VETERINARSKI ARHIV 77 (4), 365-375, 2007

Antileishmanial activity of *Peganum harmala* extract on the *in vitro* growth of *Leishmania major* promastigotes in comparison to a trivalent antimony drug

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MIRZAIE, M., S. J. NOSRATABADI, A. DERAKHSHANFAR, I. SHARIFI: Antileishmanial activity of *Peganum harmala* extract on the *in vitro* growth of *Leishmania major* promastigotes in comparison to a trivalent antimony drug. Vet. arhiv 77, 365-375, 2007.

ABSTRACT

Parasites of the genus *Leishmania* are transmitted by sandflies that ingest the parasite in the amastigote stage resident within macrophages, then inoculate the promastigote stage into other hosts. *Peganum harmala*, or Syrian Rue, has pharmacologically active compounds including several alkaloids with antiprotozoal properties, which are found especially in the seeds and the roots. In this research, *Leishmania major* were cultured *in vitro*, then by using a MTT assay, the biological activity of *P. harmala* extract in comparison to potassium antimonyl tartrate [Sb(III)] on *L. major* promastigotes was assessed. *P. harmala* extract and Sb(III) solutions for biological testing were prepared in PBS at 5000-20000 µg/mL and 62.5-500 µg/mL, respectively. All experiments were repeated at least three times in duplicate. For *P. harmala* extract and Sb(III), the concentration-response curve was plotted, from which IC_{50} values were determined. Both *P. harmala* extract and Sb(III) inhibited the growth of promastigote forms of *L. major in vitro* after 72 h. of incubation and had an IC_{50} of 1832.65 ± 89.72 µg/mL and 17.87 ± 2.05 µg/mL, respectively. Statistical analysis of the results (optical density and inhibitory percentage) of the different concentrations of *P. harmala* extract and Sb(III) showed that there was no significant difference between *P. harmala* extract and Sb(III) ($P>0.05$) but with a concentration increase of *P. harmala* extract or Sb(III), optical density decreased significantly, while inhibitory percentage increased. The different concentrations resulted in different optical densities or inhibitory percentages ($P<0.05$) so that *P. harmala* extract is effective against *L. major in vitro*.

Key words: antileishmanial activity, *Leishmania major*, *Peganum harmala*, potassium antimonyl tartrate, MTT assay

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ISSN 0372-5480
Printed in Croatia

Introduction

Parasites of the genus *Leishmania* are transmitted by sandflies that ingest the parasite in the amastigote stage resident within macrophages, and then inoculate the promastigote stage into other hosts. Current estimates indicate that parasites of the genus *Leishmania* affect people in 88 countries, with 350 million at risk of contracting the disease and with approximately 2 million new cases being reported each year (HANDMAN, 2000; HANDMAN, 2001). Proven therapies against human leishmaniasis include pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), amphotericin B, pentamidine, and paromomycin (BERMAN, 1996; BERMAN, 1997). The mentioned drugs have the disadvantages of high cost, lack of oral formulation (e.g., amphotericin B can be used only intravenously), or serious side effects that require close monitoring of the patients (BERMAN, 1997). Also, development of resistance by the parasites have been reported (EPHROS et al., 1997; LIRA et al., 1999; BOELAERT et al., 2002), so that new therapies are needed to supplement or replace currently available therapies.

Peganum harmala, or Syrian Rue, is the plant from which harmine was first isolated, as well as a source of harmaline and tetrahydroharmine. Total beta-Carboline content runs almost 4% by weight in the seeds of Syrian Rue. Ten gr. of Syrian Rue seeds provide about 400 mg of total beta-Carbolines. Syrian Rue grows in semi-arid conditions such as those which obtain in Iran. It originated in Central Asia and is held in high esteem throughout Asia Minor as a medicinal plant. The pharmacologically active compounds of *P. harmala* are several alkaloids, which are found especially in the seeds and roots. These include β-carbolines such as harmine, harmaline (identical with harmidine), harmalol and harman and the quinazoline derivatives vasicine and vasicinone. The alkaloidal content of unripe seeds is less than that of ripe ones (KAMEL et al., 1970). Alkaloids compounds illustrate well the diversity of antiprotozoal compounds found in *P. harmala* plant, (WRIGHT and PHILLIPSON, 1990) and among the several alkaloids, harmaline (harmidine, C13H14N2O) has been found to be a major active alkaloid (BUDAVARI and O'NEIL, 1996).

Several different protozoan infections have been shown to be susceptible to *P. harmala* extract in varying degrees, including *Theileria annulata, Theileria hirci, Theileria sergenti, Babesia bigemina, Anaplasma marginale, Babesia equi* and *Babesia cabali* (VECHERKIN, 1977; FAN et al., 1997; HU et al., 1997; MIRZAIEDEHAGHI, 2006). The objective of the present study was to determine the effect of *P. harmala* extract compared with potassium antimonyl tartrate, a trivalent antimony, on the *in vitro* growth and viability of *Leishmania major* promastigotes in a cell-free culture.

Materials and methods

Plant material and extraction. The aerial parts of *P. harmala* were collected around Isfahan province. The plant was taxonomically identified by botanists in the Department of Biology (Shiraz University, Shiraz, Iran). An extract of *P. harmala* was prepared from the seeds of the plant according to the method described by MANSKE and HOLMES (1952). Finally, the resulting concentrated extract was dried below 70 °C in an oven. All concentrations of the extract are based on extract dry weight.

Antileishmanial drug. Potassium antimonyl tartrate, a trivalent antimonial [Sb(III)], was supplied by Sigma (Sigma Chemical Co., St Louis, Mo.).

Parasite and culture. Leishmania major (WHO designation: MRHO/SU/59/P) promastigotes were cultured at 25 ± 1 °C to logarithmic phase in D-MEM/F-12 medium (Gibco BRL) without phenol red, supplemented by 10% heat inactivated foetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin, then washed 3 times with phosphate-buffered saline (PBS) by centrifugation at 1500 rpm for 10 min at room temperature and resuspended at a concentration of 2.5×10^6 parasites/mL in medium.

Various concentrations of compounds. Plant extract solutions for biological testing and the drug were prepared in PBS at 5000-20000 µg/mL and 62.5-500 µg/mL, respectively.

Antileishmanial activity assays (MTT assay). The antileishmanial activity of the *P. harmala* extract in comparison to potassium antimonyl tartrate was evaluated *in vitro* against the promastigote forms of *Leishmania major* using a MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide)-based microassay as a marker of cell viability. The MTT assay used was based on that originally described by MOSMANN (1983) modified by NIKS and OTTO (1990). A stock solution of MTT (Sigma Chemical Co., St. Louis, Mo.) was prepared by dissolving MIT in phosphate-buffered saline (PBS) at 5 mg/mL and storing in the dark at 4 °C for up to 2 weeks before use. For the antileishmanial activity assays, 100 µL/well of the culture which contained 2.5×10^6 cells/mL promastigotes was seeded in 96-well flat-bottom plates. Then, 10 µL/well from various concentrations of potassium antimonyl tartrate and *P. harmala* extract were added to duplicate wells and plates were incubated for 72 hour at 25 ± 1 °C. The first well of 96 wells was as a blank well which only contained of 100 µL culture medium without any plant extract, drug or parasite. At the end of incubation, 10 µL of MTT was added to each well and plates were incubated for 3 hour at 25 ± 1 °C. Enzyme reaction was then stopped by the addition of 100 µL of 50% isopropanol and 10% sodium dodecyl sulfate. The plates were incubated for an additional 30 min. under agitation at room temperature. Relative optical density (OD) was then measured at a wavelength of 570 nm using a multiwell scanning spectrophotometer (ELISA reader). The background absorbance of multiwell plates was measured at 690 nm and substract from 570 nm measurement. The absorbance of the formazan produced by the action of mitochondrial dehydrogenases of metabolically

active cells is shown to correlate with the number of viable cells (MOSMANN, 1983; NIKS and OTTO, 1990; MAAROUF et al., 1997; SERENO and LEMESRE, 1997; AVLONITIS et al., 2003). All experiments were repeated at least three times.

Data analysis. The results of each experiment were analyzed by the method described by HUBER and KOELLA (1993). For calculation of IC_{50}, they used the following mathematical formula:

$$\log(IC_{50}) = \log(x1) + [(y1 - y0/2)/(y1 - y2)] [\log(x2) - \log(x1)].$$

Briefly, HUBER and KOELLA (1993) proposed finding two concentrations, $x1$ and $x2$, such that the parasite density, $y1$, at concentration $x1$ (and all lower concentrations) was more than half of the density found in the control, $y0$, and that the parasite density, $y2$, at concentration $x2$ (and all higher concentrations) was less than half of $y0$. The IC_{50} was then found by linear extrapolation between $x1$ and $x2$.

The percentage of non-viable organisms which failed to metabolize MTT and therefore did not produce the formazan product was determined by applying the following formula (BANSAL et al., 2004): Percentage of non-viable organisms or inhibitory percentage at each compound concentration = 100 - (Test OD - Blank OD/ Control OD - Blank OD) × 100.

Statistical analysis. The univariate analysis of variance (Univariate ANOVA) and the Student's *t*-test, with significance at P values of <0.05, were used to compare the antileishmanial activity of the *P. harmala* extract with potassium antimonyl tartrate. For *P. harmala* extract and potassium antimonyl tartrate, the concentration-response curve was plotted, from which IC_{50} values (50% inhibitory concentrations) were determined.

Results

Antileishmanial assays. Both *P. harmala* extract and potassium antimonyl tartrate inhibited the growth of promastigote forms of *L. major in vitro* after 72 h. of incubation, and had a 50% inhibitory concentration (IC_{50}) of 1832.65 ± 89.72 µg/mL and 17.87 ± 2.05 µg/mL, respectively. Details of the *in vitro* inhibitory effect of different concentrations of *P. harmala* extract and potassium antimonyl tartrate against *Leishmania major* promastigotes are presented in Figs. 1. and 2. Also, details of reducing optical density, caused by the antileishmanial activity of different concentrations of *P. harmala* extract and potassium antimonyl tartrate on the *in vitro* growth of *Leishmania major* promastigotes, are presented in Figs. 3. and 4.

Using univariate ANOVA statistical analysis on the results (optical density and inhibitory percentage) of the different concentrations of *P. harmala* extract and potassium antimonyl tartrate it was shown that there was no significant difference between *P.*

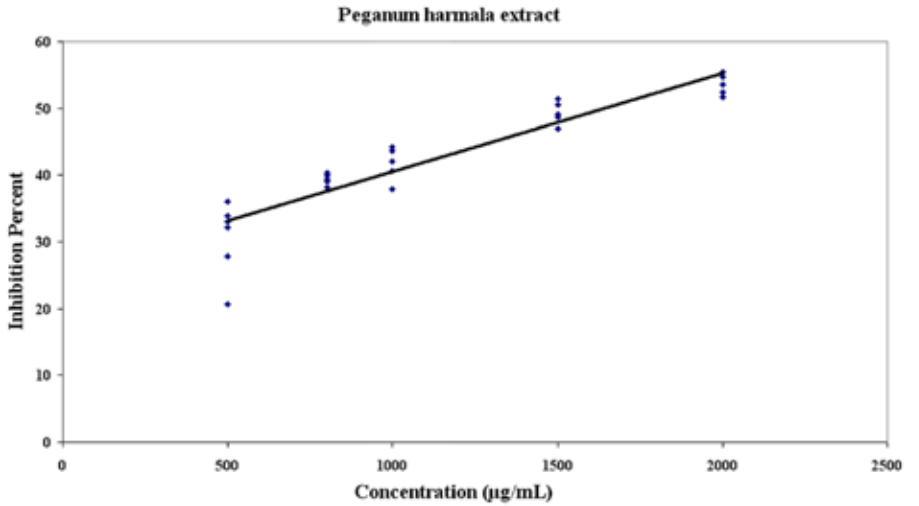

Fig. 1. Inhibitory effects of different concentrations of *P. harmala* extract on the *in vitro* growth of *L. major* promastigotes

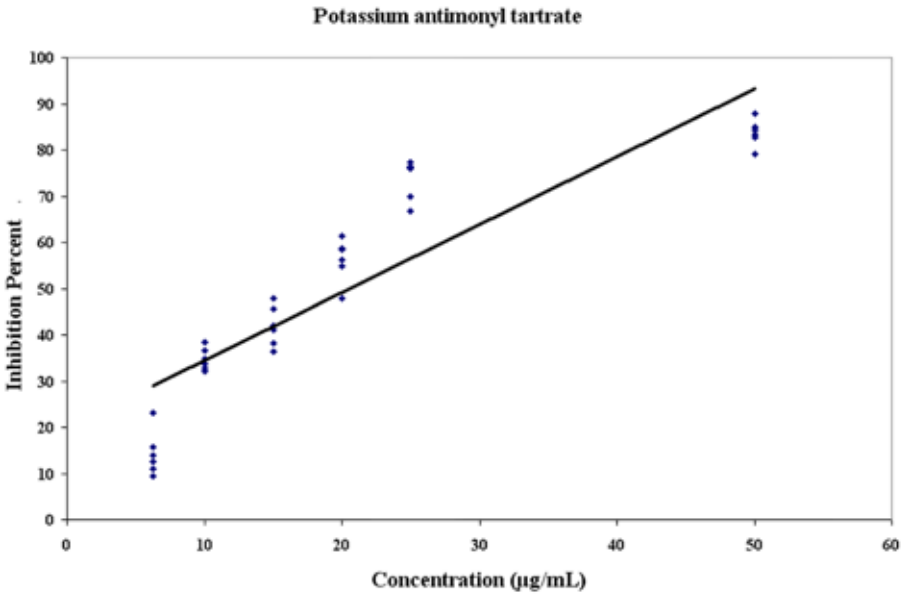

Fig. 2. Inhibitory effects of different concentrations of potassium antimonyl tartrate on the *in vitro* growth of *L. major* promastigotes

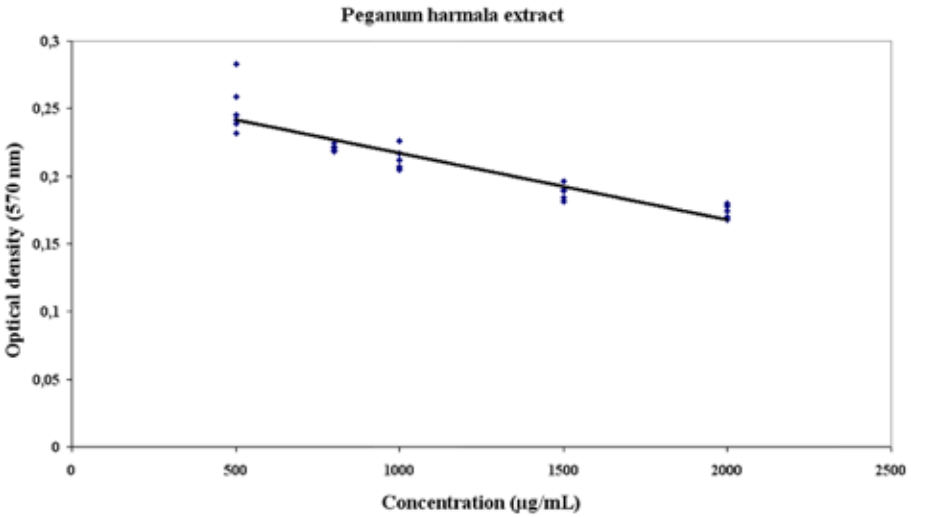

Fig. 3. Reducing optical density caused by antileishmanial activity of different concentrations of *P. harmala* extract on the *in vitro* growth of *L. major* promastigotes

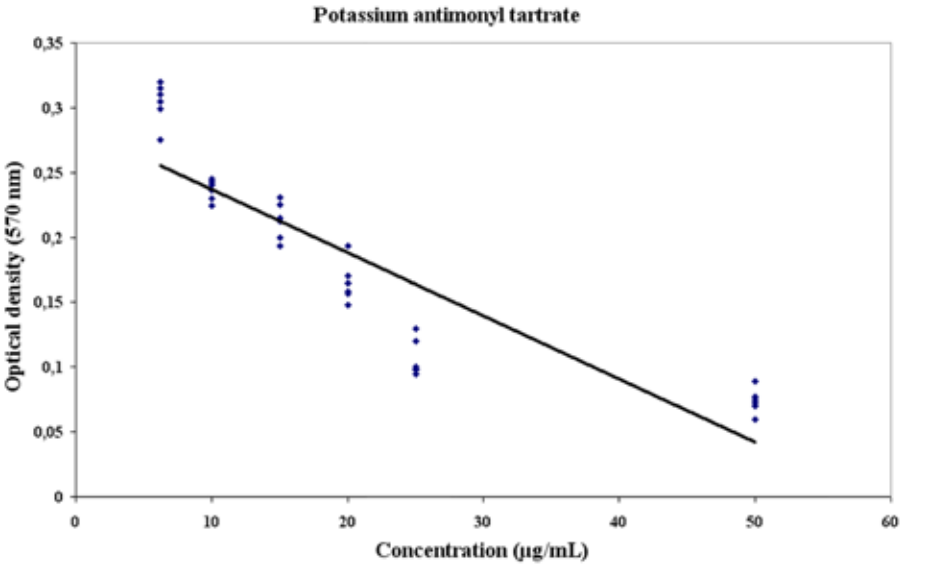

Fig. 4. Reducing optical density caused by antileishmanial activity of different concentrations of potassium antimonyl tartrate on the *in vitro* growth of *L. major* promastigotes

harmala extract and potassium antimonyl tartrate (P>0.05). However, with a concentration increase of *P. harmala* extract or potassium antimonyl tartrate, optical density significantly decreased and inhibitory percentage increased, and the different concentrations resulted in different optical densities or inhibitory percentages (P<0.05). Also, the Student's *t* test analyzed on the inhibitory percentages of the different concentrations of *P. harmala* extract and potassium antimonyl tartrate on the *in vitro* growth of *L. major* promastigotes, showed that there was no significant difference between *P. harmala* extract and potassium antimonyl tartrate (P>0.05).

Discussion

The results of this research show the antileishmanial activity of *P. harmala* extract (Syrian Rue) against *Leishmania major in vitro*. To our knowledge, based on a search of the literature, no studies have been conducted on the effects of *P. harmala* extract (Syrian Rue) on the *in vitro* growth of *Leishmania major* promastigotes.

There is a general lack of effective and inexpensive chemotherapeutic agents for the treatment of leishmaniasis. Although trivalent antimonial [Sb(III)] like potassium antimonyl tartrate and pentavalent antimonial drugs are the first-line treatment for this disease, with amphotericin B and pentamidine being used as alternative drugs, all of these have serious side effects and resistance has become a severe problem. Therefore, new drugs are urgently required. Natural products have potential in the search for new and selective agents for the treatment of important tropical diseases caused by protozoans (WRIGHT and PHILLIPSON, 1990).

The *in vivo* efficiencies of drugs have been reported to be under the control of different parameters, such as pharmacokinetic parameters (SERENO and LEMESRE, 1997), so that for various reasons, including simplicity in *in vitro* culture maintenance, routine screenings of antileishmanial chemotherapeutic agents are often based on promastigote susceptibility assays (GUPTA et al., 2001). In this report, a relevant viability test (MTT) was used to investigate the inhibitory effect of *P. harmala* extract on the *in vitro* growth of *Leishmania major* promastigotes. Previous *in vitro* experiments with *L. mexicana* promastigotes demonstrated that antimony sodium gluconate (Triostam), a trivalent analog of sodium stibogluconate, had a 50% lethal dose of 20 µg of Sb(III)/mL (MOTTRAM and COOMBS, 1985). Other investigators have shown that trivalent antimonial compounds were highly toxic to different *Leishmania* species in the promastigote form at concentrations ranging from 1.58 to 35.00 µg of Sb(III)/mL (MOTTRAM and COOMBS, 1985; ROBERTS and RAINEY, 1993; ROBERTS et al., 1995). Potassium antimony (III) tartrate was shown to be substantially more potent than sodium stibogluconate against promastigotes (ROBERTS and RAINEY, 1993; ROBERTS et al., 1995), so that in this research potassium antimony (III) tartrate at concentrations ranging from 6.25 to 50.00 µg of Sb(III)/mL was used

to compare the antileishmanial activity of *P. harmala* extract against *Leishmania major* promastigotes.

Although the antiprotozoan mechanism of *P. harmala* extract is as yet unknown, several studies have shown that different protozoan infections have been susceptible to *P. harmala* extract in varying degrees, including *Theileria annulata, Theileria hirci, Theileria sergenti, Babesia bigemina, Anaplasma marginale, Babesia equi* and *Babesia cabali* (VECHERKIN, 1977; FAN et al., 1997; HU et al., 1997; MIRZAIEDEHAGHI, 2006). Alkaloid compounds illustrate well the diversity of antiprotozoal compounds found in *P. harmala* plant (WRIGHT and PHILLIPSON, 1990), and among the several alkaloids (harmine, harmaline, harmalol, harman, vasicine and vasicinon) derived from *P. harmala* extract, harmaline (harmidine, C13H14N2O) has been found to be major active alkaloid and quite soluble in dilute acids (BUDAVARI and O'NEIL, 1996). In this research, distilled water and acetic acid have been used for the plant extraction (MANSKE and HOLMES, 1952) and it is shown that this plant extract contains a high quantity of harmaline and is therefore very effective against *Leishmania major* promastigotes. Also, we demonstrated that *P. harmala* extract demonstrated excellent antileishmanial activity against *Leishmania major* promastigotes *in vitro*. Potassium antimonyl tartrate at concentrations ranging from 6.25 to 50.00 µg of Sb(III)/mL, which has been used in this research, is highly toxic and effective against *Leishmania major* promastigotes (MOTTRAM and COOMBS, 1985; ROBERTS and RAINEY, 1993; ROBERTS et al., 1995) and statistical analysis of the results showed that there was no significant difference between *P. harmala* extract and potassium antimonyl tartrate ($P>0.05$). Consequently, *P. harmala* extract is very effective against *Leishmania major* promastigotes *in vitro*.

In this study, the results showed that with a concentration increase of *P. harmala* extract or potassium antimonyl tartrate, while the inhibitory effect on the growth of *Leishmania major* promastigotes will be increased, relative optical density will be decreased. The reason for OD decrease is a decrease of formazan, which is produced by the action of mitochondrial dehydrogenases of metabolically active cells and is shown to correlate with the number of viable cells (MOSMANN, 1983; NIKS and OTTO, 1990; MAAROUF et al., 1997; SERENO and LEMESRE, 1997; AVLONITIS et al., 2003).

Therapeutic evaluations for medicinal plants are essential because of the growing interest in alternative therapies and the therapeutic use of natural products. Natural products can be lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development, and the discovery of new therapeutic properties not yet attributed to known compounds (HAMBURGER and HOSTETTMANN, 1991). Natural products have made, and are continuing to make, an important contribution to this area of therapeutics. Perhaps their future potential will be even greater. In this study we report the inhibitory effect of *P. harmala* extract on the *in vitro* growth of *Leishmania*

major promastigotes. This activity represents an exciting advance in the search for novel antileishmanial agents from natural sources, since a significant and important effect against the promastigote form of the protozoan was demonstrated. Although this plant showed significant activity against *Leishmania major* promastigotes *in vitro*, further synthesis and *in vivo* studies are indicated to validate these results.

Acknowledgements

The authors wish to thank Dr. Gholamreza Hatam, medical parasitology department of the faculty of medical sciences (Medical University of Shiraz, Iran) for his kind cooperation, and Mr. Mansour Aminzadeh (Shahid Bahonar University of Kerman) for his technical assistance.

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Received: 6 September 2006
Accepted: 2 July 2007

MIRZAIE, M., S. J. NOSRATABADI, A. DERAKHSHANFAR, I. SHARIFI: Protulišmanijski učinak iscrpka biljke *Peganum harmala* na *in vitro* rast promastigota *Leishmania major* u odnosu na trovalentne pripravke antimona. Vet. arhiv 77, 365-375, 2007.

SAŽETAK

Parazite roda *Leishmania* prenose papatači preko makrofaga zaraženih amastigotima, a nakon razvoja nastale promastigote prenose na druge domaćine. Biljka *Peganum harmala* ili sirijska rutvica posjeduje farmakološki aktivne sastojke uključujući nekoliko alkaloida s protuprotozojskom učinkovitošću, koji se osobito nalaze u sjemenkama i korijenu biljke. Za potrebe ovog istraživanja, protozoon *Leishmania major* uzgojen je *in vitro*. Učinak iscrpka biljke *P. harmala* na promastigote *L. major* u odnosu na kalijev antimonil-tartarat [Sb(III)] određen je MTT metodom. Iscrpak *P. harmala* bio je pripravljen u PBS-u u koncentraciji od 5.000-20.000 µg/mL. Antimonski pripravak bio je pripravljen u PBS-u u koncentraciji od 62,5-500 µg/mL. Svaki pokus *in vitro* bio je ponovljen najmanje tri puta. Dobiveni rezultati prikazani su krivuljom učinkovitosti kojom su određene vrijednosti inhibitorne koncentracije (IC_{50}). Oba su pripravka kočila rast promastigota *in vitro* nakon 72 sata. Vrijednost IC_{50} za iscrpak *P. harmala* iznosila je 1832,65 ± 89,72 µg/mL. Vrijednost IC_{50} za antimonski pripravak iznosila je 17,87 ± 2,05 µg/mL. Statističkom obradom rezultata (optička gustoća i postotak inhibicije) dobivenim inkubiranjem protozoa u različitim koncentracijama pripravaka nisu utvrđene značajne razlike ($P>0,05$). Više koncentracije iscrpka *P. harmala* imale su za posljedicu značajno smanjenje optičke gustoće te istovremeno povećanje postotka inhibicije. S obzirom da su različite koncentracije rezultirale različitom optičkom gustoćom ili postotkom inhibicije ($P<0,05$) zaključuje se da iscrpak *P. harmala* djeluje *in vitro* na vrstu *L. major*.

Ključne riječi: protulišmanijska učinkovitost**,** *Leishmania major*, *Peganum harmala*, kalijev antimonil-tartarat