Original Article

In vitro Anti-Leishmanial Activity of *Satureja khuzestanica* Jamzad and *Oliveria decumbens* Vent. Extracts on *Leishmania major* and *Leishmania infantum* Promastigotes

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Abstract

Background and Objectives: Leishmaniasis is the main health problem and affects millions of people, especially in developing countries. On the other hand, there is no immunoprophylaxis (vaccination) accessible for the treatment of *Leishmania* infections and commercial drugs are unsatisfactory. Therefore, there is an effort to find alternative herbal remedies. The objective of the present study was to state the antileishmanial activity of two herbal medicines such as *Satureja khuzestanica* Jamzad and *Oliveria decumbens* Vent. leaf extracts on the promastigotes of *Leishmania major* and *Leishmania infantum*. **Materials and Methods:** The hydroethanolic extracts of each plant were extracted and their antileishmanial effects evaluated in different concentrations (0–156 µg/ml) and at various hours (24, 48, and 72 h) using colorimetric (3[4, 5dimethylthiazol2yl]2, 5 diphenyltetrazolium bromide) assay. The concentrationresponse curves of tested extracts and glucantime solutions as a reference were designed, and 50% of inhibitory concentration (IC50) values were recorded. **Results:** Antileishminal activity of *S. khuzestanica, O. decumbens*, and glucantime drug on *L. major* and *L. infantum* promastigotes were revealed with IC50 values of 4.3 and 5.5 µg/ml for *S. khuzestanica,* 0.85 and 0.23 µg/ml for *O. decumbens*, and 40.2 and 18.5 µg/ml for glucantime after 72 h incubation. **Conclusion:** These results revealed that compounds from *S. khuzestanica* and *O. decumbens* have antileishmania properties that necessary to survey the effects of these extracts on *leishmania* genus in animal models in the future.

Keywords: *Antileishmanial, Leishmania infantum, Leishmania major, Oliveria decumbens, promastigote, Satureja khuzestanica*

Introduction

Leishmaniasis is a chronic disease caused by protozoan parasites belonging to the genus *Leishmania* transmitted by sandflies. Leishmaniasis is an important disease with three main clinical manifestations of leishmaniasis are recognized as follows: cutaneous leishmaniasis (CL), muco-CL, and visceral leishmaniasis.[1,2] Antileishmanial drugs such as pentavalent antimonial compounds, meglumine antimoniate (glucantime), sodium stibogluconate (Pentostam), amphotericin B, pentamidine, and paromomycin have been used against human leishmaniasis.[3,4] The mentioned drugs have limitations for being used due to the prolonged term of treatment, high cost, high toxicity, painful implementation, the absence of oral formulation (e.g., amphotericin B can be used only intravenously), or important side effects and high-resistant parasites against these drugs.[4,5] In addition, other probable complications of these drugs are damage to the heart, liver, pancreas, hematopoietic tissues, and renal failure.[5] Other drugs include oral drug miltefosine have been used for the treatment of human visceral *Leishmania* infections, and fluconazole has also been effective against CL.[6] Since medicinal plants contain valuable components, easily accessible and cheap, the use of such native plants could be surveyed as wealthy sources of antileishmanial compounds.[7-10] *Satureja khuzestanica* Jamzad leaf extracts (Marzeh Khuzestani in Persian), family of (*Lamiaceae*) is an endemic plant that it grows in the Southern and Western parts of Iran, especially Khuzestan and Lorestan provinces.[11] The extract of *S. khuzestanica* has biological properties such as antibacterial,[12] antifungal,[13] antiviral,[14] and antileishmanial

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activity.[10] Other plant, *Oliveria decumbens* Vent., family of (Umbelliferae) is an ancient herbal medicine which has been used in Behbahan (Khuzestan, South West of Iran). The essential oil of aerial parts of *O. decumbens* was analyzed, and antimicrobial properties of this plant carried out against Gram-positive bacteria, *Escherichia coli*, *Aspergillus niger,* and *Candida albicans*.[12] The present study aimed to evaluate the *in vitro* antileishmanial activity of two hydroalcoholic extracts of *S. khuzestanica* and *O. decumbens* in different concentrations (0–156 μg/ml) and at various hours (24, 48, and 72 h) on *L. major* and *L. infantum* promastigotes by (3-[4, 5-dimethylthiazol-2-yl]-2, 5 dipheny ltetrazolium bromide) (MTT) colorimetric assay.

Materials and Methods

Ethics statement

The protocol of this study was approved by the Ethics Committee of Abadan School of Medical Sciences, Abadan, Iran. The University Ethics Committee code number was IR.SBMURECH.1395: No. 93U-029.

Plant materials and extract

The medicinal plants of *S. khuzestanica* and *O. decumbens* were collected from Shiyon Dezful district in Khuzestan province, and the specimens were identified by agricultural and natural resources center (ANRC), Ahvaz, Iran and deposited at the Herbarium of ANRC with voucher numbers of 7936 and 5288 for *S. khuzestanica* and *O. decumbens*, respectively. For the preparation of hydroethanolic extracts, 10 g of shade-dried and powdered plant material was macerated in 100 mL ethanol with 85% of (Merck) solution on a rotary shaker for 72 h.[15] The plant extracts were filtered through Whatman No. 1 and the filtrated hydroethanolic extracts were evaporated and dried at the room temperature. Dried extracts were obtained and stored at 4°C for further assay.

Parasite culture

Parasites-Leishmania (L.) major (MRHO/IR/75/ER) and *Leishmania (L.) infantum* (MCAN/IR/96/LONDON 49) promastigotes strains were obtained from the School of health, Tehran University of Medical Sciences, Iran and were maintained in RPMI-1640 (Sigma, Chemical company) medium and supplemented with 10% of fetal calf serum sigma, 100 IU/mL penicillin and 100 μg/ml streptomycin. All promastigotes (10^6 parasites/ml) were incubated at 26°C for 24, 48, and 72 h in fresh RPMI-1640.[16]

Promastigote viability using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide assay

MTT is colorimetric assay measured reduction of MTT dye (tetrazolium) into formazan by mitochondrial enzymes in viable cells. To prepare the stoke solution MTT (Sigma Chemical Co., St. Louis, Mo.), 5 mg from yellow powder was soluble in 1 ml phosphate-buffered saline and kept in −20°C. At working, a freshly solution was prepared with mixing one volume of MTT stock solution in 9 ml of RPMI-1640 medium containing 2.5% of colony-stimulating factor, the final concentration of MTT reaches to 0.5 mg/ml.[17]

Add promastigotes, selected plants extract and medicine

Aliquot 100 μl *L. major* and *L. infantum* promastigotes (10^6 parasites/ml) added to per well of 96-well microplate and incubated for 6 h. Afterwards, 10 μl the two-fold dilutions of selected plant extracts ranged from 0 to 156 μg/ml and 20 μl working MTT reagent were added to per well of 96-well microplate. Negative control well was contained only 100 μl culture medium plus 20 μl working MTT reagent and glucantime drug was used as positive control. After 3–4 h incubation and centrifugation, separated the supernatant solution and added 100 μl pure dimethyl sulfoxide to per well for dissolving of purple formosan crystals. After 15 min of shaking, optical density (OD) by ELISA reader in wavelength of 570 nm and reference wavelength of 630 nm.[16,18]

Statistical analysis

Statistical analyses of the differences in mean values between different experimental groups were performed with the two-tailed Student's *t*-test. The significance of difference was measured using analysis of variance and with a confidence interval of 95%, $P = 0.05$ or less were considered as statistically significant.

Results

In this study, the antileishmania activity of hydroethanolic extracts of selected herbals was determined using MTT assay. Inhibitory concentration (IC_{50}) values were for the hydroethanolic extract of *S. khuzestanica* at 24, 48, and 72 h for *L. major* 41.2, 13.3, and 4.3 μg/ml and for *L. infantum* promastigote 42.1, 30.2, and 5.5 μg/ml, respectively, whereas these values were for *O. decumbens* at 24, 48, and 72 h for *L. major* promastigote 22.3, 2.7, and 0.85 μg/ml and for *L. infantum* promastigote 7.1, 1.13, and 0.23 μg/ml, respectively. The findings demonstrated that the hydroethanolic extracts of *S. khuzestanica* and *O. decumbens* had potent antileishmanial activity against the forms of *L. major* and *L. infantum* promastigotes *in vitro* after 24, 48, and 72 h of incubation ($P < 0.05$). These results also revealed that the hydroethanolic extract of *O. decumbens* leaf in comparison with the hydroethanolic extract of *S. khuzestanica* leaf had significantly ($P < 0.05$) higher leishmanicidal effect on the promastigotes of *L. major* and *L. infantum* since it exhibited lower IC_{50} values for the tested promastigotes. The effect of the antileishmanial activity of the *S. khuzestanica* leaf extract against the forms of *L. major* and *L. infantum* promastigotes in

various concentrations and at different hours (24, 48 and 72 h) are shown in Table 1 and Figure 1. Furthermore, antileishmanial activity of the *O. decumbens* leaf extract on *L. major* and *L. infantum* promastigotes in different concentrations and at various hours (24, 48, and 72 h) are shown in Table 1 and Figure 2. Glucantime drug also revealed IC_{50} values of 40.2 and 18.5 µg/ml for *L. major* and *L. infantum* promastigotes after 72 h incubation, respectively. The comparison of means of antileishmanial activity of *S. khuzestanica*, *O. decumbens,* and glucantime drug against the forms of *L. major* and *L. infantum* promastigotes after 72 h revealed statistically significant ($P < 0.05$).

Table 1: IC_{50} of *S. khuzestanica*, *O. decumbens* leaf extracts and glucantime against *L. major* and *L. infantum* promastigotes after 24, 48 and 72 h incubation

Compounds	IC_{50} (µg/ml)		
	24 h	48 h	72 h
S. khuzestanica on *L. major*	41.2	13.3	4.3
S. khuzestanica on *L. infantum*	42.1	30.2	5.5
O. decumbens on *L. major*	2.3	2.7	0.85
O. decumbens on *L. infantum*	7.1	1.13	0.23
Glucantime on *L. major*	104.45	61.4	40.2
Glucantime on *L. infantum*	99.7	45.6	18.5

S. khuzestanica: Satureja khuzestanica, L. major: Leishmania major, O. decumbens: Oliveria decumbens, L. infantum: Leishmania infantum, IC_{50}: 50% inhibitory concentration

IC_{50} value of *S. khuzestanica*, *O. decumbens,* and glucantime at 72 h after treatment were 50.62 ± 30.31 µg/ml, 34.43 ± 28.79 µg/ml, and 60.15 ± 34.36 µg/ml, respectively. The *P* values for each plant and drug are presented in Table 2.

Discussion

The CL is also a major health problem in Iran, especially in Mashhad, Northeast of Iran.[19] Natural products, such as herbal extracts, have been used as pure compounds for the treatment of various diseases such as infectious

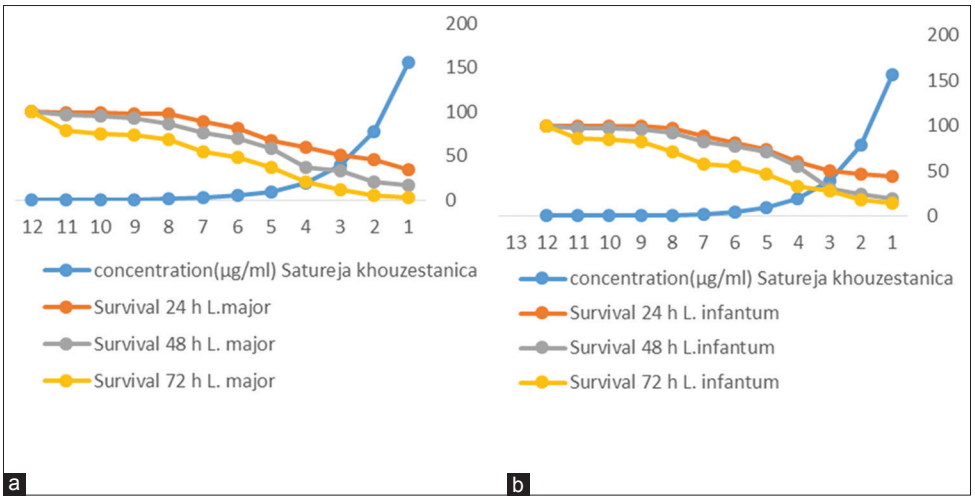

Figure 1: a) Viability of *L. major promastigotes* and b) Viability of *L. infantum promastigotes* in the various concentrations of *S. khuzestanica* after 24,48,72 h

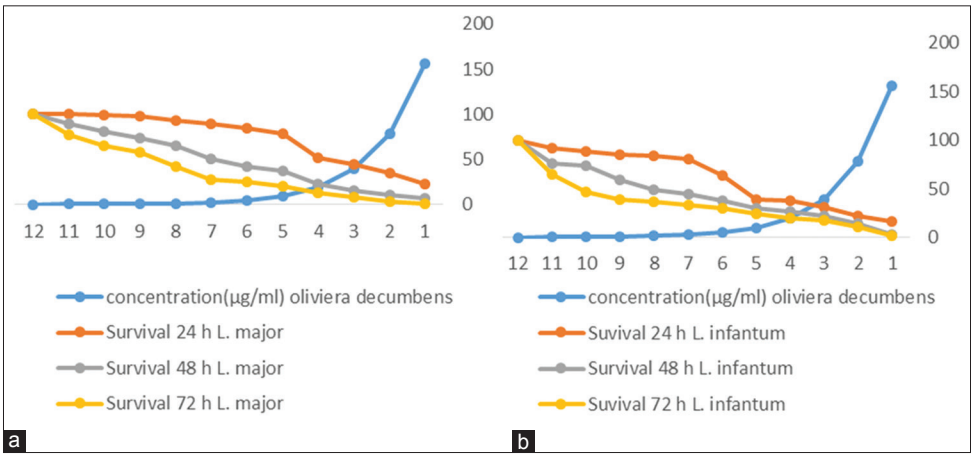

Figure 2: a) Viability of *L. major promastigotes* and b) Viability of *L. infantum promastigotes* in the various concentrations of *O. decumbens* after 24,48,72 h

Table 2: Results of comparison of paired-samples *t*-test for each plants and drug against tested promastigotes after 72 h

Plant and drug	Mean±SD	Significant (two-tailed)
S. khuzestanica - *L. major* and *L. infantum*	50.62±30.31[a]	0.001, $P<0.05$
O. decumbens - *L. major* and *L. infantum*	34.43±28.79	0.001, $P<0.05$
Glucantime - *L. major* and *L. infantum*	60.15±34.36	0.008, $P<0.05$

[a]µg/ml. Significance level $P<0.05$. S. *khuzestanica: Satureja khuzestanica, L. major: Leishmania major, O. decumbens: Oliveria decumbens, L. infantum: Leishmania infantum*, SD: Standard deviation

diseases. The use of herbal medicines in Asia for the remedy of skin diseases returns back to the past. One of the important points is that the side effects of herb medicines are less than that of the chemical drugs.[20] Until now, several studies have been demonstrated the potential antileishmania effects of various herbal extracts on parasites-leishmania in the past.[21-26] These studies showed that a number of medicinal plants have moderate-to-strong antileishmanial activity. However, to the best of our knowledge, based on the previous literature, no studies have been carried out on the effects of *S. khuzestanica* and *O. decumbens* plants extract on the *in vitro* growth of *L. major* and *L. infantum* promastigotes by MMT assay. In the present study, the hydroethanolic extracts of *S. khuzestanica* and *O. decumbens* significantly ($P < 0.05$) inhibited the growth rate of promastigotes forms of *L. major* and *L. infantum*. These results also revealed that promastigotes forms of *L. infantum* were more susceptible to the hydroethanolic extract of *O. decumbens* and glucantime drug while the hydroethanolic extract of *S. khuzestanica* was more sensitive to *L. major* than *L. infantum* promastigote after 72 h of incubation. Allahdin *et al*. studied the leishmanicidal activity of *Camellia sinensis* against promastigotes forms of *L. major* and *L. infantum*. The results of the mentioned study revealed that *C. sinensis* had IC_{50s} of 19 µg/ml, 12 µg/ml and glucantime had IC_{50s} of 21.8 µg/ml, and 10.16 µg/ml for *L. major* and *L. infantum* promastigotes after 72 h of incubation, respectively. These results were found to be *C. sinensis* was more sensitive *to L. infantum* promastigotes than *L. major* promastigotes.[27] There is the consistency between the results of the aforementioned study and those of our research. In general, based on our findings, antileishmanial efficacy of *O. decumbens* extract was significantly higher than *S. khuzestanica* extract. In the present study, the results showed that by increasing the concentrations of tested plant extracts or drug, while the inhibitory effect on the growth of *L. major* and *L. infantum* promastigotes will be increased, OD decreases. The reason for the reduction of OD is the furosian reduction, which is produced by the action of the mitochondria dehydrogenases of the active metabolic cells and is shown to be linked to the number of live cells.[28] In addition, previous phytochemical studies have also reported that the *S. khuzestanica* contained saponins, reducing sugars, flavonoids, tannins, phenols, proteins, and triterpenes.[29] Furthermore, the essential oil of aerial parts of *O. decumbens* contained γ-terpinene, myristicin, thymol, ρ-cymene, and carvacrol.[30] Moreover, in several studies, strong antibacterial, antifungal, and anti-parasitic activities of these compounds and their derivations such as α-pinene, 1, 8-cineole, limonene, thymol, and carvacrol against some pathogenic microorganisms have been confirmed.[31-34] Therefore, phytocompounds in these plants can be responsible for their antileishmanial efficacy, while the precise mechanism of their activity is unknown. However, Sikkema *et al*. investigated the antimicrobial mechanisms of some terpenoid compounds, such as monoterposes[35] and reported that terpenoid compounds damage the structures of the organs of microorganism's body. On the other hand, in other studies, indicated that antimicrobial activity of plants according to the ability of the terpenes, not only affect penetrability but also other cell membrane functions. These compounds may pass through the cell membrane, therefore, penetrating into the cell and interacting with vital intracellular sites.[36,37] *S. khuzestanica* leaf extracts contain active compounds, which could be used as suitable herbal drug in the treatment of experimental CL *in vivo*[38] and used as leishmaniacidal agent *in vitro*.[10,39] Kheirandish *et al*. studied the cytotoxic and antileishmanial activity of *S. khuzestanica* essential oil (SKEO) by the MTT method. The present research revealed the potent antileishmanial activity of SKEO and this plant had no toxic effect on mammalian cells.[40] Furthermore, Moghaddas *et al*. studied Iranian native plants, including *S. khuzestanica* on the treatment of CL.[19] The findings of this study reported that carvacrol-rich essential oil of *S. khuzistanica* showed strong antibacterial activity. Given that the strong antimicrobial properties of carvacrol contain in *S. khuzestanica* has already been reported,[12] the antileishmanial properties of this plant may be due to this compound.

Conclusions

According to mentioned results, the antileishmanial effects of *S. khuzestanica* and *O. decumbens* extracts may be recommend in the treatment of CL as a complement or substitute treatment. However, further studies are necessary and should be investigated in cell culture and *in vivo* assays in animal and human models to emphasis it.

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Conflicts of interest

There are no conflicts of interest.

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