

The activity of (Allium sativum and Peganum harmalla) plant extracts on the promastigote and amastigote in vitro stages of leishmania major

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Abstract

The effect of two aqueous extracts Bulbs of Allium sativum (Lillaceae) and seeds of Peganum harmala (Zygophyllacea) was studied against the invitro activity and growth of both Promastigotes and axenic amastigotes of leishmania (Leishmania magor) strain which was isolated from Iraqi patient infected with leishmania parasite. The results of the study showed variation between the effect of the aqueous extract of Bulbs of Allium sativum and seeds of Peganum harmala as inhibiting activity of the growth of Leishmania magor in both promastigote and axenic amastigote stages. The values of the median effective dose (ED50) had shown that the promastigote are more resistance to high concentration of plant extract, While the axenic amastigote were more sensitive to the lower concentration of these plant extracts mentioned above. This was provided by the statistic analysis that showed significant differences at ($P < 0.01$) between Promastigote and axenic amastigote for the different plant extracts.

Key Word: Leishmania major, treatment, plant extracts

Introduction

Leishmaniasis is considered as an important disease of man since long time ago. It is caused by the intracellular parasite called Leishmania which produce a long term medical consequence in the body of the patient or even the death of the victim.

The distribution of this disease is usually occurring in large areas of the tropical and subtropical regions depending on the convenient environmental conditions⁽¹⁾. More over the distribution of this disease is depending on the availability of the infected female sand fly insect in the different areas. Therefore; the geographical distribution of this insect is considered as a factor of the distribution or limitation of this disease.

Recently the disease Leishmaniasis was distributed widely in spite of the presence of a large number of the control centers every where. This was of course , due to the environmental changes made by man in addition to the elevation of the level of the immune-deficient diseases

such as (HIV), which contributing in the transmission of this disease in different parts of the world⁽²⁾.

In addition, the disease caused by this parasite producing a big and general health problems due to the ability of the fast distribution which causes a dangerous ecological consequences⁽³⁾.

The statistical results of WHO, during the last years, revealed that (350) million persons are exposing to the infection each year⁽⁴⁾, and more than 12 millions were already infected with this disease with a possibility of infection of another two more million persons each year⁽⁵⁾. It has been found that cutaneous Leishmaniasis disease more dangerous than the other clinical types as it is causing infection of about (1 – 1.5) million persons or about (50 – 70%) of the total new infections⁽⁶⁾.

For the treatment of this disease, the pentavalent antimony compounds as (Pentostam and Glucantim) are considered the best drugs for treatment and first drugs of choice. While

Pentamidin and Amphotericin B are of a second choice drugs⁽⁷⁾.

The treatment with these drugs is not always of clear effect on the parasite, but some times, they produce dangerous side effects as the heart and kidney failure⁽⁸⁾, more over, these drugs are not producing complete cure, but rather they are only causing the disappearance of the symptoms of this disease and might be the reappearance of the parasite in the body later on specially in those with immuno- deficient diseases.

Therefore; in a hope that some other sources of treatment are used as alternative compounds, these suggested compounds are plant extracts⁽⁹⁾. These plant extracts have different chemical compound that have direct effect on the parasite with less side effects compared to the chemical drugs⁽¹⁰⁾.

Accordingly, this work concentrated on the finding of some aqueous plant extract from plants that are cheap, available in Iraq such as P.harmala, and A. sativium which have efficient extract against the parasite with little toxicity and strong affectivity. Therefore; the objectives of this work is as follow:

1. The efficiency of the above mentioned plant extract on the density , growth index %GI% of the promastigote and amastigote stages (Axenic amastigote) stages of leshmania major strain.
2. Calculation of the (ED 50) medium effect dose of the plant extract on the promastigote and the amastigote (Axenic amastigote) stages of this parasite.
3. Counting the generation time to know the effect of these plant extracts on the time of division of this parasite to promastigote and amastigote stages.

Materials and Methods

Type and source of parasite: Leishmania Major Strain was isolated and diagnosed from patients who were attended Al Nahrain Teaching Hospital, Al-Nahrain University, and Baghdad. The isolate was (MHOM/IQ/93/MRC5) and diagnosed in the unite of Leishmania, Medical Research Centre, Al-Nahrain Medical College. For the maintenance of the virulency of the parasite. They were injected to experimental animals (Balb/C) mice each two months and then cultivated on a semi solid media each 21 days.

The cultivating media: Semi Solid Media: This is usually used for the routine maintenance of the parasite to get new active dose and also used for the subculturing. It is the most convenient media for this parasite (11).

- a. Biphasic medium Nicole-The Nove-MacNeal (NNN).

This phase was discovered long time ago and still till now used to produce large number of parasites (12). It is consisting of the following two phases:

1. Solid phase (13) and 2. Liquid phase or called (Locks solution) (14).

- b. RBLM (liquid culture media). This is a modified media (15).

In vitro cultivation of parasites:

- a. cultivation of promastigote: Inoculum of the parasite in 5-10 ml of the semi solid medium is transferred to a Biphasic media for the cultivation. These are incubated at 26C for 7-10 days to get high density of parasites. 5-15 ml of the Biphasic media is transferred to sterile containers containing 10 ml of the liquid media at 25C and incubated in a shaker incubater for 24 hrs then examined each 2 days (15).
- b. Cultivation of the amastigote (Axenic amastigote): The amastigote

stage is cultivated by the same procedure mentioned above for the promastigote except by the changing of the PH to be (2, 5), under 37C with the presence of 5% CO₂ (15).

Stains used:

- a. Trypan blue stain (16).
- b. Erythrocin B stain (17).

Collection and Diagnosis of plants:

- a. *Allium sativum*: collected from the local market. These were cleaned and used as fresh samples.
- b. *Peganum harmalla*: Seeds of *P.harmalla* were collected from Rabiah-Ninava, cleaned and grinded with blinder and stored at 4C. These plants were diagnosed by Prof. Ali Al mosawy/ University of Baghdad.

Preparation of *Allium sativum* extract: The method of Al-saedi (18) was used for this purpose with a few modifications. The filtered solution was collected in a container and considered as a stock solution of concentration of 500 mg/ml. A series of concentrations of (31.25, 6.25, 1.25, 0.25, 0.05, 0.01) mg/ml were prepared from the stock and sterilization was then performed and then kept at 4C until used.

Preparation of *P.harmalla* seeds extracts: One hundred (100) gms of powder were mixed with 250 ml distilled water, mixed well and filtered. The filtered solution was lyophilized and the stock solution was prepared by adding 2.5 gm of the lyophilized materials to 5 ml of the liquid medium to become a final concentration (500) mg/ml then concentrations of (0.4, 4, 40, 400) mg/ml were prepared and kept till the time of experimental work.

Tests of activity of plant extracts on the viability of *L. magor* isolate (promastigote and amastigote in vitro stages).

a. Trypan blue stain: (0.4%) was used for the promastigote stage (16).

b. Erythrocin B: For the amastigote stage (17). The same steps of test were used for the promastigote and the amastigote stages except the use of 25C for the promastigote and 37C for the amastigote with addition of 5% CO₂. Neubauer heamocytometer was also used for the counting of the number of cells.

The total $N=N \times 10 \times 1000 \times 10$, N = number of counted cells, 10 = number of cells in one cubic mm, 1000 = number of cells in one ml, 10 = dilution index. A control samples were also cultivated without the addition of extracts.

Calculation of the generation time:

This was performed according to(19).

$$N=3.33(\log N-\log N_0) = t/n$$

N = number of generation, $\log N$ = Final number, $\log N_0$ = The first number, G = generation time, t = time hrs.

Calculation of the Median Effective Dose (ED₅₀): For this purpose, (ED₅₀) was calculated according to Healy, 1988 (20).

Results

In this study, the activity of plant extracts against the promastigote and amastigote invitro stages of *L. magor* was used .The following parameters (Generation time, GI% and ED₅₀) were used for the estimation of the activities .To avoid the toxic doses, a series of experiments was conducted to find the lower toxic doses of both plant extracts on the peritoneal macrophages .The results were 40 mg/ml for *Allium sativum* and 500 mg/ml for *Peganum harmala*.

The effect of Allium sativum extract:

A: on the promastigote stage: The results revealed the inhabiting effect of *A. sativum* extract on the promastigote stage of *L. major* after 5 days of the experiment table (1).

It has been revealed that in the control group, the density of the isolate was (13.8 x 1000000) cells/ml compared to (zero) with higher concentration (31.25)mg/ml and (4.4x10) cell/ml with the lower concentration (0.05)mg/ml. Also the clear decrease of (GI%) which declined from (100%) with (zero) time and concentration to become after 5 days (3.8%) with the highest concentration(31.25)mg/ml. Moreover, the value of (ED50) after 3 days became (0.09)mg/ml.

B. On the amastigote in vitro stage: The results revealed the direct and clear decrease of the total density of the amastigote In vitro within 3days of the cultivation at 37C under 5% CO₂ and the use of different concentrations of *A. sativum*(0.01, 0.1, 1) mg/ml. These concentrations affected directly the time of reproduction which lasted 15.3 hrs for the control group, while it was 26.3 hrs with the smallest concentration 0.01 mg/ml. Therefore, this was reflected on the total density of the parasites which was (zero) cells/ml with the highest concentration and (16.9x10) cell/ml with the lowest concentration compared to the control group which became after 3 days (36.3x10) cells/ml as it illustrated in table(2). These results revealed the sharp decrease of the growth index during the 3 days of the exposure of the amastigote to different concentrations of *A. sativum* which become (zero) with the highest concentration. Also, the value of the (ED50) after the 3 days became 0.009) mg/ml.

A. on the promastigats: The use of the different concentrate of *P. harma* water extracts (0.4, 4, and 40,400) mg/ml caused a clear decrease in the number of

the parasites after 5 days compared to the control group table (3) as the total density of the parasites in the control group was (24.8x10) cells/ml. While the total density of the highest concentrations (6.24x10)ccls/ml in addition to the decrease of the total density of the parasites with the increase of the concentration of water extract of the plant. The generation time increased from (26.1) hrs in the control group to become after 5 days (25.7%) with the highest concentration (400) mg/ml and (92.3%) with the lowest concentration. The (ED50) value was after 3 days of exposure to the water extract of *P. harmalla* (5.4) mg/ml.

The results revealed the decrease of amastigote density within 3 days of the incubation at (37C) with 5% CO₂. This density was (13.2)x10) cells/ml in the control group to become (6.39x10) cells/ml with (40) mg/ml the highest concentration and (8.89x10)cells/ml with (0.4) mg/ml lowest concentration, table(4).

Discussion

From the above results, it could be concluded that *A. sativum* extracts has strong effect on the growth and reproduction of the amastigote stage of *L. major*, as the low concentrations killed 50% of the parasites. Also there was a clear statistical differences between the efficiency of the extracts on the promastigote and the amastigote in vitro stages at $P < 0.05$. These results are similar to the results of other studies which confirmed the significant differences of effect of chemical compounds on the promastigote and amastigote In vitro stages(20, 21, 22, 23)..

The high value of ED50 for the promastigote stage compared to that of the amastigote in vitro stage ,confirmed that the promastigote was more resistance to the extracts of *A. sativum* than the amastigote in vitro .This is also in

agreement with the results of (24 and 25) which confirmed that the absorption of the chemical compounds is different from phase to phase of leishmania. The effect of *A. sativum* extracts is not known precisely on both promastigote and amastigote in vitro stages, although workers mentioned that *A. sativum* extracts containing some effective chemical compounds and mineral salts (26,27,28). The compound (Allicin) as a liquid containing (Thiosulfinate) group considered to be very important compound which has strong effect on the different microbes as on virus (27). These compound also inhibiting (acetyl – coA) enzyme, which preventing the formation of fatty acids and sterols which leads finally to the destruction of the cell wall (28) and proved by (29) when studied the effect of (Allicin) on candida albicans

It has been noticed the clear decrease of the viability of the parasites, indicated by the decline of (GI%) from (100%) at (zero) time to become (49%) with high concentration (40)mg/ml and (67.7%) with the lowest concentration (0.4)mg/ml. Moreover, the value of (ED50) after 3 days of exposure was (6)mg/ml. There was a significant differences at $P < 0.01$ between the effect of *P. harmalla* extracts efficiency on the promastigote and amastigote stages, as the promastigote stage was more resistant than the amastigote In vitro stage, which was more sensitive. This might be due to the presence of some alkaloid materials in the seeds of *P. harmalla* such as (Harmine, Harmactine, Deoxy Vascine, Vascine, Harmalol, Harmol, Harmane (24,25). Also glycoside, Flafonate, Sabonines, Tynins and Amino acids and Fats (26).

The activity of water extracts of *P. harmalla* is attributed to the reason that it contained Indol alkaloid which affect directly the micro-organism (27). Moreover the Indol alkaloid which extracted from *Pescliera australis* has

significant effect on both promastigote and amastigote in vitro of *L. amazonensis* (28). The Indole compounds have also direct effect on the mitochondria, consequently the effect on the metabolic activity of carbohydrates and the effect of respiration and death of the parasites. Also Harmine compound may interfere with the production of (DNA) of cells of protozoa as *Trypanosoma* and *Plasmodium* (29). Lamchouri, et al., (30) noticed that Harmalol prevented the cancer cells through the inhibition of (DNA) synthesization and prevention of cell division

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Table (1) The effect of Various concentration of *Allium Sativum* on the In vitro culture of *L. major* promastigote

Days after Plant extract exposure	Total No. of Parasite cells / ml (x 10 ⁶)					
	plant extract concentration (mg/ml)					
	31.25	6.25	1.25	0.25	0.05	Control
Day 1	0.1 ± 0	0.78 ± 0.49	1.5 ± 0.81	1.63 ± 0.49	2 ± 0.17	2.3 ± 1.8
Day 2	0.03 ± 0.1	0.7 ± 0.55	1.9 ± 0.42	2.55 ± 0.44	2.9 ± 0.39	4 ± 0
Day 3	Zero	0.68 ± 0.62	1.7 ± 0.62	2.6 ± 0.55	3.3 ± 0.44	6.6 ± 2.12
Day 4	Zero	0.73 ± 0.5	1.85 ± 0.64	3.3 ± 0.63	3.6 ± 0.4	8.5 ± 2.8
Day 5	Zero	0.68 ± 0.62	2 ± 0.82	3.2 ± 0.59	4.4 ± 0.57	13.8 ± 8.8

Table (2) the effect of various concentrations of *Allium sativum* on the in vitro culture of *L. major* A. amastigote

Days after Plant extract exposure	Total No. of Parasite cells / ml (x 10 ⁶)			
	plant extract concentration (mg/ml)			
	1	0.1	0.01	Control
Day 1	2.13 ± 0.42	2.5 ± 1	2.93 ± 0.9	4 ± 1
Day 2	7.3 ± 0.91	9.27 ± 0.35	10.9 ± 0.53	15.9 ± 5.2
Day 3	Zero	9.9 ± 1.28	16.9 ± 6.1	36.3 ± 5.5

Table (3) The effect of Various concentration of *Peganum harmala* on the In vitro culture of *L. major* promastigote

Days after Plant extract exposure	Total No. of Parasite cells / ml(x 10 ⁶)				
	plant extract concentration (mg/ml)				
	400	40	4	0.4	0 Control
Day 1	2.3 ± 1.1	2.63 ± 0.36	2.76 ± 0.49	2.9 ± 0.36	2.9 ± 0.36
Day 2	5.1 ± 1.39	5.99 ± 1.62	6.31 ± 1.55	6.91 ± 1.9	7 ± 1.77
Day 3	7.71 ± 1.02	9.2 ± 1.34	10.37 ± 1.9	11.64 ± 1.69	12.2 ± 1.64
Day 4	11.2 ± 1.2	14.2 ± 2.1	18.5 ± 2.9	21.2 ± 2.69	22.5 ± 2.2
Day 5	6.24 ± 1.34	13.4 ± 2.91	18.6 ± 1.20	22.9 ± 2.3	24.8 ± 2.4

Table (4) The effect of various concentration of *Peganum harmala* on the In vitro culture of *L. major* axenic amastigote

Days after Plant extract exposure	Total No. of Parasite cells / ml (x 10 ⁶)			
	plant extract concentration (mg/ml)			
	40	4	0.4	0 Control
Day 1	2.6 ± 0.86	2.83 ± 1.01	3.15 ± 1.15	3.4 ± 1.25
Day 2	5.44 ± 1.75	5.99 ± 1.36	6.65 ± 0.88	7.87 ± 1.40
Day 3	6.39 ± 0.9	7.87 ± 1.49	8.89 ± 0.72	13.2 ± 1.25