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Antileishmanial Activities of Greek Juniper (*Juniperus excelsa* M.Bieb.) Against *Leishmania major* Promastigotes

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Abstract

Petroleum ether, chloroform, ethyl acetate, and *n*-butanol fractions of Greek juniper (*Juniperus excelsa* M.Bieb. from the family Cupressaceae) were evaluated for antileishmanial activities against *Leishmania major* promastigotes compared to meglumine antimoniate (Glucantime). In vitro toxicity assay was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and microplate ELISA reader. Extracts were prepared in ethanol/dimethyl sulfoxide (80/20) at 10 to 0.62 mg/mL. The standard was prepared in phosphate-buffered saline at 500 to 15.62 mg/mL. Both leaf and fruit extracts and related fractions showed strong inhibitory effects against promastigotes, significantly different from that of the standard. The leaf extract and the respective petroleum ether fraction showed maximum effectiveness compared to other fractions and also fruit extract and fractions ($IC_{90} = 1.89 \pm 0.03$ and 0.90 ± 0.03 mg/mL, respectively). Regarding the potent activities of nonpolar fractions of Greek juniper leaf extract, these fractions can be suggested for further investigation.

Keywords

antileishmanial activity, *Juniperus excelsa*, *Leishmania major*

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Leishmaniasis is a vector-borne disease caused by a protozoan parasite *Leishmania* and is transmitted by the bite of certain types of sandflies.[1] This disorder can be presented in 3 ways: cutaneous, mucocutaneous, and visceral leishmaniasis.[2] The cutaneous type is associated with skin ulcers, whereas the mucocutaneous form presents with skin, mouth, and nose ulcers. The visceral form, which starts with skin ulcers, presents with fever later, decline in red blood cells, and causes splenomegaly and hepatomegaly. Leishmaniasis is reported as an endemic disease in about 90 countries where 350 million people are at risk. It is reported that more than 12 million people are currently infected by the parasite.[3,4] Among those prevalent types of leishmaniasis, the cutaneous type is common in some parts of Asia, Africa, and central and south America.[5,6] In Iran, cutaneous leishmaniasis has been increasing during the past 10 years and more than 70% of this disease is distributed in the rural parts of the country.[7]

Treatment lines for leishmaniasis are generally application of pentavalent antimony compounds, pentamidine, and numerous antifungal formulations, such as amphotericin B, and currently, alkylphosphocholine compounds such as miltefosine.[8] Additionally, nonpharmacological interventions such as cryotherapy, localized controlled heat, and photodynamic therapy are also used to treat the cutaneous type.[9-11] In spite of various chemical medicaments applied for the treatment of this disease, results have not yet been satisfactory. Thus, seeking for new and safe medication is considered crucial.[12]

Other than the conventional remedies, natural medicaments were also investigated. With reference to herbal and complementary medicine, antileishmanial activities of plants, such as feverfew,[13] onion,[14] and *Zhumeria majdae* Rech.f. & Wendelbo[15] were studied.

Traditional Persian medicine is a summation of development of ideas and knowledge from ancient civilizations and Iranian scientists (*Hakim*) during the medieval era (Islamic

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golden ages of medical knowledge). This medical system is founded on 4 humor concept, namely, phlegm (*Balgham*), blood (*Dam*), yellow bile (*Ṣafra*), and black bile (*Sauda*). Balance in the proportion of these humors sustains health preservation, whereas lack of this balance results in the incidence of various diseases.[16] This school of medicine, which is not only a collection of previous integrative medical information but also a summation of its own scholars and practitioners,[17] contains numerous medical suggestions for these various types of diseases. In this regard, the present study aimed to assess the antileishmanial activities of Greek juniper or *Juniperus excelsa* M.Bieb. (Family Cupressaceae) extracts yielded from its leaves and fruits on *Leishmania major* in comparison with meglumine antimoniate. The herb is native to Iran and has been recently evaluated for some activities such as antimicrobial, antioxidant, and antitumor.[18,19]

Materials and Methods

Extraction and Fractionation of Fruits and Leaves of the Herb

Fruits and leaves of Greek juniper were collected from Genu Mountain, northern parts of Bandar-Abbas (Hormozgan Province, Iran). Specifying a voucher number (PM-947), the sample was identified by S. Khademian, Herbalist of the Department of Traditional Pharmacy, School of Pharmacy, Shiraz University of Medical Sciences. Dried leaves (9000 g) and fruits (500 g) were separately subjected to alcoholic extraction via the percolation procedure. Both extracts were subsequently concentrated using a rotary evaporator under reduced pressure. The time consumed for the extraction of fruits and leaves was 48 hours and 10 days, respectively.

To fractionate the fruit extract, 88 g of crude extract was dissolved in 225 mL of methanol (80%) and extracted with petroleum ether (3 × 225 mL) using a separating funnel. The petroleum ether fraction was separated and the residue was dissolved in 67 mL distilled water. Subsequently, this phase was mixed and extracted with chloroform (3 × 270 mL) using the same method. The chloroform fraction was collected and the residue was later dissolved in distilled water up to 270 mL. This phase was then mixed and extracted with ethyl acetate (3 × 270 mL). The aqueous phase that separated from the ethyl acetate fraction was mixed eventually with *n*-butanol and the *n*-butanol fractions were separated. The same procedure was performed for leaves extract, and all 4 prepared fractions related to both leaf and fruit extracts were subjected to antileishmanial activity. Figure 1 schematically represents the fractionation procedure for the fruit extract.

Growth Media and Parasite Culture

Blood agar–based biphasic Novy-MacNeal-Nicolle (NNN) as well as brain-heart infusion (BHI) media were employed for the recovery and mass cultivation of the parasite, respectively. Reference strain of *Leishmania major* (MRHO/IR/75/ER) was purchased from Pasteur Institute in Iran and was cultivated on NNN medium having BHI as the liquid phase. The promastigotes produced were then transferred to the fresh culture for further processing.[20] The number of parasites was adjusted to 1×10^6/mL for inoculation.

Evaluation of the In Vitro Leishmanicidal of Fractions and Meglumine Antimoniate

Fractions were dissolved in an appropriate solvent involving dimethyl sulfoxide (DMSO)-ethanol (20:80) with proper dilutions. The employed concentrations for the fractions and meglumine antimoniate were at different ranges of 0.625 to 10 and 15.625 to 500 mg/mL, respectively. Briefly, 10 mg of each fraction was dissolved in 1000 µL of the solvent. In parallel, 500 mg of meglumine antimoniate was dissolved in phosphate-buffered saline (PBS) to the concentration of 500 mg/mL. Each fraction was then treated with promastigotes in 5 different concentrations.

Counting the Parasites

Approximately, 100 λ of the parasites was transferred to a neobar lam. The WBC chamber of the lam was used to count the parasites.

MTT Method for Assessment of Antileishmanial Activity

MTT, or 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide, was applied for in vitro promastigotes cell toxicity assay of all fractions and extracts of leaf and fruit of Greek juniper as well as that of the meglumine antimoniate. This substance is used as a marker to determine the cell viability.[21] Briefly, 100 µL of BHI culture (2.5×10^6 promastigotes) was transferred to each well of a 96-well standard microplate. Subsequently, 10 µL of different concentrations (10-0.625 mg/mL) of fruits and leaves of the herb as well as related fractions were added to the respective well. The plate was then incubated at 25°C for 48 hours. Blank and control wells contained medium and promastigotes plus solvent, respectively. Following incubation, the wells containing fluid were centrifuged and, subsequently, the supernatant was removed. Afterwards, 50 µL of MTT (2 mg/mL) in PBS was added to the wells. The plate was then incubated in darkness at 25°C for 3 hours. At the end, 100 µL of DMSO, as stopper, was added to the wells to stop the enzymatic reaction. The plate was left standing for 5 minutes. Finally, the optical density was determined at 570 nm using a microplate ELISA reader to read continuously. Lysis of the parasites (%) by extracts and fractions as well was meglumine antimoniate was determined using the following formula:

$$\text{Lysis } \% = 100 - [(\text{test} - \text{blank})/(\text{control} - \text{blank})] \times 100$$

The values of IC_{90} were determined by plotting the amounts of promastigotes lysis (%) with respect to that of the control against treatment concentrations.

All tests were performed in triplicate, and the average data were reported as the final results. GraphPad InStat software was used to compare the resulting data. Statistical analysis was done by using one-way ANOVA and Tukey post hoc tests. In this study, $P < .05$ was considered statistically significant.

Results

Results of the lethal effects and leishmanicidal activities of Greek juniper leaves and fruits were determined in 5 different concentrations. As the parasite had shown sensitivity to the extracts, the employed concentrations were 50 times diluted compared to those of the standard (meglumine antimoniate). The standard was assessed for the same activity in 6 different concentrations. Data related to the evaluation of leaves and

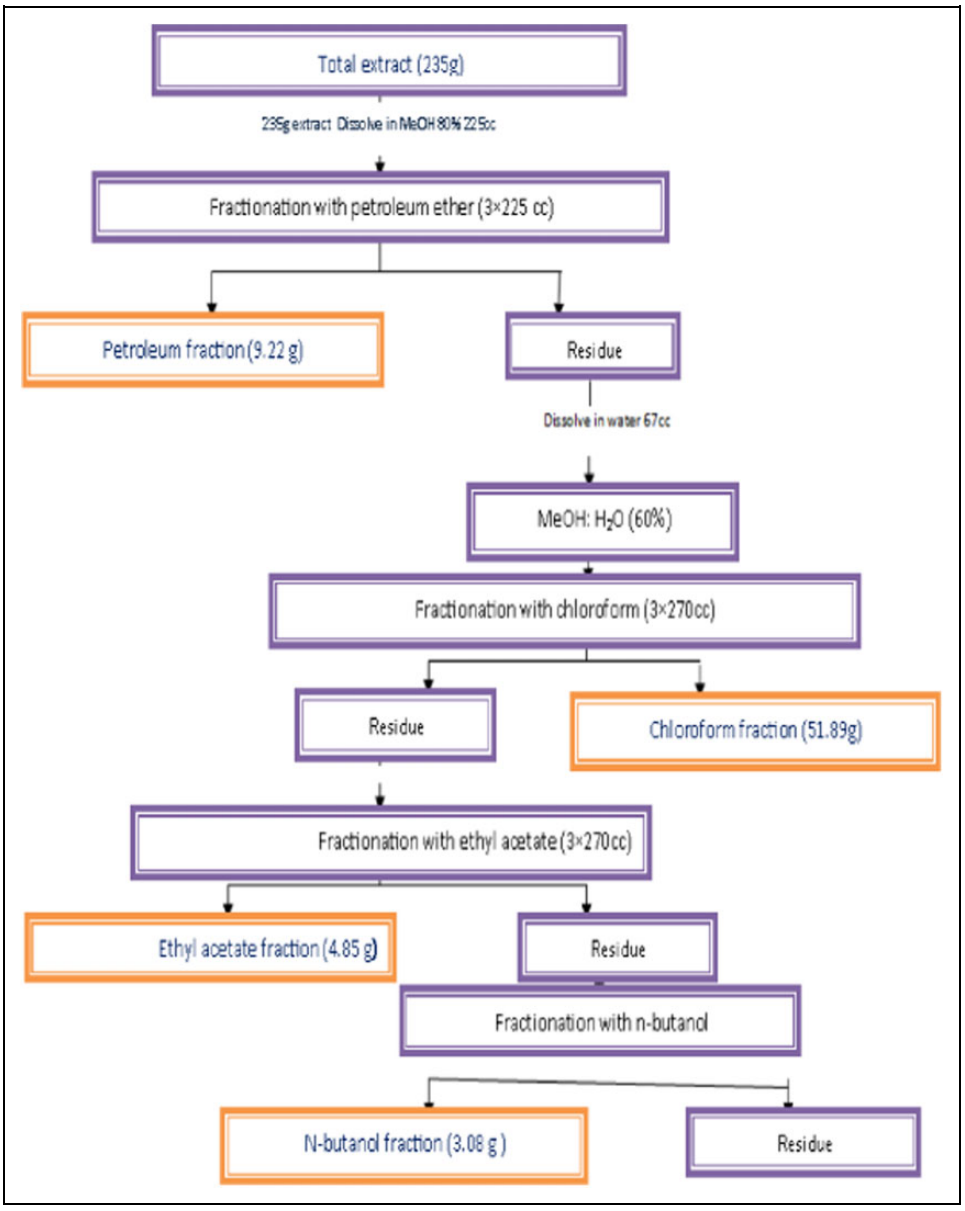

Figure 1. Fractionation of Greek juniper fruits extract via liquid-liquid extraction.

fruits extract and related fractions are shown in Table 1. Data related to the antileishmanial activity of meglumine antimoniate are presented in Table 2. Lethal effect values which were calculated fewer than 20% were considered inactive. To have a better outline, Figures 2 and 3 present profiles related to the lethal effects of extracts and respective fractions of Greek juniper fruits and leaves compared to those of the standard, respectively. Furthermore, values related to IC_{90} of leaf and fruit extracts and also respective fractions are presented in Table 3.

Discussion and Conclusion

In the current study, a potent extract and respective fractions of a medicinal plant has been applied for the efficacy assessment on leishmaniasis. According to the medical and pharmaceutical manuscripts of Persian medicine, this disorder appears as a wound, either dry or wet, and could be contagious as well. The nearest term for this disorder is defined as *Rish-e-balkhi* (*Balkh* wound). The mentioned signs and symptoms are in line with cutaneous leishmaniasis in current medicine.[22] With reference to the documents of Persian medical and pharmaceutical manuscripts and prior experimental findings, Greek juniper was selected for the current research. In *Makhzan al-adviyah* (*The Storehouse of Medicaments* by *Alavī Shīrāzī*, 18th century), a main pharmacopeia of Persian medicine, fruits and leaves of this plant have been strongly recommended for the treatment of such wounds.[23]

This medicinal plant has shown to exert various pharmacological activities. With regard to skin diseases, investigations have demonstrated considerable cytotoxic activities for Greek

Table 1. Data of Lethal Effects of Leaves and Fruits Total Extracts and Respective Fractions.[a,b]

	Concentration (mg/mL)				
	10	5	2.5	1.25	0.625
Fruits extract					
Total extract	100	87.11 ± 1.98	76.84 ± 1.04	62.84 ± 1.75	46.88 ± 2.88
Petroleum ether	100	100	100	99.64 ± 0.25	92.41 ± 1.62
Chloroform	89.98 ± 2.76	76 ± 3.48	53.32 ± 0.91	42.36 ± 1.12	31.1 ± 2.04
Ethyl acetate	66.47 ± 9.74	57.17 ± 1.71	42.34 ± 6.00	35.44 ± 1.68	27.66 ± 2.22
n-Butanol	Not active	Not active	Not active	Not active	Not active
Leaves extract					
Total extract	100	100	98.43 ± 0.01	84.51 ± 2.07	65.59 ± 1.02
Petroleum ether	100	100	98.08 ± 0.40	98.01 ± 0.19	82.90 ± 0.90
Chloroform	100	100	99.77 ± 0.07	92.72 ± 1.81	73.10 ± 2.28
Ethyl acetate	100	100	99.22 ± 0.98	86.14 ± 1.62	72.26 ± 0.36
n-Butanol	Not active	Not active	Not active	Not active	Not active

[a]Results are presented as lethal effects (%) ± standard deviation.
[b]The values which were calculated as less than 20% were considered as "not active."

Table 2. Data of Lethal Effects of Meglumine Antimoniate as Standard.

	Concentration (mg/mL)					
	500	250	125	62.5	31.25	15.625
Lethal effects (%)	98.14 ± 1.21	96.55 ± 0.77	68.08 ± 3.19	47.34 ± 0.78	23.24 ± 8.51	3.10 ± 3.83

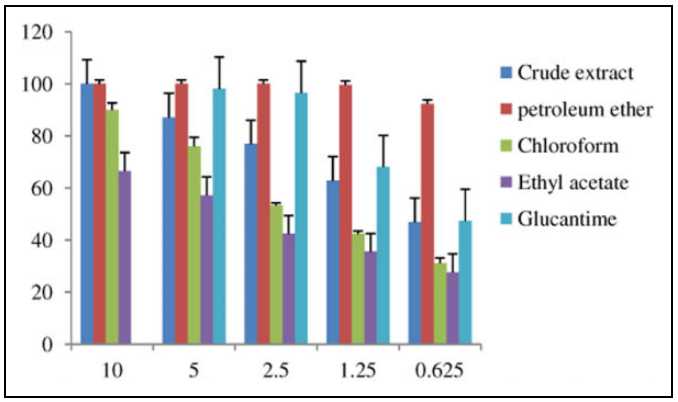

Figure 2. Lethal effects of Greek juniper fruits extract and respective fractions on promastigotes, compared to meglumine antimoniate.

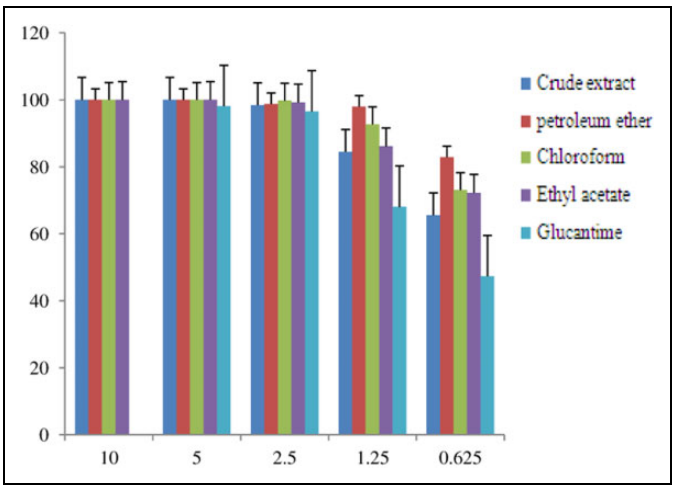

Figure 3. Lethal effects of Greek juniper leaves extract and respective fractions on promastigotes, compared to meglumine antimoniate.

juniper. A study revealed the cytotoxic activity of *J sabina* berries against tumor cell lines.[24] Another investigation showed that the cytotoxic activity of a hydroalcoholic extract of fruits of this plant was similar to that of *Taxus baccata*, a popular cytotoxic plant.[25] Greek juniper, on the other hand, has repeatedly exerted antimicrobial activities. A potent fraction of the plant's essential oil, terpenes, was effective against microbial strains.[26] Antimicrobial investigation of the herb essential oil showed considerable effectiveness (minimum inhibitory concentration = 0.625-2.5 µg/mL) on both gram-positive and gram-negative strains.[18] On the other hand, the essential oil of a *Juniperus* species was assessed for antileishmanial activities and the result was satisfactory.[27] With regard to the role of antioxidants in wounds and skin ulcers, Greek juniper has exhibited antioxidant and radical scavenging activities, which could be effective in wound healing. A study showed that essential oil of leaves and fruits of this plant could effectively inhibit lipid peroxidation in the employed cells.[28] Using 2,2′-azobis(2-amidinopropane)dihydrochloride in another assessment, the essential oil exerted antioxidant activity as compared to vitamin E.[29]

Table 3. Data Related to IC_{90} of Leaves and Fruits Extracts and Respective Fractions.

	$IC_{90} \pm SD$ (mg/mL)	
	Leaves Extract	Fruits Extract
Total extract	1.89 ± 0.03	6.96 ± 0.11
Petroleum ether	0.90 ± 0.03	0.83 ± 0.01
Chloroform	1.14 ± 0.05	9.08 ± 0.13
Ethyl acetate	1.76 ± 0.07	15.57 ± 3.71

To get more active constituents extracted, percolation was selected as the extraction procedure. Subsequently, the MTT method was employed for the determination of antileishmanial activities. In this method, living promastigotes can convert MTT to formazan via an enzymatic reaction. Later, DMSO can produce a uniform purple solution. The absorption of this solution can be determined by a microplate ELISA reader. The amount of produced formazan corresponds with number of living promastigotes.[30,31]

In this assessment, the promastigotes form of the parasite was employed due to the simplicity of medium and preservation as well as the usefulness of this form for chemotherapeutic screening.[3,31]

According to the findings of this study, the petroleum ether fraction of the fruit extract showed the highest activity among fruit total extract and respective fractions at the concentration of 0.312 mg/mL. Similar results were achieved for the leaf extract. It is also considerable that the potency of the aforementioned fraction from the leaf extract was found higher than that of the fruit ($P < .001$). On the other hand, these extracts and fractions were used at 1/50 concentration of meglumine antimoniate. These findings, thus, show that these extracts and fractions can be introduced as good candidates for clinical research, in combination or as an alternative to meglumine antimoniate.

In summary, the current study assessed the antileishmanial activity of fruits and leaves of Greek juniper at the concentration range of 0.625 to 10 mg/mL. The results revealed that activities of leaves and respective fractions were higher than those of the fruits. However, in the aforementioned concentration range both extracts were highly active. Since the lethal effects of both extracts were found to be higher than 50%, determination of IC_{90} was considered instead of IC_{50}.

Previously, other medicinal plants were evaluated for antileishmanial activity. Plumbagin isolated from bark of *Pera benensis* Rusby showed considerable effects against some *Leishmania* species ($IC_{90} = 5$ μg/mL).[32] Berberine, with a concentration of 10 μg/mL, was found effective on *Leishmania*-infected golden hamsters.[33] Other compounds such as senegalene, squamocine, and asimicine (25-100 μg/mL) isolated from *Annona senegalensis* Pers. showed antileishmanial effects against related promastigotes.[34] Alkaloids of *Galipea longiflora* K. Krause leaves has shown antileishmanial effects ($IC_{90} = 25$ μg/mL) against *Leishmania braziliensis*.[35] In another investigation, *Peganum harmala* L. extract was checked for activity. The value of IC_{50} for the extract (concentration range of 5-20 mg/mL) was calculated as 1832.65 μg/mL compared to that of the potassium antimonyl tartrate (62.5-500 μg/mL) as the standard ($IC_{50} = 17.87$ μg/mL).[3] In our study, the IC_{90} values of Greek juniper leaf extract and respective petroleum ether fractions were calculated as 1.89 and 0.90 mg/mL, respectively. Therefore, it can be concluded that the antileishmanial activity of Greek juniper from the current study is higher than that of the *Peganum harmala* L. It is also notable that fractions with antileishmanial activity may be parallel with reduction in polarity. This fact may be due to the increase in terpenoids in the petroleum fraction.

Regarding the potent activities of nonpolar fractions of Greek juniper leaf extract, these fractions can be suggested for further investigation. In addition, concerned fractions can be purified and subjected to in vivo and also human comprehensive studies.

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Author Contributions

MM and GH defined the research theme and designed methods and experiments. TMR carried out the laboratory experiments under the supervision of the other authors. MM, MMZ, and GH analyzed the data and interpreted the results. MMZ wrote the draft of the article. All authors have reviewed and confirmed the final draft.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

As this work was an experimental assessment, no ethical approval was required.

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