n = 10): mice treated with cream 5% of *J. excelsa*, placebo group (n = 10): mice treated with placebo cream, Glucantime group (n = 10): mice treated with reference drug, control group (n = 10): mice infected and untreated control. * Significance difference between the test group with other groups (placebo, Glucantime and control) in the sixth week ($P < 0.05$).

centration of glucantime was four times higher compared with extracts and fractions. The leaf extract with $IC_{50} = 0.97$ mg/mL had the highest efficacy against *L. major* amastigotes. Similarly, in a research by Moein et al. the anti-leishmanial activity total extract of leaves, fruit, and fractions against promastigotes of *L. major* at concentrations of 0.625 to 10 mg/mL showed a potent anti-leishmanial effect (22). Since the metabolic pathways, culture conditions, pH, and temperature vary in the amastigotes and promastigotes, the sensitivity to the drug is different. For the screening of drugs, axenic amastigotes are used as substitutes for intracellular forms of the parasite, because of the similarity of axenic amastigotes to the life cycle of *leishmania* in humans compared to promastigotes (30, 31). In an investigation in Dios region of Peru, extracts of seven medicinal plants were specifically evaluated against promastigote and axenic amastigote forms of *L. amazonensis*; among them, ethanol extract of *Himatanthus sucuuba* showed considerable activity against axenic amastigotes ($IC_{50} = 5\ \mu g/mL$) (32). Also, in this research, among leaf fractions, ethyl acetate had the most potent anti-leishmanial activity, followed by petroleum ether, chloroform, and n-butanol, which were effective, respectively. In other studies, the anti-leishmanial effect of these fractions has been reported, which is in concordance with the current study (33-35).

Another research was conducted on the extract and fractions of *Atropa acuminate*, and ethyl acetate have shown anti-leishmanial effect (36). Study of the effect of methanol and chloroform extracts of *Valeriana wallichii* root showed positive activities against *L. donovani* and *L. major* promastigote and amastigote, while in the current study, the chloroform fraction of *J. excel*sa extract showed anti-*L. major* amastigotes activity (37).

In an animal model of the current study, 5% cream from leaf extract did not serve any effect during five weeks of administration as compared to Glucantime. However, the anti-leishmanial activity appeared in the sixth week. In a clinical trial study, Parvizi et al. topical cream 5% of *J. excelsa* reduced the lesion size in patients with CL without side effects (23). Another investigation examined a Copaiba oil sample on *L. major* induced in BALB/c mice. Using subcutaneous, oral, and topical treatment with Glucantime as a standard, it was shown that topical administration (cream 4%) was effective (38).

According to the polarity of fractions in the current study, it was found that active components in both groups of polar and nonpolar compounds must be responsible for the anti-leishmanial activity. Among all fractions in the current study, n-butanol fraction demonstrated the lowest anti-leishmanial activities. The most potent anti-leishmanial activity was related to leaves of *J. excelsa* and among prepared fractions, ethyl acetate, and petroleum ether exhibited high anti-leishmanial effect.

The MTT assay was employed to carry out this evaluation. This assay is used in many fields, specifically in screen-

ing for drug discovery This method is a fast and accurate calorimetric assay yet the probability of drug interactions with tetrazolium should be considered (39, 40).

The current study should pay attention that various strains of *L. major* may present a different degree of pathogenicity (41). Using dominant native strain of *L. major* for study of the effect of anti-leishmanial compounds could be very critical and important, in the current study, the researchers used the most prevalent zymodeme of the parasite in the region isolated from the patients and identified by isoenzyme electrophoresis (5). So far no study has been conducted about the anti-leishmanial activity of *J. excelsa* on axenic amastigote and animal model. The limitations that were not covered in this study were the effect of *J. excelsa* extract and fractions on the intracellular amastigotes, the use of a more active ingredient in the topical form of the drug and a more extended treatment period.

5.1. Conclusions

The finding of this study demonstrated that *J. excelsa* had potent anti-leishmanial activity by in vitro and in vivo model, therefore, further studies with various concentrations, comprehensive, and long-term therapy assessment with this *J. excelsa* is needed to introduce it as a natural anti-leishmanial medicine with less side effects, greater cost-effectiveness, and safety.

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Footnotes

Authors' Contribution: Study concept and design: Gholamreza Hatam and Mahmoodreza Moein. Performing the experiment: Somayeh Mirzavand. Data analysis and interpretation of results: Somayeh Mirzavand, Gholamreza Hatam, Mahmoodreza Moein, and Mohammad Zarshenas. Drafting of the manuscript: Somayeh Mirzavand. Critical revision of the manuscript: Gholamreza Hatam and Mohammad Zarshenas.

Conflict of Interests: The authors declared no potential conflict of interests.

Ethical Approval: This experimental study was approved by the Ethical Committee of Shiraz University of Medical Sciences, Shiraz, Iran. The animals were handled and used in accordance with their moral guidelines during the experiment (IR.SUMS.REC.1392-6486).

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