International Journal of Molecular and Clinical Microbiology 7(1) (2017) 761-768

International Journal of
Molecular and Clinical Microbiology

# Antileishmanial effect of Crataegus microphylla leaf extract on Leishmania major (MRHO/IR/75/ER) promastigotes

**Samira Shirazi[1] and Monir Doudi[2*]**

*1. Medical Biotechnology Research Center, Ashkezar Branch, Islamic Azad University, Yazd, Iran.*
*2. Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran.*

ARTICLE INFO

*Article history:*
Received 20 November 2016
Accepted 20 April 2017
Available online 1 June 2017

*Keywords:*
*Leishmaniasis,*
*Leishmania major,*
*Crataegus microphylla,*
*MTT,*
*GC/mass.*

ABSTRACT

Various chemical drugs have been used for leishmaniasis treatment, but their side effects and drug resistance have led to look for new effective compounds. *Crataegus microphylla* the traditional and medicinal herb is a valuable source of new Pharmaceutical agents. The extract was prepared The extract obtained by maceration method, and diluted with 5% DMSO. *Leishmania major* promastigotes were cultured RPMI- 1640, enriched with 10% fetal calf serum and Penicillin- Streptomycin.Then the biological activity of herb extract and drug susceptibility was evaluated on *L.major* promastigotes compared to Glucantime (Sb III) drug using MTT colorometry. The optical density was measured with Eliza reader set, and the $IC_{50}$ value was calculated. In this study, we used the GC / mass. IC50 of Glucantime (Sb III) was 616.18 µg/ml, and alcoholic extracts of *Crataegus microphylla* 1094 µg/ml. Although Glucantime was more effective than plant extracts, all extracts had profound effects on promastigotes of *L.major*. The studied herb extract had considerable antileishmanial effects compared to Glucantime (Sb III) In vitro, the necessity of conducting more experiments to investigate its effect on the parasite in animal model is also appreciated.

## 1. Introduction

Leishmaniasis is a group of tropical diseases caused by protozoa belonging to the *Leishmania* genus. It is an ailment that affects 2-3 million new cases each year and it is considered that about 350 million people are facing the risk of infection worldwid (Doroodgar et al., 2008; Peixoto et al., 2011). Leishmaniases are transmitted by sand flies of the genus *Phlebotomus.* It is found in most tropical and subtropical countries, but 90% of the new cases of cutaneous lesishmaniasis (CL) occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria (Almeida et al., 2005). The CL is one of the most common forms that found in many countries (Minodier et al., 2004). CL has been seen clinically in two rural (humid wound) and urban (dry wound) types in Iran. The agent of rural CL is *L. major*, and the agent of urban CL is *L. tropica* (BabaeeKhoo et al., 2007).

Various studies have shown that the CL is increasing in Iran and the world. Also in recent years, the leishmaniasis treatment has faced with

*Corresponding author: Dr. Monir Doudi
E-mail address: Monirdoudi@yahoo.com

many problems due to appearance of resistance against the standard drugs which are mostly the pentavalent antimony compounds.. Glucantime and Pentosetam are consumed as the first selective drugs in most parts of the world; however; the effectiveness of the drugs has been decreased to 20-25% during a few recent years (Babaeekhoo et al., 2007). Despite of performed studies to find new anti-leishmanial drugs, Amphotericin B and Pentamidine are the first-line medications that have unpleasant side-effects (Doroodgar et al., 2008; Peixoto et al., 2011).The emergence of resistant strains caused to introduce the new antileishmanial agents such as Miltefosine, Amphotericin B, Ketoconazole and Paromomycin, and other chemicals which non of them are without side effects. Moreover, the intoxication agents and their side effect resistance even after improving dose and long term treatment are considered as their shortcoming. On the other hand, the treatments is not appropriate especially in rural areas due to expensiveness and non- accessibility (Khademvatan et al., 2011).

Herbs are a potential source of anti-protozoan. Biological activity of herb extracts is attributed to compositions belongs to several chemical groups such as alkaloids, flavonoids, phenylpropanoids, steroids and terpenoids (Mikus et al., 2000; Tiuman et al., 2011). Natural extract of different plants such as *Euphorbia* spp., *Gossypium herbacium* and *Berberis vulgaris* are directly used on skin lesions as well as on the parasite in NNN medium (Doroodgar et al., 2008). In one study, the turmeric plant of compounds has induced curcumin, indium curcumin, gallium curcumin and diacetyl curcum in against *L. major* promastigotes was checked by MTT assay In vitro. *Gallium carcumin* and *Indium curcumin*, with a lower $IC_{50}$ compared to Diacetyl curcuminanalogue, were stronger factors against *L. major* promastigotes (Barazesh et al., 2012).

Since the Aloevera plant is widely used in medicine, the effectiveness of the exudate of Aloevera leaves on leishmaniasis was investigated in a study (Dutta et al., 2008). We investigated the in vitro antileishmanial activity of aerial extract parts of *Crataegus microphylla* leaves on promastigotes of *L. major* (*MRHO/IR/75/ER*) by MTT assay.

## 2. Materials and Methods

This experimental study has been done in the laboratory of infectious diseases and tropical medicine research center of Isfahan. This study was considered antileishmanial effects of essential parts of the *Crataegus microphylla*, (including stems and leaves) on promastigotes of *L. major* (*MRHO/IR/75/ER*).

### *2.1. Plant material*

Red herb hawthorn (Crataegus microphylla) were collected in Babol, Mazandaran, north of Iran, in September 2014. Crataegus microphylla (001/01/074) have been deposited in the Herbarium Falavarjan Branch, Islamic Azad University. The stems and leaves of plant were prepared, washed with distilled water, dried by an electric fan in room temperature (20-25°C) and extracted by Clevenger (Bhogireddy et al., 2013).


**Figure 1.** Schematic of the surface morphology of *Crataegus microphylla*,.(www..henreettesherbal. com).

*2.2. Plant extraction*

The dried stems and leaves of the plant (50 g) was powdered and macerated with 50 ml of 80% ethanol for 5 days at room temperature. After evaporation of the solvent under reduced pressure at 45°C, the respective ethanolic extracts were obtained. Stems and leaves of the plant were collected in sterile conditions under the hood and rinsed with distilled water, then dried at room temperature (20-25°C) by an electric fan in the shade. For aqueous extract , extraction was done by distillation using Clevenger apparatus. For every time of extraction, 100 gr of considered plant' dry powder was extracted for 2 hours. 100 gr of the plant dry powder was provided and the extraction performed 3 times. The extracts were poured into the matte lidded glass and kept in the refrigerator (4°C) until testing.

*2.3.Gas chromatography-mass (GC/mass)*

In this study, were used the GC/mass device includes Aglient 5975 C mass detector with electron ionization source (EI) coupled with Aglient 7890 gas chromatography composed of HP- 5MS column with length 30 meters, diameter of 25.0 mm, and film thickness of 25.0 mm. The temperatures of Gas Chromatography device injectionsite,C mass detector ionization, and Analyzers (Kuadruple) were 280°C, 150°C, 230°C respectively, and the growth medium temperature between MS and GC was adjusted to 280°C.

*2.4. Antileishmanial assay*

The antileishmanial activity was carried out according to a previously described method (Mbongo et al., 1997) In brief, promastigotes of *L. major* were cultured in Schneider's medium and supplemented with 10% fetal bovine serum at 24°C. The screening was done in 96-well tissue-cultured plates. Promastigote in logarithmic phase. since after counting, the cell were suspended to reach the proper number by Neubauer chamber.

Each well was filled with the parasites suspension, and the plates were incubated at 24°C for 1 h. Then extract of plant drug was added. The extracts were dissolved in 1% (v/v) DMSO and added to each well.

To evaluate the impact of different dilutions of extracts (12.5, 25, 50, 100, 200, 400, 800, 1600 and 3200 µg/ml) on the parasite serial dilutions were prepared using RPMI- 1640 medium. Then 40 µl of various dilution of Glucantime ( Sb III) were added in triple form to growth medium containing parasites and growth medium without parasite, incubated for 24, 48 and 72 hours at the temperature of 33-34°C. Each concentration was screened in triplicate. The viability of promastigotes of *L.major* was assessed by tetrazolium-dye (MTT) colorimetric method. The results were expressed as the concentrations inhibiting parasite growth by 50% (IC50) after 3 days incubation period. The optical density of plate was investigated at a wavelength of 630- 540 nm using ElisaReader device.

*2.5. Statistical analysis*

The IC50 values at 95% confidence interval were calculated, for *Crataegus microphylla* ethanol extract, using a non-linear regression curve, using the GraFit 5 statistical software.

## 3. Results

In this study for natural products with antileishmanial, we examined leaves and steams of plant extract belonging *Crataegus microphylla*. The were showed that the GC/mass results alcoholic extract ingredients of *Crataegus microphylla* leaves and stems were presented in Table 1.

As shown in Table 2, using the MTT assay and serial dilution method, ethanol extracts from *Crataegus microphylla*, Glucantime (Sb III) and aqueous extracts showed antleishmanial activity with IC50 for 24, 48 and 72 hours.

Our data in Table 2, showed that comparison among ethanol extract and Glucantime (Sb III) tested against *L.major* promastigote forms. The most effective extract against *L.major* with IC50 of 1094µg/ml. The extract of plant showed a moderate activity against *L.major* promastigote forms with IC50 of 1601.1µg/ml.

*L.major* promastigote cells were observed with 100× magnification at 24, 48 and 72 hours which had showed some changes. These changes began after facing with glucantime (Sb III) and ethanol and aqueous extracts, also including cell

shrinkage, circulation, cytoplasm densiyfying, and cells size decreasing (figure 1).

Further analysis was performed comparing the amount of transformed protmastigotes in each concentration with control groups.

The results of GC/mass screening of the extracts of the steams and leaves of the studied plant appears to be due to substances such as, Sulfurous acid, hexa methyl cyclo trisiloxane, bis (trimethyl silyl) diethyl cyclotrisilox, 4-trimethyl silyl) acetanilide, 1,1,1,3,5,5,5- hepta methyl trisiloxane, alpha, alpha-(1- methyl ethylene dllmlno) di-ortho-cresol in the plant extracts acid by using gaschromatography- mass in ethanol extract of *Crataegus microphylla*.

Table 2. Antileishmania active IC50 ethanol and aqueous extracts of *Crataegus microphylla* , Glucantime (Sb III) after 24,48,72 hours.

**Table 1.** Ethanol extract compounds of *Crataegus microphylla* leaves and stems after gas chromatography mass

| Substance | Amount (%) |
|---|---|
| Sulfurous acid, diethyl ester | 14.6% |
| Alpha, alpha-(1-methyl ethylene edllmlno) di-ortho-cresol | 15.4% |
| 4-Tri methyl silyl) acetanilide | 14. 6% |
| Bis (trimethyl silyl) diethyl cyclotrisilox | 16.04% |
| Hexa methyl cyclo trisiloxane | 16.7% |
| 1,1,1,3,5,5,5-Hepta methyl trisiloxane | 18.2% |
| Other components | 4.1% |

**Table 2.** Antileshmanial activity IC50(µg/ml)

| | **24h** | **48h** | **72h** |
|---|---|---|---|
| **Ethanol extract** | 1601.1 | 1094 | 680.35 |
| **Aqueous extract** | 2825.92 | 2383.3 | 1785 |
| **Glucantime** (Sb III) | 680 | 616.18 | 484.39 |

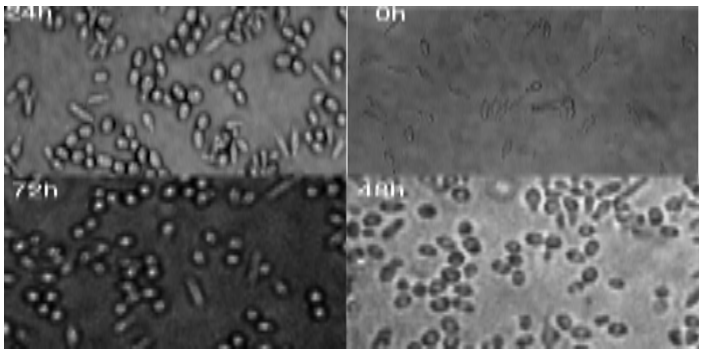


**Figure 2.** Morphology of the standard *L.major* strains treated with *Crataegus microphylla* at zero, 24, 48 and 72 hours at 100 × magnification of optical microscope. The circulation and decreasing the size of cells are observed in 48 and 72 hours .

**Table 3.** The study of morphological and proliferation L. major promastigotes affected by glucantime (Sb III)

| The percentage of transformed promastigotes | 24 hours | 48 hours | 72 hours |
|---|---|---|---|
| The percentage of transformed standard *L.major* amastigotes affected by 19 µg/ml of glucantime (Sb III) | 58% | 86% | 97% |
| The percentage of transformed clinical *L.major* promastigotes affected by 12 µg/ml of glucantime (Sb III) | 49% | 71% | 87% |

**Table 4.** The study of morphological and proliferation *L.major* promastigotes affected by Crataegus microphylla.

| The percentage of transformed promastigotes | 24 hours | 48 hours | 72 hours |
|---|---|---|---|
| The percentage of transformed standard *L.major* promastigotes affected by 360 µg/ml of *Crataegus microphylla* | 29% | 53% | 79% |
| The percentage of transformed clinical *L.major* promastigotes affected by 680 µg/ml of *Crataegus microphylla* | 21% | 49% | 61% |

## 4. Discussion

Leishmaniasis refers to a spectrum of diseases caused by protozoa of the Leishmania genus. According to WHO, there are about 12 million cases of the disease in different parts of the world and 350 million people are at risk to be stricken with this disease. The first line treatment drugs, are the Pentavalentantimony compounds, none of them are without side effects (Groft et al., 2006; Gharavi et al., 2011). Their effects include the toxicity and sustainability of their side effects on the heart and kidney. The recurrence rate, high cost, duration of treatment, and in recent years, increasing of parasite resistance to these medicines has been seen. One of the substituted treatment methods is using the medical herbs which are more accessible and cheaper, also have fewer side effects due to harmony with nature and natural flora (Rocha et al., 2005).

The photochemical study of *Purslane* extracts showed that the herb composed of substances such as Alcaloids, Saponins, Tannins, Cardiac glycoside, Steroids, Flavonoids, Flavonoid epigenin (Baloch et al., 201; Nayka et al., 2014; Almeida et al., 2005). But in this research the existence of substances such as, Sulfurous acid, hexa methyl cyclo trisiloxane, bis (tri methyl silyl) diethyl cyclo trisilox, 4-tri methyl silyl) acetanilide, 1,1,1,3,5,5,5-hepta methyl tri siloxane, alpha, alpha-(1-methyl ethylene edllmlno) di-ortho-cresol *Crataegus microphylla*, according to Table 1.

In a study aimed to determine the possibility of inducing apoptosis of garlic essence on *L. major* promastigotes, it was found that garlic has powerful antioxidant compounds, such as *Allicin.* The compounds have created antibacterial and anti-parasitic featurs in *Garlic* herb. The promastigotes of this cultured parasite in vitro of RPMI-1640 were influenced by *Garlic* various concentrations,and IC50 was calculated by MTT assay.It was found in the study that *Garlic* has a dose-dependent cytotoxic effect withal most 100% mortality in concentration of 93 µg/ml (Khademvatan et al., 2011; Feily, et al., 2012).

In one study, the assessment of antileishmanial activity of *Curcumin* and its derivatives, *Indium curcumin, Gallium curcumin* and diacetyl *Curcumin* against *L. major* promastigotes was checked by MTT assay in vitro.*Curcumin* is the active ingredient in herbal treatments and is responsible for many biological effects of turmeric plant. It has strong antioxidant, anti-inflammatory and anti-cancer properties. The IC50 for *Curcumin, Gallium carcumi*n, *Indium curcumin*, diacetyl *Curcumin*, and Amphotericin B (control medicine) was calculated as 38, 32, 26, 52 and 20 g/ml respectively. *Gallium carcumin* and *Indium curcumin*, with a lower IC50 compared to diacetyl *Curcumin* analogue, were stronger factors against *L. major* promastigotes (Barazesh et al., 2012).

Since the Aloevera plant is widely used in medicine, the effectiveness of the exudate of Aloevera leaves on leishmaniasis was investigated in a study. Promastigotes of species causing visceral, mucosal and cutaneous leishmaniasis were susceptible to Aloevera leaf and IC50 of the plant extract was 100 to 180 µg / ml. This data revealed that the Aloevera leaf can cause the better activity of the host macrophages through direct antileishmanial activity, and we can use it as an effective antileishmanial agent in pharmaceutical researches (Dutta et al., 2008).

A research group examined the antileishmanial effects of extracts of *Zatariamultiflra, Peganumharmala, Myrtle,* and tartaratic control drug by MTT assay in vitro.The results were calculated as IC50 for each extract separately. It was obtained for the extraxts of *Zatariamultiflra, Peganumharmala,* and *Myrtle* 5.8 µg/ml, 7.2 µg/ml, and 5.8µg/ml respectively.

Tartaratic IC50 amount was calculated 4.7 µ g/ml of Myrtle extract. The Myrtle extract with the minimum IC50 had a better effect compared to the other extracts. All of these extracts showed significant antileishmania leffects (Mirzaie et al., 2007).

No study has been conducted so far to examin the effect of *Crataegus microphylla* ethanol and aqueous extracts on *L.major* parasite promastigotes growth. The case was studied in this project and according to results presented in graps 1, it was observed that the *Crataegus microphylla* ethanol and aqueous extracts has IC50 equal to 360 µg/ml against the standard *L.major* promastigotes and 680 µg/ml against the clinilal strain of the parasite after 48 hours.

According to the results presented in Tables 3 and 4, it can be concluded that compared with Glucantime (Sb III) , the minimum number of modifiedcells was related to promastigotes which were affected by *Crataegus microphylla* ethanol and aqueous extracts. Also with comparing the clinical and standard strain *L.major* promastigotes, we can say that in all cases the number of transformed cells in the standard strains was more than the clinical ones.

## Acknowledgements

The authors would like to express their gratitude to the personnel of Laboratory of Infectious and Tropical Diseases Research Center Isfahan Seddigheh Tahereh, resectable staff of parasitology laboratory of Isfahan University of Medical Sciences. Here we confirm that all authors participated in the research design and contributed to sections of the research.

## Refereces

Adami, Y.L., Daniel-Ribeiro, C.T., Espinola-Mendes, E., Figueiredo, M.R., Ferreira-da-Cruz, M., 2000. The intraperitoneal Plasmodium berghei-Pasteur infection of Swiss mice is not a system that is able to detect the antiplasmodial activity in the Pothomorphe plant extracts that are used as antimalarials in Brazilian endemic areas. Export Parasitology. 94: 243–247.

Almeida, J.R., Barbosa-Filho, J.M., Macedo, R.O., Rocha, G., 2005. A review of natural products with antileishmanial activity. Phytomedicine. 12(6-7):514-35.

BabaeeKhoo, L., Mohebali, M., Nikan, L., 2007. MehrabiTavana A. The therapeutic effect ofeucalyptus Myrtus, ferula, aretmisia, Allium and Urtica extracts against cutaneous leishmaniasis caused by Leishmania major in small white mice. Hakim Research J. 10(2): 21-27.

Baloch, N., Nabi, S., Al Kahraman Yasser, M.S.A., 2013. In vitro antimicrobial, insecticidal, antitumor activities and their phytochemical estimation of methanolic extract and its fractions of Medicago lupulina leaves. World Applied Journal. 23(4): 500- 506.

Barazesh, A., Farrokhzad, F., Fouladvand, M., 2012. Evaluation of in vitro anti-leishmanial activities of curcumin and its derivatives "gallium curcumin, indium curcumin and diacetylecurcumin. Int J Infect Dis. 16(1): 151-152.

Bhogireddy, N., Gaddaguti, V., Naga, V.K.A., Pradeep, K.M., Raj, K.K., Ramesh, B., Reddy, O.V.S., Venkataraman, B., 2013. Anti inflammatory and anti-diabetic activities with their other ethnomedicinal properties of the plants. J Med Plant. 1(5): 87-96.

Derakhshanfar, A., Mirzaie, M., Nosratabadi, S. J., Sharifi, I., 2007. Antileishmanial activity of Peganumharmala extract on the in vitro growth of Leishmania major promastigotes in comparison to a trivalent antimony drug. VeterinarskiArhiv. 77 (4): 365-375.

Doroodgar, A., Arbabi, M., Razavi, M.R., Mohebali, M., Sad,r F., 2008. Treatment of Cutaneous Leishmaniasis in Murine Model by Hydro Alcoholic Essence of Artemisia sieberi. Iranian J Arthropod-Borne Dis. 2(2): 42-47.

Dutta, A., Sarkar, D., Gurib-Fakim, A., Mandal, C., Chatterjee, M., 2008. In vitro and in vivo activity of Aloevera leaf exudate in experimental visceral leishmaniasis. Parasitology Research. 102(6):1235-1242.

Feily, A., Khademvatan, S., Maraghi, S., Moosavi, Z., Saki, J., Siahpoosh, A., 2012. In vitro activity of green tea against Leishmania major promastigotes. Inter J Clin Farma . 50(3): 233-236.

Gharavi, M.J., Khademvatan, S.H., Rahim, F., 2011. Miltefosine induced apoptotic cell death on Leishmania major and L.tropica strains. Korean Journal of Parasitology. 49(1): 17–23.

Groft, S.L., Seifert, K., Yardley, V., 2006. Current scenario of drug development for leishmaniasis. Indian J Med Res. 123(3): 339-410.

Khademvatan, S.H., Gharavi, M.J., Rahim, F., 2011. MilteFosine induced apoptotic cell death on Leishmania major and L.tropica strains. Korean Journal of Parasitology. 49(1): 17–23.

Mbongo, N., Loiseau, P.M., Lawrence F., Bories, C., Craciunescu, D.G., Robert-Gero, M., 1997. In vitro sensitivity of Leishmania donovani to organometallic derivatives of pentamidine. Parasitol Res. 83(5):515-7.

Mikus, J., Steverding, D.A., 2000. simple colorimetric method to screen drug cytotoxicity against Leishmania using the dye Alamar Blue. 48(3): 265-9.

Minodier, P., Parola, P., 2004. Cutaneous leishmaniasis treatment. J Travel Med infect Dis. 5(3):150-8.

Mirzaie, M., Nosratabadi, S.J., Derakhshanfar, A., Sharifi, I., 2007. Antileishmanial activity of Peganumharmala extract on the in vitro growth of Leishmania major promastigotes in

comparison to a trivalent antimony drug. Veterin arski Arhiv. 77 (4): 365-375.

Nayaka, H.B., Londonkar, R.L., Umesh, M.K., Tukappa, A., 2014. Antibacterial attributes of apigenin, isolated from Portulaca oleracea L. Hindawi Publishing Corporation International Journal of Bacteriology, Article ID . 75851: 8.

Peixoto, J.A., Andrade, E., Silva, M.L., Crotti, A.E., Cassio Sola Veneziani, R., Gimenez, V.M., et al., 2011. Antileishmanial Activity of the Hydroalcoholic Extract of Miconia langsdorffii, Isolated Compounds, and Semi SyntheticDerivatives. Molecules. 16, 1825-1833.

Rocha, G., Almeida, J.R., Macedo, R.O., Barbosa-Filho, J.M., 2005. A review of natural products with antileishmanial activity. Phytomedicine. 12(6-7):514-35.

Tiuman, T.S., Santos, A.O., Ueda-Nakamura, T., Filho, B.P., Nakamura, C.V., 2011. Recent advances in leishmaniasis treatment. Inter J Infect Dis. 15(8): 525- 532.