Synergistic effect of *Lawsonia inermis* and *Peganum harmala* aqueous extracts on *in vitro* growth of *Leishmania tropica* promastigotes comparison to Sodium Stibogluconate

Niran Al-Ogaili
Alyarmouk University College, Baghdad, Iraq

خلاصة البحث

السوطيات من جنس الليشمانية تنتقل بواسطة عضة ذبابة الرمل من جنس الفليبوتوماس. التهاب الليشمانيا الجلدي او مايسمى بحبة بغداد تعد من الامراض المنتشرة في المناطق الجنوبية من العراق. نباتي الحنة والحرمل يحتويان على عدة مركبات مضادة للبروتوزواز. استخدم فحص ام تي تي (MTT) لتقيم الفعالية البايولوجية المختبرية للمستخلص المائي لنباتي الحنة والحرمل على سوطيات الليشمانيا تروبكا مقارنة بالادوية خماسية التكافؤ الانتمونية. حضر المستخلص لنبات الحنة بالتراكيز(5%،2,5% و 1,25%) و لنبات الحرمل بالتراكيز (10%،5% و 2,5%). كما حضرت تراكيز من النباتين بدمجهم مع بعض لتقيم التاثير التناغمي للنباتين على السوطيات. التجربة كررت ثلاث مرات. تم حساب معدل التثبيط لكل مستخلص نباتي منفرد، اضافة الى المستخلصات المندمجة او مركبة. التحليل الاحصائي وضح بان الخلاصات بالتراكيز القليلة و المتوسطة ثبطت السوطيات بينما التركيز الاعلى لم يكن له فعالية التثبيط مقارنة بدواء الصوديوم الستيبوكلوكونيت. دمج الخلاصات ادت الى معدل تثبيط عالي مقارنة بالمستخلصات المنفردة لكل نبتة و هذا يدل على وجود تاثير تناغمي على السوطيات.

Abstract

Promastigotes of genus *Leishmania* are transmitted by *Phlebotomus* sandflies bites. Cutaneous leishmaniasis (CL) is endemic in southern parts of Iraq. *Lawsonia inermis* (henna) leaves contain several active compounds that have antiprotozoal activity, as well as, *Peganum harmala* possess several alkaloids with antiprotozoal properties. In this study, MTT assay was used to assess the antileishmanial activity of *L. inermis* and *P. harmala* aqueous extracts in comparison to pentavalent antimonial drug(sodium stibogluconate) on *in vitro* promastigotes of *Leishmania tropica. L. inermis* and *P. harmala* extracts were prepared in concentrations of (5%, 2.5% and 1.25%) and (10%, 5% and 2.5%) respectively. Also, combinations of various concentrations were prepared to assess the synergistic effect of both plants on promastigotes. Inhibition rate was calculated for each extract concentration and their combinations. Statistical analysis showed a significant($P<0.01$) inhibition of promastigotes of *L.tropica* by both extracts of low and moderate concentrations, while higher concentrations had no inhibitory effect in comparison to sodium stibogluconate solution.. The combination of extracts showed a strong inhibitory effect in comparison to individual extracts of plants. Synergism was obvious when both extracts were combined.

Key words: *Leishmania tropica*, *Peganum harmala*, pentavalent antimonials, MTT assay, Synergism, *Lawsonia inermis*

Introduction

Cutaneous Leishmaniasis (CL),also known as Baghdad boil, an endemic in all tropical and subtropical areas of the world(1). Cutaneous Leishmaniasis is a widespread disease in Iraq, except for the three provinces in the northeast, bordering Turkey and Iran, where cases are rare, continues to present serious treatment problems(2). *Leishmania* is a genus of trypanosomes and spread by sandflies of the genus *Phlebotomus*(3). The disease, although self-limiting, can cause considerable morbidity and may result in severe disfigurement. The manifestation can be greatly variable depending on the strain of the infecting organism, the host's immunological status and the probable secondary infection. Pentavalent antimonials such as sodium stibogluconate, have been the mainstay for therapy in the endemic regions because of its efficacy and cost effectiveness (4,5). The disadvantages of the anti monials are their requirement for intramuscular or intravenous injection each day for 20-28 days, their toxicity and the growing incidence of resistance in endemic and non-endemic regions(5,6). The development of new safer and more efficacious drugs against leishmaniasis is needed. Recent

investigations focused on plants have shown an alternative way to get potentially rich source of drugs against leishmaniasis(5).

Lawsonia inermis L. is a biennial dicotyledonous herbaceous shrub commonly known as Henna or Mhendi belonging to family Lytheraceae(6). It is abundantly available in tropical and subtropical areas, a native of North Africa and South- West Asia(7). Henna leaves, flowers, seeds, stem bark and roots are used in traditional medicine to treat a variety of ailements as rheumatoid artheritis, headache, ulcers, diarrhea, leprosy, fever, leucorrhea, diabetes, cardiac diseases, hepato protective and coloring agents(5).

Peganum harmala belongs to the family Zygophylaceae is a medicinal herb with a long history of folkloristic use in Iraq. *Peganum harmala* extract have been reported to have antimicrobial(7), antifungal(8), antiprotozoal(9) and anticancer(10). The pharmacologically active compounds of *P. harmala* are beta- carbolins (harmine, harmaline, harmalol and harman) and the quinazoline deravitives (vasicine and vasicinone)(11). Harmaline has been found to be a major active alkaloids(12).

The objectives of this *in vitro* study is to assess the synergistic effect of *Peganum harmala* and *Lawsonia inermis* on *Leishmania tropica* promastigotes in comparison to conventional antileishmanial treatments.

Materials and Methods

The seeds of *Peganum harmala* and leaves powder of *Lawsonia inermis* were purchased from local market. The henna aqueous extract was prepared by macerating 20 grams of powder in 200 milliliters of distilled water at room temperature for 24 hours. The extract was filtered through two layers of guaze then through Whatman filter paper (No. 1). The concentration of the crude extract obtained was 10% w/v. Three serial dilutions of the extract were prepared (5%, 2.5% and 1.25%). The *P.harmala* seeds were grinded by an electrical grinder. Fifty grams of the plant macerated in 250 milliliters of distilled water for 24 hours at room temprature. The crude extract was filtered firstly by a piece of guaze and secondly by filter paper Whatman (No. 1). The final concentration of the extract was 20% w/v from which three serial dilutions were prepared (10%,5% and 2.5%).

Antilieshmanial drug, a pentavalent antimonial (sodium stibogluconate injection 100/ml)was used as a positive control, supplied by GSK(GlaxoSmithKline), UK.

Leishmania tropica promastigotes were supplied by Biotechnology Research Center, Al-Nahrain University. The strain was isolated from cutaneous leishmaniasis (CL) cases in the southern parts of Iraq, where CL is endemic. The promastigotes will be used to evaluate the effect of the antileishmanial activity of the plant extracts.

L.tropica promastigotes in late log phase were incubated in RPMI(Roswell Park Institute Park Memorial) medium enriched by 12% fetal calf serum, at an average of 10^5 parasites/ml.

Preparation of concentrations of plant extracts and drug: Plant extract solutions for biological testing of *L.inermis* prepared in concentrations of 500 µg/ml, 250 µg/ml and 125 µg/ml and *P.harmala* concentrations were 1000 µg/ml, 500 µg/ml and 250 µg/ml.

The sodium stibogluconate (100 mg/ml) as a positive control, prepared by diluting 1 ml of drug upto 10 ml of distilled water to obtain a concentration of 10 mg/ml (1000 µg/ml). Six microliters were inoculated separately in the wells with 1 ml of RPMI media and 1 ml of inoculum. The tests were repeated three times to assess reproducibility.

For the synergistic effect assessment of the extracts on the antileishmanial activity , a combination of different concentrations of *L. inermis* and *P. harmala* extracts were prepared.

For the antileishmanial activity assays, 100 µl/well of culture which contained 10^5 cells/ml, promastigotes were seeded in 96-well flat-bottom plates. Then 10 µl/well from various concentrations of both aqueous extracts and sodium stibogluconate were

added to triplicate wells, as well as , a combination of various concentrations of plant extracts, the plates were incubated for 24 hours at 25 ± 1˚C. The first well of 96 wells is a blank well which only contained 100 µl of culture medium without any plant extract, drug or parasite. Negative control well contained only medium and parasite. At the end of incubation, 10 µl of MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide), to assess cell metabolic activity, was added to each well and plates were incubated for 4 hours at 25 ± 1˚ C. Dimethyl sulfoxide (DMSO), as a solubilizing solution added and incubated for 30 minutes. Relative optical density (OD) measured at a wavelength of 490 nm using a multi well scanning spectrophotometer (ELISA reader). The absorbance of the formazan produced by the action of mitrochondrial dehydrogenases of metabolically active cells is shown to correlate with the number of viable cells (14,15,16,17, 18). All experiments were repeated three times.

Results

Phytochemical investigations of active constituents: The active constituents of both *Lawsonia inermis* and *Peganum harmala* extracts were identified using tests for alkaloids, flavonoids, saponins and polyphenols.

Data analysis: The percentage of non-viable organisms which failed to metabolize MTT and therefore did not produce the formazan product determined by applying the following formula (19): The inhibitory percentage of each compound's concentration=100-(Test OD- Blank OD/ Control OD- Blank OD)× 100.

Statistical Analysis: Statistical analysis was performed using Statistical Analysis System(SAS) 2012 program to show the effect of different concentrations of extracts on promastigotes activity and Least Statistical Difference (LSD) test was used for statistical significance at P< 0.01(20).

Concentrations of P. harmala Extract(20%)	Inhibition rate-IR% (Mean ± SD)
2.5 %	6.50 ± 0.27 c
5.0 %	33.00 ± 2.74 a
10.0 %	0.00 ± 0.00 c
Positive Control (+ve)	19.40 ± 1.66 b
Negative Control (-ve)	0.00 ± 0.00 c
LSD value	7.285 **
P-value	0.0073

Both *L. inermis* of 1.25% and *P. harmala* of 2.5% and 5% extracts inhibited the growth of *L. tropica* promastigotes *in vitro* after 24 hours of incubation. The inhibitory effect of various concentrations of both plants extracts and sodium stibogluconate (+ve) control against the promastigotes of *L. tropica* are shown in details in (Tables 1 and 2) and (figures 1 and 2).

Table 1 and Table 2. The effect of various concentrations of *L. inermis* and *P. harmala* extracts on inhibitory rate

Concentrations of L. inermis Extract (10%)	Inhibition rate-IR% (Mean ± SD)
1.25 %	9.70 ± 0.82 b
2.50 %	0.00 ± 0.00 c
5.00 %	0.00 ± 0.00 c
Positive Control (+ve)	19.40 ± 1.66 a
Negative Control (-ve)	0.00 ± 0.00 c
LSD value	4.619 **
P-value	0.0133

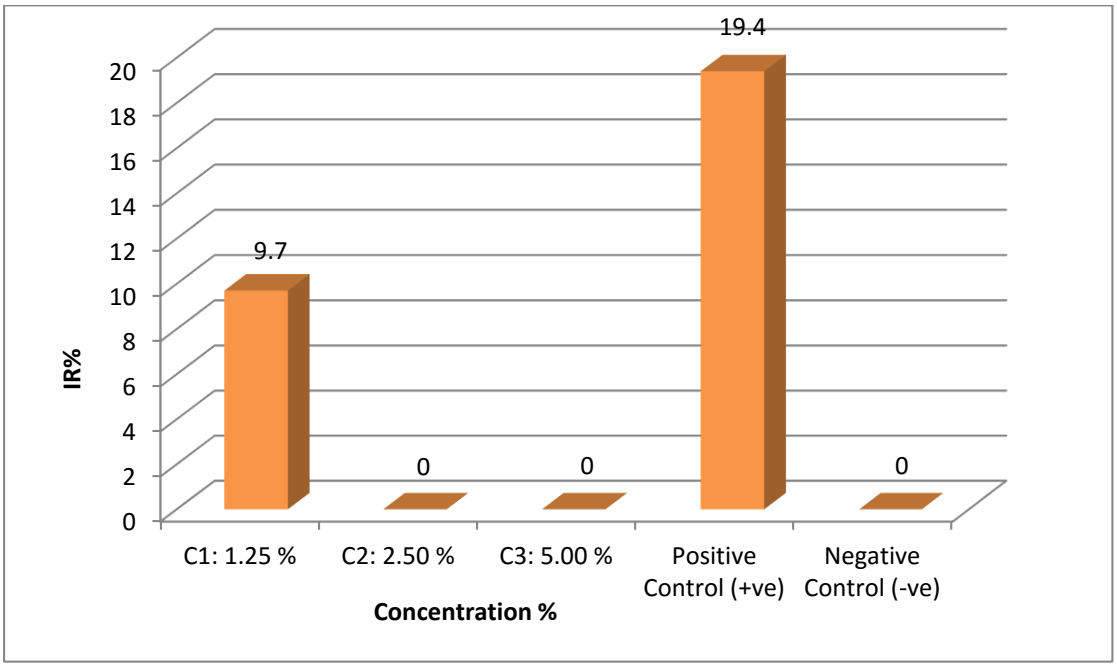

Figure 1. Inhibitory effect of various concentrations of *L. inermis* extract against *Leishmania tropica* promastigotes

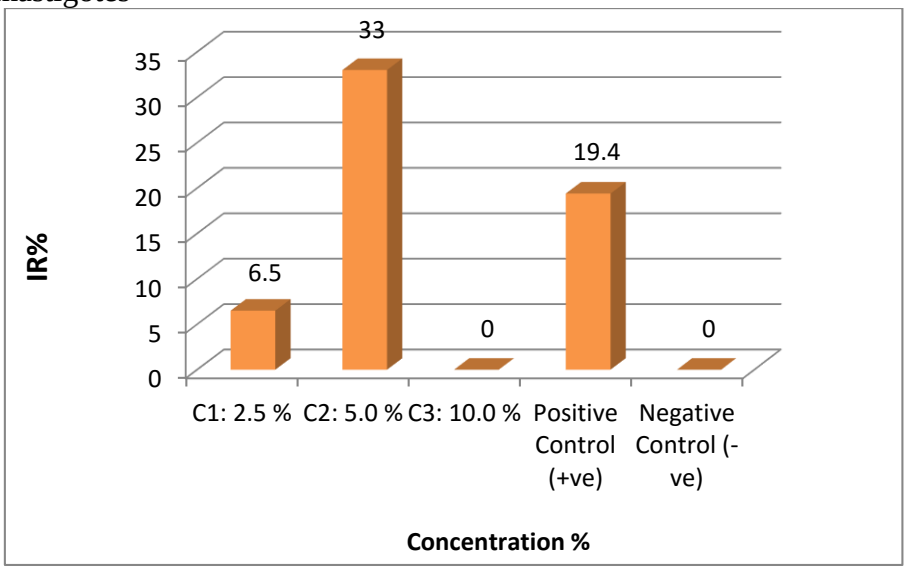

Figure 2. Inhibitory effect of various concentrations of *P. harmala* against *Leishmania tropica* promastigotes

The

synergistic effect of the combination of different concentrations of extracts of *L. inermis* and *P. harmala* against *L. tropica* promastigotes compared to (+ve) control are shown in (Table 3) and (figure 3).

Table 3. The effect of combinations of various concentrations of *L. inermis* and P. *harmala* extracts

Concentrations (%) of *L.inermis* and *P. harmala*	Inhibition rate-IR% (Mean ± SD)
1.25 %+ 2.5%	23.00 ± 1.07 b
2.50 %+ 5.0%	99.20 ± 4.39 a
5.0 %+ 10%	0.00 ± 0.00 c
Positive Control (+ve)	19.40 ± 1.66 b
Negative Control (-ve)	0.00 ± 0.00 c
LSD value	7.813 **
P-value	0.0068

P<0.01**

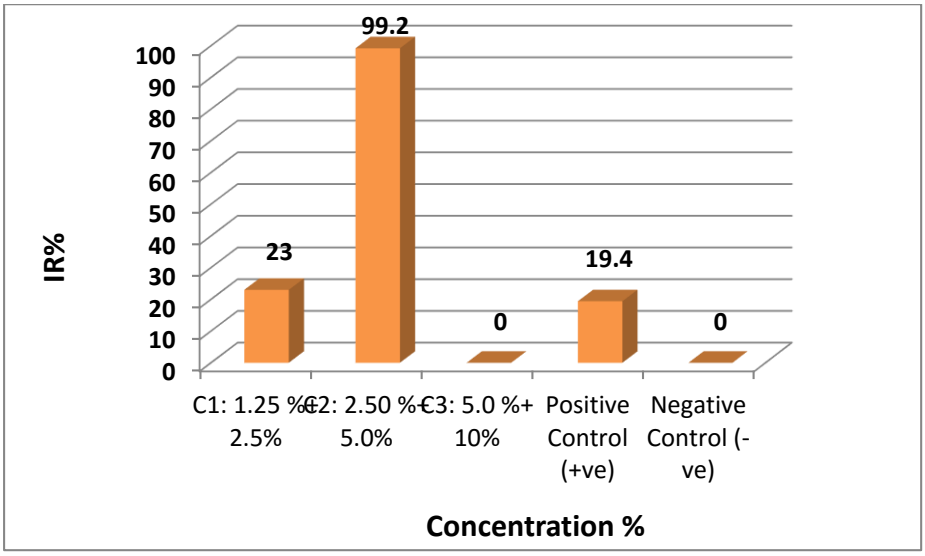

Figure 3. The effect of various combinations of concentrations of *L.inermis* and *P. harmala* aqueous extract against *Leishmania tropica* promastigotes.

promastigotes at lower concentrations of both extracts, while higher concentration did not have any effect. *L. inermis* and *P. harmala* extracts in combination exerted a strong inhibitory effect at moderate concentrations. Major active constituents identified in both plant extracts are shown in Table 4.

The inhibition rate of promastigotes of *L. tropica* by different concentrations of *L. inermis* and *P. harmala* individually and their combinations compared to sodium stibogluconate were significant with a P-value< 0.01, using least significant difference(LSD) test. The results of the study also showed an *in vitro* inhibition of

Table 4: Phytochemical Investigation of *L. inermis* and *P. harmala*

Active Constituents	Test	*L.* inermis 10% Extract	*P.* harmala 20% Extract
Alkaloids	Dragendroff	_	+
Saponine	Foam Formation	traces	+
Flavanoids	NaOH reagent	+	+
Polyphenols	FeCl3 3% reagent	+	+

Discussion

As far as my knowledge, no studies have been conducted on the synergistic effect of *L. inermis* and *P. harmala* aqueous extracts on *in vitro L. tropica* promastigotes inhibition. The study showed strong synergism in inhibiting promastigotes of *L. tropica* when extracts are combined together at certain concentrations than extracts tested individually .

Since the only antileishmanial treatment available in Iraq is sodium stibogluconate (Pentostam®) that have serious side effects and resistance, development of new drugs are needed. Natural products have potential in the search for new and selective agents for the treatment of important tropical diseases caused by protozoans(21).

Routine evaluation of antileishmanial chemotherapeutic agents are often based on promastigotes susceptibility assays(22). The MTT assay was used to assess the inhibitory effect of *L. inermis* and *P. harmala* aqueous extracts on the *in vitro* growth of *L. tropica* promastigotes. The current *in vitro* study showed significant ($P<0.01$) inhibition of promastigotes of *L.tropica* by *L. inermis* aqueous seed extract compared to positive control. A study conducted by(Serakta *et al.*, 2013) showed a significant reduction in promastigotes of *Leishmania major* by *L.inermis* hydroalcoholic extract.

Almost a hundred of phytoconstituents, representing a variety of classes, have been identified from all parts of *L. inermis.* Phenolic compounds, including coumarins, flavonoids and naphthaquinones are particularly prevalent in henna extract(24).

Lawsone a naphthaquinolone derivative is the dyeing principle in henna is particularly concentrated in leaves(25). Many biological properties displayed by the plant have been attributed to lawsone. Henna have a wide range of biological activities including antifungal, antibacterial, viracidal, antiparasitic, antinflammatory, analgesic and anticancer properties(26).

This study also showed significant results($P<0.01$) in *in vitro* inhibition of promastigotes of *L. tropica* by *P. harmala* aqueous seed extract. General chemical identification for alkaloids of the aqueous extract showed positive results. The alkaloid content of the plant includes beta-carbolines(harmaline, haramine, harmalol, harmane) and quinazoline derivatives (vasicine and vasicinone)(11). Among the several alkaloids derived from *P.harmala* extract, harmaline has been found to be the major alkaloid(12). A study conducted by(Moghaddan *et al.*, 2011) found that harmaline was present in the highest concentration in the extract followed by harmine. Several studies have shown that different protozoan infections have been susceptible to *P. harmala* extract in varying degrees. Alkaloid compounds illustrate well the diversity of antiprotozoal compounds found in *P. harmala* plant(21). Evens and Croft(1987) showed that harmaline exerted *in vitro* and *in vivo* antileishmanial activity. One study showed the quinazoline derivatives of *P. harmala,* vasicine(peganine) exhibited *in vitro* activity against both extracellular promastigotes as well as intracellular amastigotes within murine macrophages in *L. donovani*(29). These findings may explain the strong inhibition of *in vitro* promastigotes of *L. tropica* by *P. harmala* extract compared to *L. inermis* extract and positive control in the present study.

The present study showed a high inhibitory rate of promastigotes of *L. tropica* when the aqueous extracts of both plants with moderate concentrations were combined and it may be a demonstration of synergism.. Synergistic effect describes the effect of drugs working together where one drug increases the other's effectiveness. Phytochemical investigation of the current study showed positive results for flavonoids in both plant extracts. Previous study reported significant antiprotozoal activity of flavonoids has been reported against *Trypanosoma* and *Leishmania* species(30). In another study synergism has been demonstrated between various combinations of flavones and flavanols and suggested that a combination of both extracts are more active than its individual component

compounds(31) and this may explain the results in the present study.

In this study, the moderate and lower concentrations of extracts showed significant inhibition of promastigotes, while higher concentrations did not exhibit any inhibitory effect. This study is inconsistent with a study conducted by (Mirzaie *et al.*, 2007) that showed an increase in the concentration of *P. harmala* extract increased the inhibitory effect on the growth of *Leishmania major* promastigotes.

Many studies have been conducted on antileishmanial activity of *L. inermis* and *P. harmala* extracts individually. In the current study, a combination of aqueous extracts of both plants augmented the antileishmanial effect against promastigotes of *L. tropica* due to synergism. Further studies needed to investigate the mechanism of synergistic effect of the extracts on promastigotes of *L. tropica*.

References

1- Aoun, K.; Bouratbine, A. "Cutaneous Leishmaniasis in North Africa: a review. Parasite 2014; 21: 14.

2- Al-Khafaji, A.W. CDC, Baghdad and El- Twijri, S. Zoonotic Disease, Ministry of Health, Baghdad. WHO Consultative meeting on cutaneous leishmaniasis in EMRO countries, Geneva, May 2007.

3- Ryan KJ, Ray CG (2004). Sherris Medical Microbiology(4th ed.) McGraw Hill.pp.749-54.

4- Markle WH, Makhoul K. Cutaneous leishmaniasis: recognition and treatment. Am Fam Physician 2004; 69(6): 1455-60.

5- Mishra BB, Kale RR, Singh RK, Tiwari VK. Alkaloids future prospective to combat leishmaniasis.Fitoterapia 2009; 80(2): 81-90.

6- Lavhate MS, Mishra SH. A review: nutritional and therapeutic potential of *Ailanthus excels.* Pharmacognosy Rev. 2007; (1): 105-113.

7- Chaudhary G, Sandeep G, Priyanka P. *Lawsonia inermis Linnaeus*: A Phytopharmacological Review. International Journal of Pharmaceutical Sciences and Drug Research. 2010; 2(2): 91-98.

8- Harsh ML, Nag TN. Antimicrobial principlesfrom in vitro tissue culture of *Peganum harmala*. J Nat Prod. 1984; 47(2): 365-7.

9- El-Bahri L, Chemli R. *Peganum harmala* : A poisonous plant of North Africa. Vet Hum Toxicology. 1991; 33(3): 276-7.

10- Arshad N, Zitter- Eglseer K, Hasnain S, Hess M. Effect of *Peganum harmala* or its beta carbolin alkaloids on certain antibiotics resistant strains of bacteria and protozoa from poultry. Phytother Res. 2008; 22(11): 1533-8.

11- Kamel, S., L. Ibrahim, A. Afifi, S. Hamza (1970): Major alkaloidal constituents of the Egyptian plant. *Peganum harmala.* UARJ, Vet. Sci. 7, 71-86.

12- Budavari, S., M. J. O'Neil (1996): The merck index, 12rd ed., CRC Press, New Jersey. pp. 4644-4645.

13- Lamchouri F, Settaf A, Cherrah Y, Zemzami M, Lyoussi B, Zaid A et al. Antitumor principles from *Peganum harmala* seeds.Therapie. 1999; 54(6):753-8.

14- Mosmann, T.(1983). Rapid colorimetric assay for cellular growth and survival:

15- Niks, M. Otto(1990): Towards an optimized MTT assay. J. Immunol. Methods 130, 149-151.

16- Maarouf, M., Y. Kouchkovsky, S. Brown, P. X. Petit, M. Robert-Gero (1997): *In vivo* interference of paromomycin with mitrochondrial activity of Leishmania. Exp. Cell Res. 232, 339-348.

17- Sereno, D., J. L. Lemesre (1997): Axenically cultured amastigote forms as an *in vivo* model for investigation of antileishmanial agents. Antimicrob. Agents Chemother. 41, 972-6.

18- Avlonitis, N., E. Lekka, A. Detsi, M. Koufaki, T. Calogeropoulou, E. Scoulica, E. Siapi, I. Kyrikou, T. Mavromoustako, A. Tsotinis, S. G. Grdadolnik, A. Makriyannis (2003): Antileishmanial ring-substituted ether phospholipid. J. Med. Chem. 46, 755-767.

19- Bansal, D., R. Sehgal, Y. Chawla, R. C. Mahajan, N. Malla (2004): *In vitro* activity of antiamoebic drugs against clinical isolates of *Entamoeba dispar* . Ann. Clin. Microbiol. Antimicrob. 3, 27-31.

20- SAS. 2012. Statistical Analysis System, User's Guide. Statistical Version 9. 1st ed. SAS. Inst. Inc. Cary. N.C. USA.

21- Wright, C. W., J. D. Phillipson(1990): Natural products and the development of selective antiprotozoal drugs. Phytother. Res. 4, 127-39.

22- Gupta, N., Goyal, A. K. Rastogi (2001): *In vitro* cultivation and characterization of axenic amastigotes of *Leishmania.* Trends Parasitol. 17, 150-153.

23- Serakta, M., Djerrou, Z., Mansour-Djaalab, H., Kahlouche-Riachi, F., Hamimed, S., Trifa, W., Belkhiri, A., Edikra, N., Hamdipacha, Y.; Afrr J Tradit Complement Altern Med.(2013); 10(3):427-430..

24- Phirke, S.S., Saha, M. 2013. *Lawsonia inermis* L.: a rainfed ratooncrop. National Conferencs on Biodiversity: Status and Challenges in conservation, FAVEO, 189-193.

25- Cartwight- Jones, C., 2006. Developing guidelines on Henna: A Geographical Approach(Master's Dissertation). Masters of Liberal Studies. Kent State University, Kent, Ohio,USA.

26- Li, Q., Gao, W.Q., Zhao, Y.Q., 2013. Advances in studies on chemical constituents and biological activities of *Lawsonia inermis.* China Journal of Chinese Materia Medica 38,795-799.

27- Moghaddan,P.R., Ebrahimi, S.A., Oumarzadi, H., Selseleh, M., Karjalian, M., Hassani, G.H.,

Alimohammadian, M.H., Mahmoudian, M., Shafiei, M. J Res Med Sci 2011 Aug; 16(8): 1032-1039.
28- Evans, A. T., Croft, S.L. Antileishmanial activity of harmaline and other tryptamine derivatives. Phytother Res. 1987; 1(1):25-7.
29- Khaliq, T., Misra, P., Gupta, S., Reddy, K.P., Kant, R., Maulik. P. R., *et al.* Peganine hydrochloride dehydrate an orally active antileishmanial agents. Bioorg Med Chem Lett. 2009; 19(9):2585-6.
30- Silva AM., Tavares J., Silvestre R., Quaissi A., Coombs GH., Silva AC. Characterization of *Leishmania infantum* thoil- dependent reductase I and evaluation of its potential to induce immune protection. Parasite Immunology 2012; 34(6) 345-50.
31- Armous M., Simoes CM., Girre L., Suavagers F., Cormier M. Synergistic effect of flavones and flavanols against Herpes simplex virus type I in cell culture. Comparison with the antiviral activity of propolis. J Nat Prod 1992:55: 1732-40.
32- 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. Veterinarski Arhiv 2007; 77(4) 365-375.