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The Efficacy of Hydroalcoholic Extracts of *Prosopis farcta* Against *Leishmania major*

Prosopis farcta'nın Hidroalkolik Ekstraktının Leishmania major'a Karşı Etkinliği

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ABSTRACT

Objective: Cutaneous leishmaniasis caused by *Leishmania major* (*L. major*) is an endemic disease in Iran. The current reference drugs, including Glucantime, possess high toxicity in addition to some side-effects. Therefore, there is a growing interest in exploring biomedical plants. The goal of the present study was to evaluate the anti-leishmanial activity and cytotoxicity of hydroalcoholic extracts from *Prosopis farcta* (*P. farcta*) over promastigote and amastigote forms.
Methods: This study was performed at the Iran Birjand University of Medical Sciences, during the year 2019. In this study, the hydroalcoholic extracts of the stems, leaves (LE) and fruits (FE) of *P. farcta* were obtained. The anti-leishmanial activity was assessed against leptomonad promastigotes and intracellular amastigotes of *L. major*. The cytotoxicity of these extracts was determined in murine macrophages.
Results: The FE and LE of *P. farcta* demonstrated a significant leishmanicidal effect against *L. major* promastigotes with an IC50 of 0.9 mg/mL and 1.1 mg/mL, respectively. The FE showed the most anti-leishmanial activity and presented with the highest index of selectivity (SI=14.6) as an anti-leishmanial product. Infected macrophages treated using the FE showed a reduction in parasite burden by 97.3%.
Conclusion: The results of the present study demonstrated the leishmanicidal activity of *P. farcta* on both promastigotes and intracellular amastigotes. There is a need for performing comprehensive studies on relevant animal models and to access the effects of active components of *P. farcta* extract on the growth of L. major.
Keywords: Macrophages, *Prosopis*, *Leishmania*, extraction, *in vitro* techniques

ÖZ

Amaç: *Leishmania major'un* (*L. major*) neden olduğu kutanöz leishmaniasis, İran'da endemik bir hastalıktır. Glucantime dahil olmak üzere mevcut referans ilacın yüksek bir toksisitesi ve yan etkisi vardır. Bu nedenle, biyomedikal bitkilere ilgi artmıştır. Çalışmanın amacı, sitotoksisitenin yanı sıra promastigot ve amastigot formları üzerindeki anti-*Leishmania* aktivitesinin değerlendirilmesidir.
Yöntemler: Bu çalışma, 2019 yılında İran'ın Birjand Tıp Bilimleri Üniversitesi'nde yapılmıştır. Bu çalışmada *Prosopis farcta*'nın (*P. farcta*) saplarının, yapraklarının (LE) ve meyvelerinin (FE) hidroalkolik özleri elde edilmiştir. Anti-leishmanial aktivite, leptomonad promastigotlara ve *L. major'un* hücre içi amastigotlarına karşı değerlendirilmiştir. Bu ekstraktların sitotoksisitesi fare makrofajlarında belirlenmiştir.
Bulgular: *P. farcta*'nın FE ve LE değerleri, sırasıyla 0,9 mg/mL ve 1,1 mg/mL IC50 ile *L. major* promastigotlara karşı anlamlı bir leishmanisidal etki göstermiştir. Meyve ekstraktı en yüksek anti-leishmanial aktiviteye sahip olup, bir anti-leishmanial ürün

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olarak en yüksek seçicilik endeksini (SI=14,6) sunmuştur. FE kullanılarak, enfekte makrofajlarda tedavi etkinliği de ölçülmüş ve parazit yükünde %97,3 oranında bir azalma elde edilmiştir.
Sonuç: Bu çalışmanın sonuçları, *P. farcta*'nın hem promastigotlar hem de hücre içi amastigotlar üzerindeki leishmanisidal aktivitesini göstermiştir. *L. major'a* karşı kesin etkisini değerlendirmek için hayvan modeli ve *P. farcta* ekstresinin aktif bileşeni üzerinde kapsamlı çalışmalara ihtiyaç bulunmaktadır.
Anahtar Kelimeler: Makrofajlar, *Prosopis*, *Leishmania*, ekstraksiyon, *in vitro* teknikler

INTRODUCTION

Cutaneous leishmaniasis (CL) is a vector-borne disease with various clinical sign results in different species of Leishmania. The worldwide prevalence of CL is 1-1.5 million cases annually and over the 370 million people are at risk (1). Global warming lead to an annually increased the prevalence of zoonotic cutaneous leishmaniasis (ZCL), especially in endemic areas such as Iran (2). The existing treatment of leishmaniasis is meglumine antimoniate and sodium stibogluconate. In contrast to the therapeutic regimes, ZCL infections rate highly remains in tropical areas, mostly due to drug resistance, recurrence infections, drug complications, high cost, and long treatment periods. Therefore, a safe and effective alternative approach such as new medical herbal extracts with no side effects is in high demand (3). The *Prosopis farcta* (*P. farcta*) belongs to the Fabaceae family is grown in the tropical area of Asia, Africa, and America. In folk medicine of Iran, the different parts of *P. farcta* including stems, leaves, and fruits are usually used for rheumatism, diabetic, diarrheal and even anti-microbial purposes (4). The major chemical constituent of *P. farcta* essence are lectins, vitexin, tryptamine, tannin, and apigenin. It is determined that lectins and toxins in the *P. farcta* can kill *L. major* promastigotes in sand flies and culture media (5). While there is not any study to determine which part of *P. farcta* has a most leishmanicidal effect. So, the present study was made to assess the *in vitro* leishmanicidal effect and cytotoxicity of hydroalcoholic extract of the different parts of *P. farcta* against *L. major* as pathogenic parasitic strain.

METHODS

P. farcta Sample and Extraction

The *P. farcta* samples were obtained from rural regions of Tabas city, east of Iran, from April to June 2019, and their codes of herbarium were obtained from the School of Pharmacy, Birjand University of Medical Sciences. The plants were divided into stems, leaves, and fruits. The percolation method was used for extraction. Briefly, the plants were washed, dried in shadow, then get soaked with 70% methanol, and shaken for 48 h at room temperature. The acquired liquid was filtered and dried at 37 °C. Different dilutions (0.078-20 mg/mL) were prepared for each extract a placed in 96 well plates.

Murine Macrophages and Parasite

The murine peritoneal macrophages of BALB/C were obtained by washing the peritoneal cavity with Roswell Park Memorial Institute (RPMI), 5 days after intraperitoneal (ip) injection of 1.5 mL of 3% sterile sodium thioglycolate (6). In brief, the macrophages were plated into the cell culture flask, incubated and finally, the supernatant was pouring out.

The isolated strain of *L. major* from a patient referred to Research Laboratory of tissue and blood protozoa at the Department of Parasitology and Mycology at the Faculty of Medicine, Birjand University of Medical Sciences, was characterized before cultured (2). The clinical suspect was informed about the objectives and procedures of this study and subsequently the informed consent was obtained. The parasite was cultured in novy-macneal-nicolle medium and subculture in RPMI 1640 supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL) and 15% heat-inactivated fetal bovine serum (FBS, Sigma) and incubated at 27 °C.

Assessment of Extracts Against Leptomonad Promastigotes of *Leishmania major*

The leishmanicidal effect of *P. farcta* extracts was evaluated by the methylthiazol tetrazolium (MTT) as previously described (7). Briefly, 100 µL of the promastigote's suspension was dispensed in 96-well plate (1×10^6 cells), then 100 µL of serial dilutions of *P. farcta* extracted were added to each well, triplicate. Following incubation at 26 °C for 48 h, 10 µL of MTT (5 mg/mL) was added, and the plates were incubated for 4 h. The reactions were stopped by dimethyl sulfoxide. The absorbance was measured at 570 nm using by micro-plate spectrophotometer (BioTek, UK). A Meglumine antimoniate (Glucantime®), was used as a positive control. The calculation of formazan production was performed by subtracting of background absorbance from total absorbance. The result was expressed as the average percentage of macrophages viability reduction compared to untreated control and then 50% cytotoxicity concentration (IC50) value was determined as described (8).

In vitro Cytotoxicity Assay

The *in vitro* cytotoxicity of hydroalcoholic extract of *P. farcta* to murine macrophages was evaluated by serial dilutions of different extracts including SE, leaf extract (LE) and fruit extract (FE) (ranging from 0.078-20 mg/mL) in 96-well plate in the presence of cultivated murine macrophages (5×10^5 cell), for 48 h at 24 °C. Meglumine antimoniate (MA) (mg/mL) was used as a positive control. After that, 10 mL of MTT with a concentration of 5 mg/mL was injected to each well and plate were incubated at 37 °C for 4 h in 5% CO_2. The reactions were stopped by adding 100 µLs of 50% isopropanol-10% sodium dodecyl sulfate-hydrochloric acid. The absorbance was measured at 570 nm using by micro-plate spectrophotometer (BioRAD Benchmark Plus). The calculation of formazan production was performed by subtracting of background absorbance from total absorbance. The result was expressed as average percentage reduction of macrophages viability compare to that in the untreated control well and then 50% cytotoxicity concentration (CC50) value was determined as described (8). The selective index (SI) for the macrophages was determined as the ratio of CC50 to IC50 for all evaluated products as previously described (9). Each test was performed in triplicate in three independent experiments.

Treatment of Infected Macrophages

The any of macrophages were infected with ten leptomonad promastigotes *of L. major* in the wells and then incubated at 37 °C for 4 h. The percentage of the infected macrophages with promastigotes was evaluated by staining the prepared slides. The infected macrophages were transformed into the cultured wells and treated during 24 h with leaf extract (LE) and FE (0.078-20 mg/mL). Also, MA was used as a positive control. The slides were prepared from incubated macrophage cells, to examine intracellular parasite load by light microscope, by fixed with cold methanol, then stained with Giemsa. The number of amastigotes in 100 macrophages, as well as the percentage of infected macrophages by promastigotes, were calculated.

Statistical Analysis

All assay was repeated three times and the results were expressed as the mean ± standard deviation. All data were compared by analysis of variance (One-Way ANOVA). Moreover, to compare the IC50 values of the groups, t-test was performed difference was considered significant when $p<0.05$. Statistical analyses were done by using GraphPad Prism™ version 5.0 for Windows (San Diego, CA, USA). The IC50 and CC50 were obtained from the mean percentage reduction of leptomonad promastigotes/macrophages, compared to that in the untreated controls, respectively.

RESULTS

The leishmanicidal activity of *P. farcta* against promastigotes of *L. major* were determined by the MTT test. Several concentrations of FE and LE showed a significant decrease ($p<0.05$) in optical density as measured by the MTT method. While SE showed no antileishmanial activity (Table 1). The IC50 value of FE and LE (IC50 of 0.9 mg/mL and 1.1 mg/mL, respectively) was a little higher than MA with IC50 1.3 mg/mL. The FE showed good antileishmanial activity with IC50 0.9 mg/mL and SI of 14.6. In contrast, LE and SE collected from this plant were more toxic and less effective, respectively (Table 1).

Table 1. Leishmanicidal effect of different parts of *P. farcta* extract against promastigote, CC50 and selective index for *Leishmania major*

Samples IC50 (mg/mL)	*L. major*	Macrophages	
		CC50 (mg/mL)	Selective index
Stems	**No activity**	**Nd**	**Nd**
Fruits	0.9±1.12	13.2±1.1	14.6
Leaves	1.1±2.6	4±2.3	3.6
Glu	1.3±0.82	10.3±2.1	7.9

The FE and LE were able to reduce a significant number of intracellular forms of *L. major* within the macrophages compared to the control group. The evaluation of the capacity of FE, SE, and LE of *P. farcta* was also performed in treating infected macrophages. The result revealed that infected macrophages which follow were treated with 0.6 0.8, 1 and 1.2 mg/mL of hydroalcoholic extract of *P. farcta* presented a reduction in the parasite burden in order of 48.25%, 70.1%, 82.4% and 97.3% respectively (Table 2).

DISCUSSION

Leishmaniasis is an important vector-borne disease in the world. For reducing the cost, the side effect of chemotherapy in endemic areas, alternative strategies including the use of medicinal plant extracts are considered (10). In the last decade, many studies revealed the high effect of herbal plants on many diseases such as leishmaniasis (11). The anti-leishmanial activity of herbal extract has been demonstrated in many studies (12-15). In the current study, we examined anti-leishmanial activity and *in vitro* cytotoxicity of the different part of *P. farcta*. The result of antil-eishmanial activity, cytotoxicity against macrophages and SI are presented in Table 1. The SI can be determined relevant because value higher than 10 could propose a better safety of extract for use in the mammalian host (16). In this regard, the FE indicates a high leishmanicidal effect and low toxicity to uninfected macrophages. The results of this study showed a potent anti-leishmanial effect of *P. farcta* FE and LE against promastigote forms of *L. major* with IC50 values 0.9 and 1.1 mg/mL, respectively. While the SE has not any anti-leishmanial activity. The IC50 value for MA as a controlled drug was 1.3 mg/mL. In consistent with our study, the other studies showed that IC50 was significantly different among the different parts of herbal extract (2,17). There is no previous study on the leishmanicidal effect. According to the previous study anti-*leishmania* effects of *P. farcta* have been reported (5). In this study, we indicated that, due to the anti-leishmanial potency of hydroalcoholic extracts of *P. farcta* fruits, it can be considered as novel drugs at least for the *in vitro* model. In one meta-analysis study on herbal extract with capable of anti-leishmanial activity, it was determined that the hydroalcoholic extracts are the best way for extraction with high IC50 value (17). To conclude, the findings of this study demonstrated high antil-eishmanial activity of *P. farcta* against *L. major* by *in vitro* model that indicated the potential of *P. farcta* as a medicinal plant for open a new perspective in the

Table 2. Reduction of infection in macrophages follow the treatment with the extract of *P. farcta*. The results are shown as medium ± standard deviation of the percentages of the macrophages infected with *L. major*

Extraction	Concentration (mg/mL)	Percentage of infected macrophages after treatment[a]	Number of amastigotes per macrophage after treatment	Reduction of internalized parasites (%)[a]
Fruit extraction	0.6	49.1±4.3	8.1±0.9	48.25±8.1
	0.8	28.2±8.2	6.1±1.2	70.1±6.2
	1	16.3±7.5	5.2±2.1	82.4±4.1
	1.2	2.1±6.4	3.1±1.3	97.3±3.4

[a]: Mean reduction of internalized parasites was determined by counting 100 cell coverslips, thrice, as compared to the negative controls. In the non-treated controls, the number of amastigotes per cell and the percentage of infected macrophages was 9.51±1.1 and 96.31±4.2 respectively

research of new drug with leishmanicidal effect. However, for usage in animal models and humans, more examination will be performed.

*** Ethics**

Ethics Committee Approval: This experimental study was by the approved Ethical Committee of Birjand University of Medical Sciences, Tehran, Iran. The animals were handled and used by their moral guidelines during the experiment (IR.BUMS.REC.1398.105).

Informed Consent: Informed consent was obtained.

Peer-review: Internally peer-reviewed.

*** Authorship Contributions**

Concept: M.H.N., R.S., T.R., E.S., M.K., G.H., D.T., S.M.R., Design: M.H.N., R.S., T.R., E.S., M.K., G.H., D.T., S.M.R., Data Collection or Processing: M.H.N., R.S., T.R., E.S., M.K., G.H., D.T., S.M.R., Analysis or Interpretation: M.H.N., R.S., T.R., E.S., M.K., G.H., D.T., S.M.R., Literature Search: M.H.N., R.S., T.R., E.S., M.K., G.H., D.T., S.M.R., Writing: M.H.N., R.S., T.R., E.S., M.K., G.H., D.T., S.M.R.

Conflict of Interest: No conflict of interest was declared by the authors.

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