

## Original Article

# The Effect of *Alkanna tinctoria* and *Peganum harmala* Extracts on *Leishmania major* (MRHO/IR/75/ER) *in vitro*

Ruhallah Yousefi, \*Fatemeh Ghaffarifar, Abdolhosein Dalimi Asl

Dept. of Parasitology, School of Medicine, Tarbiat Modarres University, Tehran, Iran

(Received 6 Aug 2008; accepted 9 Jan 2009)

### Abstract

**Background:** Cutaneous leishmaniasis is an important health problem caused by *Leishmania spp.* As there is no vaccine, drug treatment is the only way to tackle leishmaniasis. In the present study, inhibitory and killing effects of *Peganum harmala* and *Alkana tinctoria* extracts on amastigotes and promastigotes forms of *Leishmania* were evaluated in-vitro.

**Methods:** The seeds of *Peganum harmala*, Stems and roots of *Alkana tinctoria* were collected and crude extraction carried out. In this experimental study, *Leishmania major* promastigotes were cultured in RPMI-1640 with 10% FBS at 22-26°C, and infected macrophages with amastigotes were cultured in RPMI-1640 with 10% FBS at 37°C in 5% CO<sub>2</sub>. Then the extracts of each plant were added to cultivated parasites and incubated for 3 days. Promastigote and amastigote assay was carried out using counting assay based on growth inhibition.

**Results:** The results indicated that both extractions can inhibit the growth of promastigotes, and in concentrations of 40µg/ml of *P. harmala*, 200µg/ml of *A. tinctoria*, and 20 µg/ml of equal combination of *P. hamala* and *A. tinctoria* are Inhibitory Concentration (IC<sub>50</sub>) for parasites growth. By adding these concentrations of the extracts to the infected macrophages in the culture, their effects were separately evaluated. The mean of amastigotes number in macrophages in the culture with *P. harmala*, *A. tinctoria*, combination and control groups were 0.7, 0.7, 0.6, 2.3 amastigotes per macrophage, respectively.

**Conclusion:** By this method, inhibition of intracellular and extracellular growth of *L. major* was demonstrated suggesting that, plant drugs with efficacy and safe products can be applied as new treatment for cutaneous leishmaniasis.

**Key words:** *Leishmania major*, *Peganum harmala*, *Alkana tinctoria*, In-vitro

### Introduction

Leishmaniasis has been identified as a major public health problem, particularly in Africa, Asia and Latin America. About 1.5 to 2 million people are affected annually by this parasitic infection (1). As there is not any applied vaccine, therefore the drug treatment is the topic way against leishmaniasis. The chemical drugs of

choices against leishmaniasis are sodium stibogluconate and meglumine antimonite (both pentavalent antimony derivatives). Pentavalent antimony and meglumine antimonate are characteristically moderately toxic and there are risks of recurrence and unsatisfactory side effects. Amphotericin B is recommended as a second-line treatment.

\*Corresponding author. Tel. : +982182883566  
E-mail: ghafarif@modares.ac.ir

The most dangerous side effect of amphotericin B is kidney damage. The lowest incidences were in the generic stibogluconate group. The efficacy and tolerance of inexpensive generic stibogluconate appears comparable to the branded formulations for the treatment of cutaneous leishmaniasis in the endemic regions.

Topical application of paromomycin in an ointment led to protozoal clearance in 76% to 86.3% of the patients with cutaneous leishmaniasis caused by *L. major* (1-3). However, in another study, the efficacy of paromomycin remains controversial (3). Recently photodynamic therapy was introduced as an attractive antiparasitic therapeutic option that offers rapid localized destruction of the lesions without affecting the adjacent normal tissues. Furthermore, in contrast to systemic treatments, photodynamic therapy has no risk of toxicity, but it is an expensive method (4).

Unfortunately, no ideal therapy for the cutaneous leishmaniasis has yet been identified (1, 3, 5).

Finding healing powers in plants is an ancient idea. The use of and search for drugs and dietary supplements derived from plants have been accelerated in recent years. Traditional healers have long used plants to prevent or cure infectious conditions. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids, which have been found in vitro to have antimicrobial properties (5).

The aim of the present study was to investigate the inhibitory and killing effects of plant extractions (*P.harmala* and *A.tinctoria*) on promastigotes and amastigotes forms of *L. major* in-vitro. *P. harmala* or Syrian Rue grows in semi-arid conditions in parts of North Africa, Mediterranean, the Middle East, Pakis-

tan and India, but later Introduced and a few areas of Southwest USA, South Africa and New South Wales in Australia. It now grows wild in Eurasia and has recently been spread to Texas, Nevada, New Mexico and Southern California. The main products of *Peganum harmala* are alkaloids (6). *P. harmala* alkaloids (1) are known for their antimicrobial (7-8) and hypothermic (9). In Moroccan traditional medicine, seeds of *P. harmala* have been used for the empiric treatment of cancers (10). It had usages in traditional medicine as an anthelmintic, lactogenic, antispasmodic and emetic (1, 11).

*Alkanna tinctoria* perennial grows to 0.2m by 0.25m. It is in flower in June. The flowers are hermaphrodite (have both male and female organs). The plant prefers sandy and loamy soils, requires well-drained soil and can grow in nutritionally poor soil. The plant prefers neutral and alkaline soils. It can grow in semi-shade or no shade. It requires dry or moist soil and can tolerate drought. The plant can tolerate maritime exposure. Habitats and possible locations are woodland, cultivated beds and dappled shade (12).

It helps old ulcers, hot inflammation and burnings by common fire. For these uses, the best way is to make it into an ointment. Also if you make a vinegar of it, it helps the morphy and leprosy. It also cures the yellow jaundice, spleen, and gravel in the kidneys. It also kills worms, bruises and falls, and is as gallant a remedy to drive out the smallpox and measles as any is; an ointment made of it is excellent for green wounds, pricks or thrusts (8, 13).

The root is antibacterial, antipruritic, astringent and vulnerary (12). It is used externally in the treatment of varicose veins, indolent ulcers, bedsores and itch-

ing rashes. The root is harvested in the autumn and can be dried for later uses (12).

## **Material and Methods**

### **Peganum harmala**

The seeds of *Peganum harmala* were collected, dried and finely ground in a spice mill. Fifty grams of the seeds powder were dissolved in 500 ml of water shook overnight, then centrifuged and the supernatants were collected. The supernatants were then filtered, evaporated under vacuum (at 100°C) and lyophilized. The powder resulting from lyophilisation was termed the aqueous crude extract and the residue was dissolved. The aqueous crude extraction was autoclaved, aliquot and froze at -20°C (11).

### **Alkanna tinctoria**

Stems and roots of *Alkanna tinctoria* were collected, dried and ground in a spice mill, then 50g of the powder was dissolved in 500ml of chloroform shook overnight, then centrifuged and the supernatants were collected. The supernatants were filtered and evaporated under vacuum (11).

### **Leishmania culture**

*L. major* (MRHO/IR/75/ER) was cultured in RPMI 1640 (Gibco) with 20% FCS (Gibco) for preparation of adequate promastigotes.

### **Promastigote assay**

Promastigote assay was carried out using a previously described direct counting assay based on growth inhibition (6). The effects of the crude extracts were evaluated in 24-well microtitre plates. The promastigotes were seeded at an initial concentration equivalent to that of

early log phase ( $2 \times 10^5$  promastigotes/ml) and allowed to multiply for 60 hrs in the medium alone (control group) or in the presence of serial dilutions of the drug until late log phase ( $10^7$  parasites/ml). Plant extract susceptibility experiments were performed in RPMI 1640 (Gibco) media with 20% FBS (Gibco). The parasites were counted daily for 3 days in a haemocytometer with a light microscope, and the results were compared with those from the controls. Each assay was performed in duplicate and repeated in separate experiments.

### **Amastigote/ Macrophage Assay**

Drug susceptibilities of the amastigotes in the macrophage BALB/c mice were determined by following a modification of the method of Chang (14). Briefly, peritoneal macrophages were collected and infected in-vitro with promastigotes in RPMI medium with 10% heat-inactivated fetal bovine serum to yield  $10^6$  cells and  $10^7$  promastigotes per ml. The cultures were incubated for about 3 days at 37°C in 5% CO<sub>2</sub> to allow phagocytosis of the promastigotes and adhesion to the surface. The number of parasites were calculated in 100 macrophages, then  $2 \times 10^5$  macrophages/well were cultured in 24-well plates with RPMI and 10% FBS. The extracts of plant with the concentration that obtained by IC<sub>50</sub>; were added to the wells after 24 h. The plates incubated in 37°C and 5% CO<sub>2</sub> incubator and the amastigotes in 100 macrophages were counted in 24, 48 and 60 h after incubation.

### **Cytotoxicity assay against macrophages**

The cytotoxic effect of the plant, expressed as cell viability, was assessed on the mice macrophages. The test was carried out in 24-well microtitre plates. A

suspension of  $5 \times 10^5$  macrophages was added to each well, and then incubated in a 37°C and 5% CO2 incubator. Viability of the macrophages was evaluated after 60 h (15).

**Results**

**Promastigote assay**

Growth inhibition of promastigotes was evaluated by four concentrations of each plant extraction (20, 40,100 and 200µg/ml) after 60 h (Fig. 1). Following the application of *Peganum harmala*, *Alkanna tinctoria* and equal combination of the two plants, promastigote IC<sub>50</sub> were measured as 40 µg/ml, 200µg/ml and 10 µg/ml, respectively.

**Amastigote-macrophage assay**

The mean of the amastigotes/macrophages before adding the extracts was 1.5 and after adding the extracts *Peganum harmala*, *Alkanna tinctoria* and combination of two extracts after 60 h was 0.7, 0.7 0.6, respectively whereas it was 2.6 for control group. The complete results for 24 and 48 h are shown in Table 1 and Fig. 2.

**Cytotoxicity assay against macrophages**

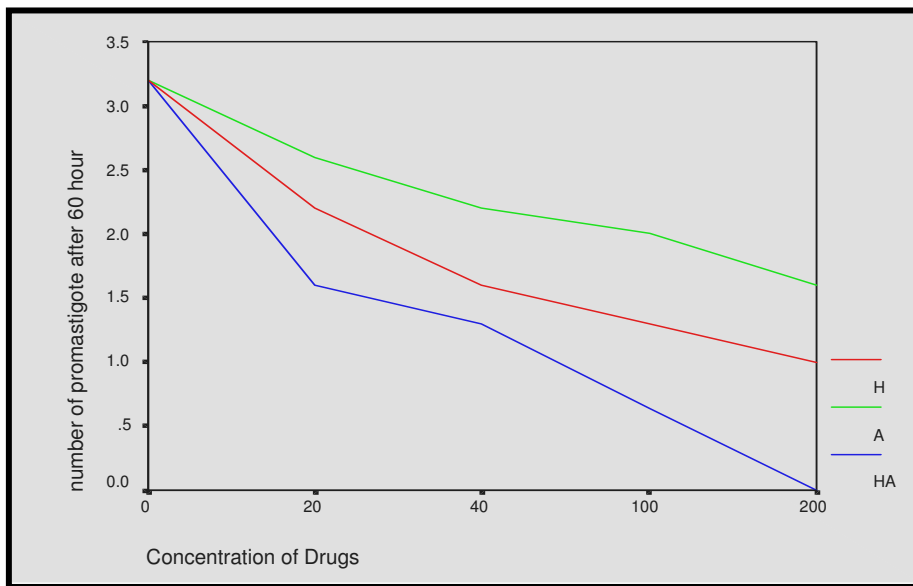
For checking the effect of cytotoxicity of these extracts on macrophages, the viability of macrophages after 60h was checked and the results are shown in Table 2.

**Table 1:** Mean number of amastigote/macrophage in 24, 48 and 60 h after adding extracts

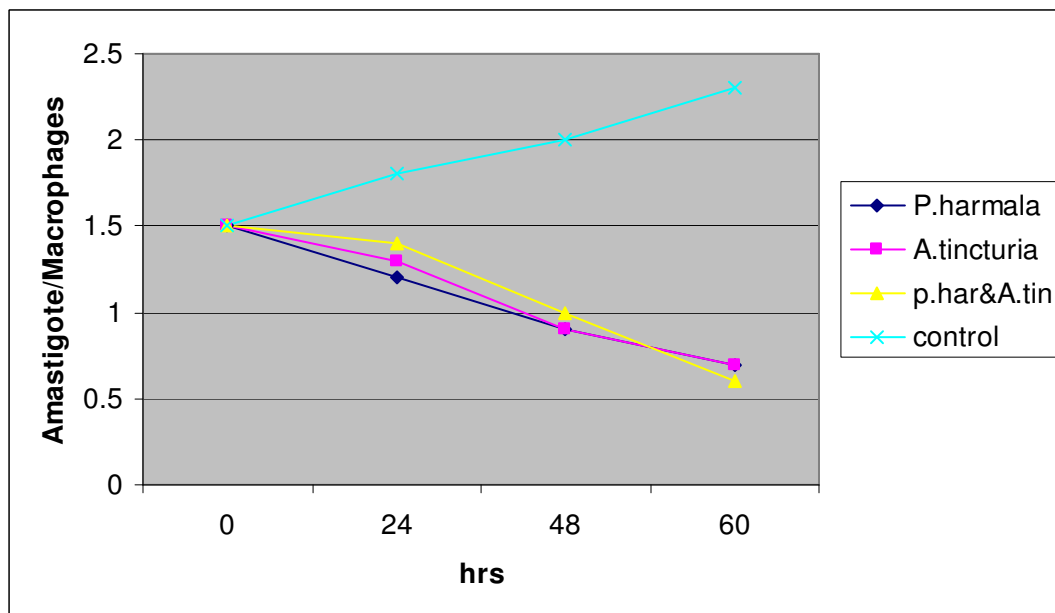
Drugs	Mean number of amastigote/macrophage during		
	24 h	48 h	60 h
<i>P. harmala</i> 40µg/ml	1.2	0.9	0.7
<i>A. tinctoria</i> 200µg/ml	1.3	0.9	0.7
<i>P. harmala</i> 10µg/ml	1.4	1	0.6
<i>A. tinctoria</i> 10µg/ml			
Control group	1.8	2	2.3

**Table 2:** Percentage of Macrophages viability after 60 h of adding extracts

Drugs	Drug concentration (µg/ml)			
	20	40	100	200
	Macrophage viability (%)			
<i>P. harmala</i>	90	85	83	80
<i>A. tinctoria</i>	90	86	82	80
<i>P. harmala and A. tinctoria</i>	90	85	82	79
Control groups	95	95	95	95



**Fig. 1:** Number of promastigotes after 60 h use of different concentration of *Pegalum harmala*(H) *Alkanna tinctoria* (A) and equal combination of *Pegalum harmala* and *Alkanna tinctoria* (HA)



**Fig. 2 :** Mean number of amastigotes in macrophages affected by *Pegalum harmala* , *Alkanna tinctoria* and combination of two extracts during 60 h

## Discussion

The recommended drugs for both visceral and cutaneous leishmaniasis are the pentavalent antimonials sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime). Both drugs, used for over 50 years, require long courses of parenteral administration and have toxic side effects (16).

Treatment of cutaneous leishmaniasis is directed toward the eradication of amastigotes and the reduction of the size of the lesions to promote healing and achieve maximum efficacy with minimal scarring and toxicity (5). However, no treatment has proved to be completely satisfactory. Pharmaceutical research in natural products represents a major strategy for discovering and developing new drugs. The use of medicinal plants for the treatment of parasitic diseases is well known and has been documented since ancient times by the use of *Cinchona succiruba* (Rubiaceae) as an antimalarial (17).

Alkaloids are one of the most important classes of natural products providing drugs for humans since ancient times. Most alkaloids are well known because of their toxicity, but many alkaloids have had a deep impact on the treatment of parasitic infections (18).

On the other hand, alkaloids have been found to have microbiocidal effects including against *Giardia* and *Entamoeba* species (19). Berberine is an important representative of the alkaloid group. It is potentially effective against trypanosomes and plasmodia (20). The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmaline (21) is attributed to their ability to intercalate with DNA (22).

The crude alkaloidal extract of *Zanthoxylum chiloperone* stem bark exhibited in vitro activity against various strains of

*Leishmania* spp. at 100 µg/ml. This drug reduced the parasite loads by 91% in the lesion (23).

These results confirmed the effect of these extracts for the treatment of cutaneous leishmaniasis in-vivo (24). Mirzaie et al. (2007) found *P. harmala* extract inhibited the growth of promastigote forms of *L. major* in vitro after 72 h. of incubation and had an IC<sub>50</sub> of 1832.65 ± 89.72 µg/mL (25).

The results of the present study showed that the plant drugs with efficacy and safe products can be applied as new treatment for cutaneous leishmaniasis. Alkaloids are one of the most important classes of natural products providing drugs for humans since ancient times. Most alkaloids are well known because of their toxicity, but many alkaloids have had a deep impact on the treatment of parasitic infections. *Peganum harmala* had better effect than *Alkanna tinctoria* (IC<sub>50</sub> for *P. harmala* 40 µg/ml and for *A. tinctoria* 200), whereas the cytotoxicity for both of them in the same concentration is alike. Combination of two extracts had a good effect with low doses (IC<sub>50</sub> for the equal combination of *P. harmala* and *A. tinctoria* was 10 µg/ml from each one) and low toxicity in the same IC<sub>50</sub>. The results also indicated that the effects of two combined extracts were better than the separated extracts.

## Acknowledgements

This study was supported by Tarbiat Modares University through pecuniary aid. The authors wish to thank Miss Ghasemi for kind helping. The authors declare that there is no conflict of interests.

## References

1. Munir C, Zaidi MI, Ahmad N, Attaur Rahman. An Easy Rapid Metal Mediated Method of Isolation of Harmine and Harmaline from *Peganum harmala*. *Fitoterapia*. 1995; 1:73.
2. Hepburn NC. Cutaneous leishmaniasis. *Clin Exp Dermatol*. 2000;25:363–70.
3. Bermann JD. Human leishmaniasis: clinical, diagnostic and chemotherapeutic developments in the last 10 years. *Clin Infect Dis*. 1997; 24:684–703.
4. Moskowitz PF, Kurban AK. Treatment of cutaneous leishmaniasis: retrospectives and advances for the 21st century. *Clin dermatol*. 1999; 17:305–15.
5. Ghaffarifar F, Jorjani O, Mirshams M, Miranbaygi MH, Hosseini ZK. Photodynamic therapy as a new method for the treatment of cutaneous leishmaniasis. *East Mediterr Health Journal*. 2006; 12(6):902-908.
6. Hewaldt BL. Leishmaniasis. *Lancet*. 1999; 354:1191–9.
7. Callahan HL, Kelley C, Pereira T, Grogl M. Microtubule inhibitors: structure-activity analyses suggest rational models to identify potentially active compounds. *Antimicrob Agents Chemother*. 1996; 40(4):947–952.
8. Chopra RN, Chopra IC, Handa KL, Kapoor LD. Chopra's Indigenous drug of India. 2<sup>nd</sup> ed. U.N. Dhar and Sons Pvt. Ltd. Calcuta; 1958.
9. Fern K. Plants for A Future: Edible & Useful Plants For A Healthier World. Permanent Publications, Hyden House Ltd; Little Hyden Lane, Clanfield, Hampshire PO8 0RU, England; 1997.
10. Abdelfattah AFM, Watanabe H. Hypothermic effect of harmala alkaloid in rats: involvement of serotonergic mechanism. *Pharmacol Biochem Behav*. 1995; 52:421.
11. Bellakhdar JL. A pharmacopée marocaine traditionnelle. *Medicine arabe ancienne et savoirs populaires*. Paris: Ibis Press; 1997.
12. Gracie and Zarkov, *Peganum harmala*. 2006. Available from: [http://www.erowid.org/plants/syrian\\_rue/syrian\\_rue\\_info4.shtml](http://www.erowid.org/plants/syrian_rue/syrian_rue_info4.shtml)
13. Polunin O, Huxley A A. *Flowers of the Mediterranean*. Hogarth Press;1987. Callahan HL. Microtubule inhibitors: structure activity analyses suggest rational models to identify potentially active compounds. *Antimicrob Agents Chemother*. 1996; 40 (4): 947–952.
14. Chang KP. Human cutaneous leishmania in a mouse macrophage line: propagation and isolation of intracellular parasites. *Science*. 1980; 209(4462):1240–1242.
15. Bodley AL, McGarry MW, Shapiro TA. Drug cytotoxicity assay for African trypanosomes and *Leishmania* species. *J Infect Dis*. 1995;172(4): 1157-1159.
16. Thakur CP, Singh RK, Hassan SM, Kumar R, Narain S, Kumar A. "Amphotericin B deoxycholate treatment of visceral leishmaniasis with never modes of admin-

- istration and precautions: a study of 938 cases". *Trans Roy Soc Trop Med Hyg.* 1999; 93: 319–23.
17. Kayser O, Kiderlen A, Croft SL. Natural products as potential antiparasitic drugs. In: Atta-ur-Rahman (Hg.), *Studies in Natural Products Chemistry, Part G: Bioactive Natural Products*, chapter 12. Vol. 26. Amsterdam: Elsevier Science BV; 2002. p. 779-848.
  18. Freiburghaus F, Kaminsky R, Nkunya MH, Brun R. Evaluation of African medicinal plants for their *in vitro* trypanocidal activity. *J Ethnopharmacol.* 1996; 55:1–11.
  19. Ghoshal S, Krishna Prasad BN, Lakshmi V. Antiamoebic activity of *Piper longum* fruits against *Entamoeba histolytica* in vitro and in vivo. *J Ethnopharmacol.* 1996; 50:167–170.
  20. Omulokoli E, Khan B, Chhabra SC. Antiplasmodial activity of four Kenyan medicinal plants. *J Ethnopharmacol.* 1997; 56:133–137.
  21. Hopp KH, Cunningham LV, Bromel MC, Schermeister LJ, Khalil SK. In vitro antitrypanosomal activity of certain alkaloids against *Trypanosoma lewisi*. *Lloydia.* 1976;39(5):375–377.
  22. Cowan MM. Plant Products as Antimicrobial Agents. *Clin Microbiol Rev.* 1999;12(4):564–582.
  23. Ferreira ME, Rojas de Arias A, Torres de Ortiz S, Inchausti A, Nakayama H, Thouvenel C, Hocquemiller R, Fournet A. Leishmanicidal activity of two canthin-6-one alkaloids, two major constituents of *Zanthoxylum chiloperone* var *Angustifolium*. *J Ethnopharmacol.* 2002; 80 (2-3): 199-202.
  24. Tabatabaie F, Ghaffarifar F, Dalimi AA. Evaluation the effect of *Alkanna tinctoria*, *Peganum harmala* and *Euphorbia mysinites* extracts on sore of BALB/c mice infected with *L. major* (MRHO-IR/75/ER) *in-vivo*. *Daneshvar Journal.*2004; 12(57):37-48.
  25. Mirzaie M, Nosratabadi SJ, Derakhshanfar A, Sharifi I. Antileishmanial activity of *Peganum harmala* extract on the *in vitro* growth of *Leishmania major* promastigotes in comparison to a trivalent antimony drug. *Veterinari Archiv.* 2007; 77(4):365-375.