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The study of synergistic antileishmanial effect of *Portulaca oleracea* herb leaves and stems and *Medicago lupulina* leaves essence and alcoholic extract on a number of clinical strain of *Leishmania major in vitro*

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ABSTRACT:

Background and objective: Leishmaniasis 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. *Portulacaoleracea* and *Medicago lupulina*, the traditional and medicinal herb- are two valuable source of new Pharmaceutical agents.
Material and Methods: The essences were prepared through water distillation and alcoholic extracts were prepared through maceration method, then these were dried, and essences diluted with DMSO 5% and extracts 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 composed of essences and extracts were evaluated on *L.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} was 9 **µ**g/ml after 48 hours respectively. After 48 hours, IC_{50} for composed of essences and extracts was 80 **µ**g/ml against *Leishmania* promastigotes respectively. Although the glucantime pharmaceutical drug was more efficient compared to investigate composed of essences and extracts, the composed of them also had significantly effects on *leishmania major* promastigotes with higher density.
Conclusion: Regarding that the studied composed of essences and extracts 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*, *Portulaca oleracea*, Schneider Medium, *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 study, the chamomile and tarragon methanol extracts effect on *L. major* had been studied in vitro. Mentioned extracts had significant antileishmanial activity in different concentrations .

In a research, the antileishmanial activity of mountain rutagraveolens 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 composed of essences and extracts of *Medicago lupulina* and *Portulaca oleracea*, on promastigotes of *L. major* parasite (*MRHO/IR/75/ER*) and a number of clinical strains of the parasite have been evaluated by MTT assay.

Portulaca oleracea

According to the papers, the active ingredients of the herb contain oxalic acid, cinnamic acid, caffeic acid, maleic acid, citric acid, coumarins, flavonoids, alanine, tannin, alpha-linolenic acid, saponins, steroids, phenols, substances such as gum substance, jelly-like, oil, fats and menotropin Glycoside and it has been found that alkaloids are among the most important chemicals of theherb. Purslane is a rich source of antioxidants such as vitamins A, B1, B2, C, E , beta-carotene and other essential amino acids. It is also a rich source of minerals such as calcium, iron, phosphorus, copper and potassium has a significant amount of necessary linoleic acid (omega-3) among food sources. Linoleic acid is an essential fatty acid that the body is unable to synthesis and it should always be ingested with food[12]. More recently a flavonoid called apigeninhas been derived from the herb. Studies have shown that flavonoids hasanti-tumor features[13].

Dhol and et al. proved the anti-microbial effect of methanol and water extracts of herb leaves and roots on gram-positive bacteria *(Bacillus subtilis, Staphylococcus aureus*), gram-negative bacteria (*Pseudomonas aeruginosa*) bacteria and *Aspergillus niger*. Agar diffusion method was used in this test. Methanol and water extracts of the herb root at a concentration of 750 µg/ml had formed inhibition haloes with large diameters. In spite that the methanol and water extracts of leaves of the herb were less sucsessful compared to roots, they had formed inhibition haloes with more significant diameters[12].

In a test conducted by Landonkar et al., the antimicrobial effect of chloroform extract of the herb onbacterias such as *Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumoniae*, *Bacillus cereus* and *Aspergillus niger*, *Aspergillus fumigatus and Neurospora crassa* was *proved*[14].

Figure 1: Purslane

Medicago lupulina

Balvch 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[15].

Figure 2: *Medicago lupulina*

Regarding that the active ingredients of two plants 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 composed of essences and extracts 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 composed of extracts and essences of *Portulaca oleracea* and *Medicago lupulina* on promastigotes of *L. major* (a number of clinical strain) by using MTT.

Portulaca oleracea with herbarium code of 001/001/151 were collected in Ahvas arable lands and *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 and stems of the plants, these organs were harvested before flowering stage.

Ahvaz, capital of Khuzestan province, is one of the metropolises of Iran. The geographical locationofthe city- which located in the central part of Ahvaz county- is 31 degrees and 20 minutesof north latitude as well as 48 degrees and 40 minutes of east longitude in the plain part of Khuzestan and 18 meters above sea level.

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.

Stems and leaves of the plants 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.

Essence extraction

Extraction was done by distillation using Clevenger apparatus. For every time of extraction, 100 grams of considered plant' dry powder was extracted for 2 hours. 300 grams of the plant dry powder was provided and the extraction performed 3 times. At the end of each extraction, the essence formed as a separate layer on the water. Finally, the essence was transferred to small sterilized jar covered with aluminum foil to be safe from the sun. The essence was kept at the temperature of 4 °C until usage time.

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 extracts were 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[17].

The Study of antileishmania effect of of composed of extracts and essences

For preparing the clinical sample, first the lesion disinfected with 70% ethanol, and then a small cut was created on the leading edge of wound by a disposable surgical blade. Some amount of tissue along with cerussite removed from the lesion and expansion was made on glass slide. Smears prepared on glass slides dried in expose of air, fixed with methanol for a few minutes and stained with Giemsa for 20-30 minutes. The slides were studied preliminary under the microscope with lens of 40X, then tested with oil lens of 100X to see amastigote forms of *Leishmania*. Direct examination of amastigotes was positive. In this way, the samples were taken from the leading edge of the wound using the scalpel, and transferred to physiological salin, and the clinical samples transferred to biphasic NNN growth medium a few hours later. However, to ensure the full growth of the samples, a part of NNN growth medium and Physiological saline transferred to Schneider growth medium containing 10% fetal bovine serums.

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 parasitesweregrowing. 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 composed of essences and extracts were 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.

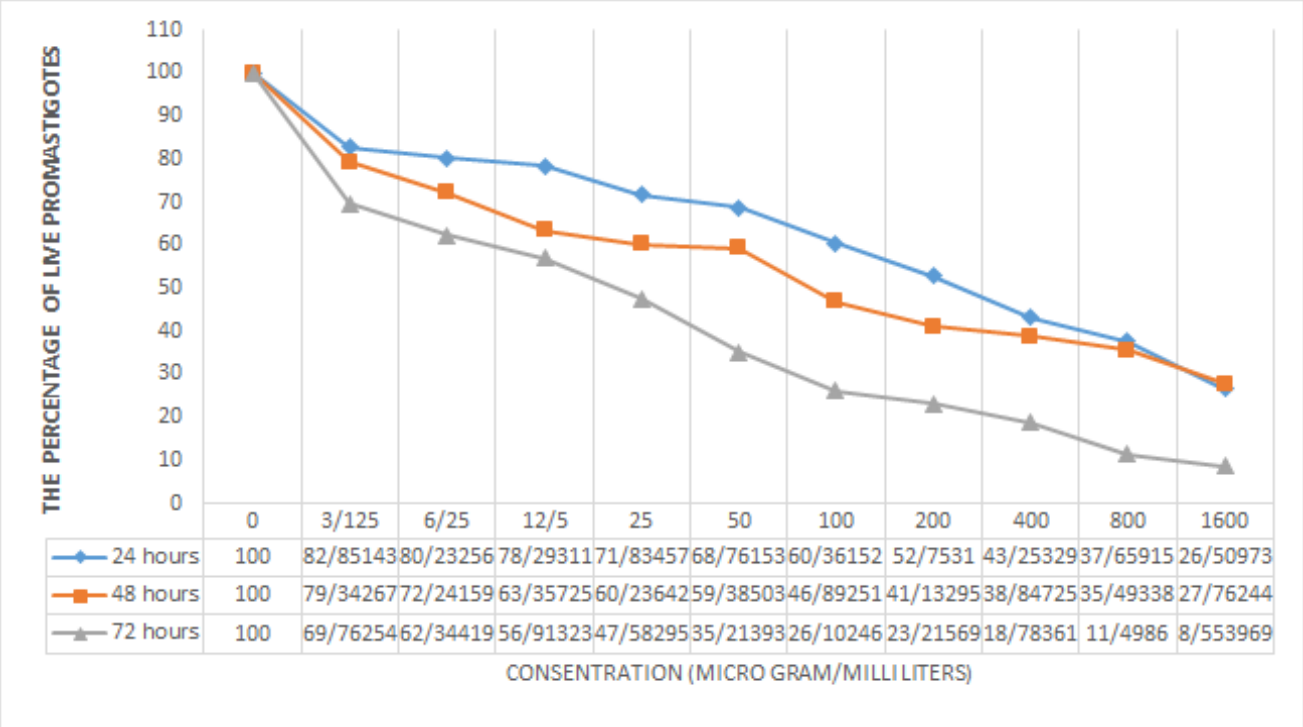
Glucantime is one of the reference drugs to kill the parasite,the necessary amount of which dissolved firstly in phosphate-buffered (PBS), and then the drug was diluted by preparing serial dilutions using the environment RPMI-1640 so that, 500 ml of Eppendorf was added to the second one at first, then 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 composed 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.

Results

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

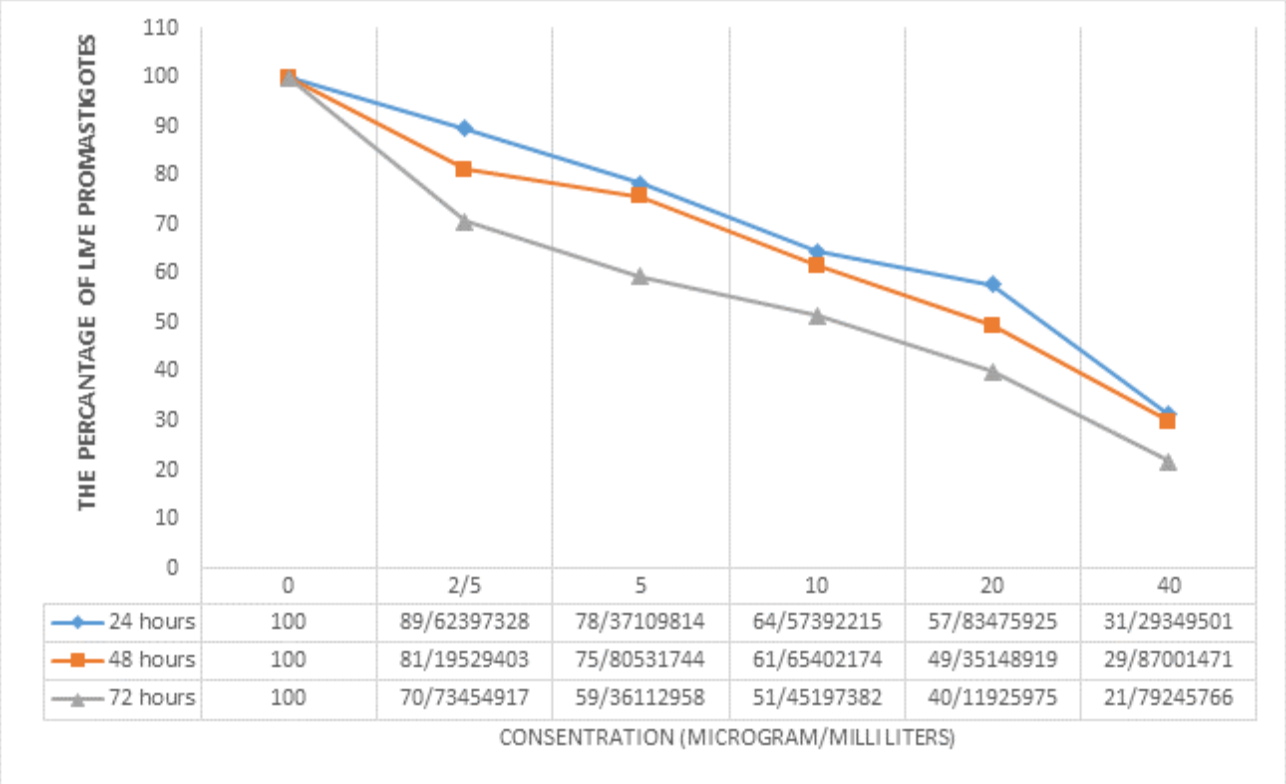


	0	3/125	6/25	12/5	25	50	100	200	400	800	1600
24 hours	100	82/85143	80/23256	78/29311	71/83457	68/76153	60/36152	52/7531	43/25329	37/65915	26/50973
48 hours	100	79/34267	72/24159	63/35725	60/23642	59/38503	46/89251	41/13295	38/84725	35/49338	27/76244
72 hours	100	69/76254	62/34419	56/91323	47/58295	35/21393	26/10246	23/21569	18/78361	11/4986	8/553969

Figure 1: The calculation of IC_{50} of composed of essences and extracts using the results of its different concentrations effect on the *L. major* promastigotes *in vitro* by MTT assay after 24, 48 and 72 hours (the blue graph: 24 hours, the red graph: 48 hours,the gray graph: 72 hours).

According to Fig 1, IC_{50} for plants composed against the *L. major* promastigotes *in vitro* after 24, 48 and 72 hours, was calculated as 260, 80, and 25 µl/ml respectively.

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



	0	2/5	5	10	20	40
24 hours	100	89/62397328	78/37109814	64/57392215	57/83475925	31/29349501
48 hours	100	81/19529403	75/80531744	61/65402174	49/35148919	29/87001471
72 hours	100	70/73454917	59/36112958	51/45197382	40/11925975	21/79245766

Figure 2: The study of IC_{50} of glucantime drug, as the control group, on the *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).

According to Figure 2, IC_{50} for glucantime drug against the *L.major* promastigotes was obtained *in vitro* after 24, 48, and 72 hours equal to 26, 19 and 11 **µ**g /ml respectively.

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[18,19]. The first line treatment drugs, are the Penta valent antimony compounds, none of them are without side effects[1,19,20]. Their effects include the toxicity and sustain ability 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[21].

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[17].

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 **µ**g/ml, 7.2 µg/ml, and 5.8 µg/ml respectively. Tartaratic IC_{50} amount was calculated 4.7**µ**g/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 purslane arial organs and *Medicago lupulina* leaves essence and extracts on *L. major* parasite promastigotes growth. The case was studied in this project and according to results presented in grap 1, it was observed that the purslane arial organs and *Medicago lupulina* leaves essence and extracts have IC_{50} 680 **µ**g/ml against the clinilal strain of the parasite after 48 hours. Glucantime drug was used as control drug in this study, and according to results presented in graph 2, it was observed that it has IC_{50} 19 **µ** g/ml against the clinilal strain of thr parasite after 48 hours.

Conclusion

In this project, the antileishmanial effect of composed of extracts and essences were 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 composed of extracts and essences of twon plants as antileshmania substance.

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