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The study of antileishmanial effect of Medicago lupulina leaves alcoholic extract on Leishmania major (MRHO/IR/75/ER) by MTT assay

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ABSTRACT: Background and objective: Leishmaniasis is caused by protozoa of Leishmania genus and can be considered as a Zoonosis. The desease has caused the world health problems with high endemicity in developing countries such as Iran. Various chemical drugs have been used for leishmaniasis treatment, but their side effects and drug resistance haveled to look for new effective compounds. Medicago lupulina, the traditional and medicinal herb- are two valuable source of new Pharmaceutical agents. Material and Methods: The alcoholic extract was prepared through maceration method and dried, Then solved in water and DMSO 5%. Leishmania major promastigotes were cultured in 2±25 °C temperature in the Schneider's medium, then in the stationary phase of RPMI-1640 culture growth medium, enriched with 10% fetal calf serum and Penicillin-Streptomycin to provide large quantity of them. Then the biological activity of extract was evaluated on Leishmania major promastigotes compared to glucantime drug using MTT colorometry. The optical density was measured with Eliza reader set, and the IC_{50} value was calculated. All tests repeated 3 times. Resalts and Discussion: Glucantime IC_{50} for standard parasite was equal to 12 µg/ml after 48 hours respectively. After 48 hours, IC_{50} for extract were equal to 98 µg/ml against standard parasite promastigotes respectively. Although the glucantime pharmaceutical drug was more efficient compared to investigate extract, the extract also had significantly effects on leishmania major promastigotes with higher density. Conclusion: Regarding that the studied extract had considerable antileishmanial effect compared to glucantime in vitro, the necessity of conducting more experiments to investigate its effect on the parasite in animal model is also appreciated.
Keywords*:* Leishmaniasis, Leishmania major, Medicago lupulina, MTT.

INTRODUCTION

Leishmaniasis is an Infectious disease which is caused by different species of leishmania parasite. People of some countries specially developing countries are stricken with this disease. From Harman point of view, leishmaniasis can be clinically categorized in four sorts of: Cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis, diffuse leishmaniasis, and visceral leishmaniasis. The cutaneous one is most common among them which found in abundance in some countries such as Iran. Different species of leishmania are transmitted through Phelebotomus papatasi mosquito bite, and some specisesof Phlebotom and Lutzomyia[1].

CL has been seen clinically in tow rural (humid wound) and urban (dry wound) types in Iran. Rural CL is a Zoonosiscalled ZCL. Urban CL is known as humanitarian disease called ACL. The agent of rural CL is L. major, and the agent of urban CL is Leishmania tropica. It is worth mentioning that ZCL type is dominant in most areas of Iran. Recorded statistics of the people striken with cutaneous type is 20,000 pesons annually and some people believe that the actual digit is 4-5 times of this number and the disease is considered as one of the most important parasite diseases after malariain Iran[2].

So far, a safe and effective vaccine for the disease has not been made, and fighting the disease has always been considered in our national plans. Inspite of national and international investments, not only the disease has not been eradicated, but also it is always out breaked with the appearance of new foci of the disease around the country. As a fundamental problem, the disease has attracted an important part ofhealth and social avtivities, and imposesirreparable damages on the society with creating socio-econimic and phsychological problems[3].

Various studies have shown that the CL is increasing in Iran and the world.Also in recent years, the leishmaniasis treatment has faced with many problems due to appearance of resietance against the standard drugs which are mostly the pentavalent antimony compounds. The reports of physicians in attendance suggest recurrence, no improvement or disproportionate impact of drugs on patients so that Lamidie et al.' study on the patients who had been returned to Latin America Showed that in spite of special care andtreatment with sodium Stibogluconate, the rate of disease recurrence is atleast 25%. Meglumineantimoniate (glucantime) and Sodium stibogluconate (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, and now the appearance of resestent one is considered as one of the fundamental problems of treatment.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 agents intoxication andtheir side effect resistance even after improving dose and long term treatment are considered as their short coming. On the other hand, the treatments is not appropriate specially in rural areas due to expensiveness and non-accessability[4].

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. Different research strategies are used to obtain a medicinal herb or an isolated active compound. Different parts of herbs and solvents are generally used to extraction process.Various polarized solvents are used for extraction normally. Promastigotes and intracellular amastigotes of Leishmania can be used to screen for biological activity of herb material[5-8].

Recent studies on natural herb compound, quinolone anti-leishmania effects, alkaloids (such as capsaicin and skimmianine), isoquinoline alkaloids (such as limacine and isotetrandrine), flavonoids (such as Luteolinandfisetin), saponins (such as alpha-Hdryn), Naphthoquinone (Lapachol and plobmbagine), terpenes and Tetralens in some Leishmania species have shown that herbs with flavonoids, alkaloids and terpenoids contain have anti-inflammatory property[9].

Antileishmania drugs which have medicalresource, we can point out artemisinin. Artemisinin is a terpene lactone isolated from Artemisia annua which is known as anti-malaria and anti-Leishmania drug. Invitro, the drug has resulted in the suspension offer L. major strainparasite with changes in metabolites related to metabolic cycles such as galactose metabolic pathway, sphingolipid biosynthesis as well as the biosynthetic pathway of valine, leucine and isoleucine[2].

In a study aimed at investigating the activity of green tea extract in vitro against Leishmania major promastigotes in comparison to glucatium, promastigote parasites had been exposed to 6 different concentrations of the extracts. The positive control group were treated with 85 mg/ml of glucantime and the control group received no drug. The green tea extract showed significant anti leishmanial activity against parasite promastigotes in different concentrations and anti-parasite effect was being increased with increasing the dose of extract. Live promastigotes average in concentration of 12 mg/ml of green tea was nearly equal to the concentration of 85 mg/ml meglumine andhigher concentrations of green tea were more effective than glucatium. In the mentioned study, the parasites death was investigated only qualitatively and by counting the number of parasites[10].

In a research, the antileishmanial activity of mountain Ruta graveolens on the growth of promastigotesof L. major parasite compared with trivalent antimony called Potassium Antimunyl tartratewas investigated using MTT assay in vitro. Both the extract and antimony drug had inhibited the parasite growth in vitro after 72 hours. In fact, the power of both agents to inhibit the growth was almost the same, so that their powers were being greater by increasing the concentration. IC_{50} was equal to 72.89±65.1832 µg/ml for extract and 5.02 ± 87.17 µg/ml for antimony drug. As a result, regarding the antimony drug side effects, the extract of this herb can be used as the main agent against leishmania major in vitro[11].

In this study, the antileishmanial effect of extract of Medicago lupulina on promastigotes of L. major parasite (MRHO/IR/75/ER) has been evaluated by MTT assay.

Medicago lupulina

Baloch and colleagues examine the effects of antimicrobial, insecticidal, anti-tumor and estimating its phytochemical fractions of methanolic extract of leaves of black alfalfa and examined. They agar diffusion method was used. For this purpose various biological tests for methanol extract and its fractions containing chloroform fractions, fractions n- hexane, ethyl acetate fraction, fraction of n- butanol and aqueous fractions were conducted. Antibacterial activity against Staphylococcus aureus with well-shaped with a diameter equal to (29.02 ± 0.18 mm) while the chloroform fraction showed strong activity and of equal diameter (26.02 ± 0.04 mm) against the bacteria showed. The methanol extract of Candida albicans and Candida glabrata antifungal activity against fungi with equal diameter (36.02± 0.2 mm) and (42.16 ± 0.09 mm) showed. Good activity against Candida glabrata with chloroform fractions of equal diameter (32.03 ± 0.09 mm) showed. Strong bactericidal activity against insect methanol extracts against insect against insect Ryzopertha dominica and

Tribolium kastaneum to 86 percent and 75 percent showed. Higher strong cytotoxic activity against insect Tribolium kastaneum chloroform fractions 70 percent showed. Methanolic extract of the plant is a super anti-tumor activity demonstrated to the 89.40 percent. The estimates showed that the phytochemical extracts of this plant have flavonoids, alkaloids, phenols, tannins and diterpenes[13].

Figure 1. Schematic of the surface morphology of Medicago lupulina[14].

Regarding that the active ingredients of this plant includes substances such as alkaloids and flavonoids, and the anti-bacterial, anti-fungal and anti-cancer features of the ingredient have been proven, it is hoped that in the future- after the acquisition of appropriate responses against promastigotes of L. major parasite in vitro, then in vivo- the extract of Medicago lupulina can be used as an antileishmanial combination on lesions of patients with cutaneous leishmaniasis in the skin and CL research centers as well as skin and beauty clinics , in order to coincide wound healing with parasite inhibition.

MATERIALS AND METHODS

The study, has been done experimentally in laboratory and at Seddigheh Tahereh Infectious Disease Research Center of Isfahan, analysed and evaluated the antileishmanial effect of extract of Medicago lupulina on promastigotes of L. major (MRHO/IR/75/ER) by using MTT.

Medicago lupulina with herbarium code of 073/009/001 were collected in Fereidan on early March, 2015. According to the provisions contained in investigated articles regardinganti-ulcer and anti-microbial wonderful effects of leaves of the plant, this organ was harvested before flowering stage.

Feriedan city in the west of Isfahan are 130 kilometers away from the provincial capital with the geographical coordinates 49 degrees 52 minutes to 50 degrees 51 minutes east longitude and 32 degrees 32 minutes north latitude and 33 degrees 22 minutes is located. The center of the city, the city is rich. The city of mountainous areas and the average height of 2390 meters above sea level is the center of the city. Long-term average annual temperature of 5.9 degrees Celsius city, the average of the minimum and maximum temperatures respectively 2 and 17 ° C. The average annual rainfall of about 350 mm city. In the division on the proposed approach Karimi climate, this city is very wet, cool climates with cold winters and temperate semi-arid climate with cold winters classified.

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.

Alcololic extraction

Alcoholic extraction was also done in accordance to maceration method. To perform this procedure, chopped plant put in a glass container, and 50 ml of 80% ethanol was poured on it. The procedure conducted away from sun light in order to be safe against chemical changes caused by sun biochemical interactions; also the extraction container lid was closed tightly in order to prevent from solvent evaporation.

The extract was treated for 5 days on a shaker; The aim of this work is creating the balance in the concentration of solvent substances and the plant tissue. Then the resulting extract was purified by a syringe filter and the plant residue was pressed by a mangle. Finally, the extracts were mixed then kept at the temperature less than 15 °C in 5 days in order to deposit the sediments and turbidities, then purified with caution about solvent evaporation[15].

The Study of antileishmania effect of of extract of Medicago lupulina

The standard strain of L.major parasite (MRHO/IR/75/ER) obtained from parasitology labratoary of Medical Science Faculty of Isfahan University. A small amount of it was transferred to a flask containing 3cc Schneidergrowth medium, 10% fetal bovine serum and 15 ml Stoke Pen-Strap in the laboratory, and placed for 3 days in 25 °C. SchneiderGrowth medium plays the auxiliary role, and provides the parasite with physiological life conditions.

Initially the L.major parasites were kept in the Schneider growth medium within the thermos for 3 days. After that, slides were prepared to see the parasite growth rate. Then the animated Promastigotes were observed in low light using a light microscope, but their numbers were very small. Obserivg promastigotes suggested that the parasiteswereгrowing. Because the parasites will produce toxin if remain in the growth medium in long term, and food is also will being declined, they were transferred to another flask containing 3cc Schneider growth medium, 10% fetal bovine serum and 15 ml of Stoke Pn-Strap, was and kept at the temperature 25 °C. The glass strain was taken again after 3 days and the animated promastigotes were observed. Passage and taking slide continued every 3 days until rosette bodies were found. The existence of rosette under a microscope announces that the parasite has been reached to log growth phase. After that, promastigotes RPMI-1640 should be transferred to the growth medium in order to reach the mass cultivation.

The dried of extract was diluted at room temperature using water and DMSO. DMSO was used as emulsifier. Then in order to evaluate the impact of composed on the parasite, dilutions of 1600, 800, 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml were prepared through serial dilution using RPMI-1640 environment.

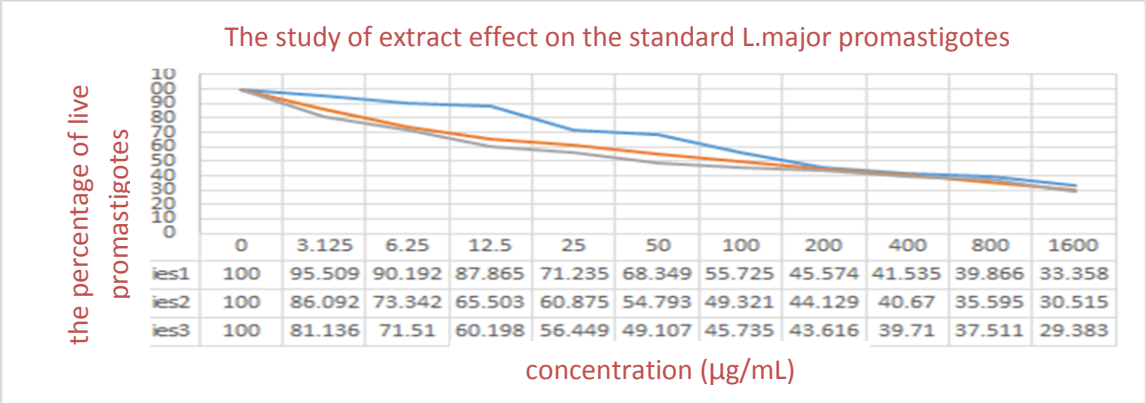
Glucantime is one of the reference drugs to kill the parasite,the necessary amount of which dissolved firstly in phosphate-buffered (PBS), and then the drug was diluted by preparing serial dilutions using the environment RPMI-1640 so that, 500 ml of Eppendorf was added to the second one at first, then the act continued until sixth one to perform the dilution. Like essences, 5 dilutions of 40, 20, 10, 5 and 2.5 µg/ml were prepared from this drug and then the extract dilutions and glucatium were passed through 0.22 micron syringe filter to be sterilized.

At first, 200 ml of a suspension containing the parasite was added to each well of the 96-well plate in the form of 2 million parasites per ml; after that, 40 ml of the of various dilution of extract and Glucantime were added in triple form to test –related wells (growth medium containing parasites) and growth medium without parasite, and the plate surface was closed with teflon, then incubated for 24, 48 and 72 hours at the temperature of 33-34 °C; Then 30 µl of MTT (0.5 mg/ml) reagents was added to each well containing the parasite promastigotes. The incubation time was 4 hours; after 1 hour the cells were examined by light microscop and invert so that the formazan crystals which formed in and tore the cell membrane are viewed under the microscope. The growth medium and MTT solution pulled gently by Puar Pasteur pipette so that the formazan crystals are not removed from the base of container.

After 4 hours of incubation at temperature of 26 °C, 100 ml solution of DMSO added to dissolve the formazan crystals, and the plate was incubated for 15 minutes in a dark room. Then the optical density of plate was investigated at a wave length of 630- 540 nm using Elisa Reader device.

RESALTS

The results of the antileishmanial effect of extract in 10 different concentrations (1600, 800, 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml) on standard Leishmania major promastigotes in vitro by MTT assay after 24, 48 and 72 hours have been presented in graph (1):



	0	3.125	6.25	12.5	25	50	100	200	400	800	1600
ies1	100	95.509	90.192	87.865	71.235	68.349	55.725	45.574	41.535	39.866	33.358
ies2	100	86.092	73.342	65.503	60.875	54.793	49.321	44.129	40.67	35.595	30.515
ies3	100	81.136	71.51	60.198	56.449	49.107	45.735	43.616	39.71	37.511	29.383

Figure 2.The calculation of IC_{50} of extract using the results of different concentrations effect on the standard L. major promastigotes invitro by MTT assay after 24, 48 and 72 hours (the blue graph: 24 hours, the red graph: 48 hours, the gray graph: 72 hours), (SPSS16 test).

According to gragh 1, IC50 for extract of plant against the standard L. major promastigotes was obtained in vitro after 24, 48, and 72 hours equal to 165, 98, and 45 µg /ml.

Glucantime drug was used as a control group to compare the effectiveness of extract on the standard L. major promastigotes in vitro by MTT assay after 24, 48 and 72 hours, and the results are presented in graph (2):

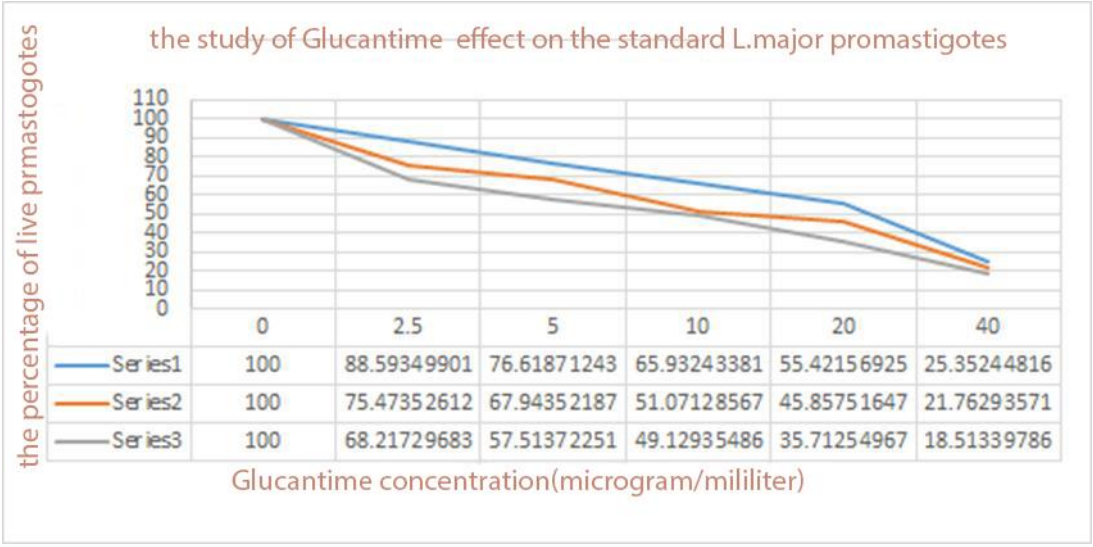


Figure 3. The study of IC_{50} of glucantime drug, as the control group, on the standard Leishmania promastigotes in vitro by MMT assay after 24,48, and 72 hours (the blue graph: 24 hours, the red graph: 48 hours, the gray graph: 72 hours), (SPSS16 test).

According to gragh 2, IC_{50} for glucantime drug against the standard L. major promastigotes was obtained in vitro after 24, 48, and 72 hours equal to 27, 12, and 8 µg /ml.

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[16,17]. The first line treatment drugs, are the Penta valent antimony compounds, none of them are without side effects[1,17,18,]. 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.Consequently, regarding the active compunds of each areanative herb, they can be considered as the source of antileishmania pharmaceutical agents[20].

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 essay. It was found in the study that garlic has a dose-dependent cytotoxic effect with almost 100% mortality in concentration of 93 µg/ml[4].

In one study, the assessment of antileishmanial activity of curcumin and its derivatives, indiumcurcumin, gallium curcumin and diacetyl curcum in 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 IC_{50} for curcumin, gallium carcumin, indium curcumin, diacetylcurcumin, and amphotericin B (control medicine) was calculated as 38, 32, 26 and 20 µg/ml respectively. Gallium carcumin and indiumcurcumin, with a lower IC_{50} compared to Diacetyl curcuminanalogue, were stronger factors against L. major promastigotes[3].

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 IC_{50} of the plant extract was 100 to 180 µg/ml. This data revealed that the Aloevera leaf can cause the better activity of thehostmacrophages through direct antileishmanial activity, andwe can use it as an effective antileishmanial agent in pharmaceutical researches[16].

A research group examined the antileishmanial effects of extracts of Zataria multiflra, Peganum harmala, Myrtle, and tartaratic control drugby MTT assay in vitro. The results were calculated as IC_{50} for each extract separately. It was obtained for the extraxts of Zataria multiflra, Peganum harmala, and Myrtle 5.8 µg/ml, 7.2 µg/ml, and 5.8 µg/ml respectively. Tartaratic IC_{50} amount was calculated 4.7µg/ml of myrtle extract. The myrtle extract with the minimum IC_{50} had a better effect compared to the other extracts. All of these extracts showed significant antileishmanial effects[11].

No study has been conducted so far to examin the effect of Medicago lupulina leaves extract on L. major parasite promastigotes growth. The case was studied in this project and according to results presented in graphes 1, it was observed that the extract has IC_{50} equal to 98 µg/ml against the standard L. major promastigotes after 48 hours. Glucantime drug was used as control drug in this study, and according to results presented in graphs 2, it was observed that it has IC_{50} equal to 12 µg/ml against the standard L. major promastigotes after 48 hours.

CONCLUSION

In this project, the antileishmanial effect of extract of Medicago lupulina was evaluated in vitro and it was found that they have relatively good antileishmanial effects.
However, the need for further tests to assess them on Leishmania parasite in animal models and human volunteers is felt, and this was the biggest limitation of the study because the main form of pathogenic parasites is intracellular (amastigote) one and these studies are required to be done in order to know extract of leaves of Medicago lupulina as antileshmania substance.

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