The effect of aqueous and alcoholic extracts of fruit pell of *Punica granutum* against *Leishmania major* in *vitro* and *in vivo*

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الخلاصة

تم دراسة تاثير المستخلص المائي والكحولي لنبات قشور الرمان على نمو وفعالية الطور المسوط لطفيلي*Leishmania major* خارج جسم الكائن الحي، تم استخدام المستخلص الكحولي موضعيا على الحيوانات المصابه بطفيلي اللشمانيا . اظهر المستخلص الكحولي فعاليه عاليه ضد الطور المسوط اكثر من المستخلص المائي .

Abstract

The effects of aqueous and alcoholic extract of *Punica granatum*, were studied against the activity and growth of promastigote of *Leishmania major in vitro*. The alcoholic extract was used by topical route against BALB/c mice infected with *L.major.*

The alcoholic extract showed high activity against *L.major* promastigote than aqueous extract.

Introduction:

Leishmania are protozoan parasites belonging to the family trypanosomatidae, genus *Leishmania* (Belding, 1965). About 22 *Leishmania spp.* are known to be pathogenic for human (Marquardt, *et al*., 2000).

Cutaneous leishmaniasis is an endemic and prevalent disease in Iraq particularly in Baghdad and is apparently on the increase (Rahim & Tatar, 1966). it is wide disseminated in different regions of world especially in tropical and subtropical regions (Goldman & Bennett, 2000).

Key word: *Leishmania major;* cutaneous Leishmaniasis; *Punica granutum;* plant extract.

Resistance of Leishmania parasites against conventional drugs like pentavalent antimony derivatives indicated the need of new treatment and prophylatic protocol (Balcioglu, 1997). The development of a new drug for the treatment of leishmaniasis has been impeded by the lack of a simple rapid drug-evaluation system that is universally applicable to the various *Leishmania* species/ strain (WHO, 1990).

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial properties (Cowan, 1999). Tradition medicine has been practiced to some degree in all cultures and other terms based on culture include Africa, Asia or Chinese medicine (Al-Rahbi, 2000).

Punica granatum belongs to family punicaceae. Fruit pell contains tannin, mucilage, resin and sugar while ripe fruit contains invert sugar, enzyme, malice acid, citric acid (Al-Rawi & Chakravarty, 1988). The main economic used of *punica granatum* is for food, fruits and pells are used for medicine purposes and tannage (Chakravarty, 1976).

Al-Bashir study the effect of gallotannic and gallic acid (phenolic substances) isolated from fruit pell of *p.granatum* against promastigote and axenic amastigote of three species of *Leishmania* (*L.major*, *L.tropica* and *L.donovani*) (Al-Bashir, *et al*., 2000) .

Successive petroleum ether, chloroform, methanol and water extracts of *punica granatum* were tested *in vitro* for their antibacterial activity. The methanolic extract was found to be most effective against all tested microorganisms (Prachanth, *et al*., 2001).

Materials and methods:

Sample source and culture media

The strain of cutaneous leishmaniasis, which used in the present study was clinically identified as *Leishmania major*. This strain was isolated from female patient aged 35 years by specialized clinic of dermatology belong to dermatologists Dr. Kathem K. Al-Rubiay.

Diphasic (NNN) medium was used in this study. The diphasic medium is made of 2 phases a solid and a liquid phase

(Kagan and Norman, 1970; Meredith, *et al*., 1995). The promastigotes were cultivated in diphasic media at (26-28)°C then harvested on the 6th day either for animals infection or for sub-culturing in new media. The number of promastigotes per ml was determined by counting in hemocytometer and adjusted to $1x10^7$/ 0.1ml for inoculation. (Hazra, *et al*., 1989). BALB/c mice, 8-10 weeks old with a body weight of approximately 20-25 gm, were used in this study.

Infection of mice with *L. major* Promastigote:-

Group I: Each mouse in this group was inoculated with a dose $1x10^7$/ 0.1 ml of promastigotes at the hind footpad (each footpad received 0.05ml).

Group II: Each mouse in this group was inoculated subcutaneously with a dose $1x10^7$/ 0.1 ml promastigotes in a shaved area above the tail.

Post infection:-

Group I:Mice in this group were used at 4 weeks post-infection for the treatment study. Mice with 4mm. footpad thickness were selected and the amastigotes were checked in stained smears.

Group II: Mice in this group were used for the treatment study after 8 weeks post-infection with *L.major* promastigote when the ulcerative lesions were appeared, ulcer size ranged (5x5 – 7x10) mm. in diameter and amastigotes were seen in smear.

Plant extraction:

The aqueous and alcoholic extract of fruit pell of *punica granatum* were prepared according to (Harborne, 1984; WHO, 1998), different plant concentrations of aqueous and alcoholoc extracts were prepared and tested (2,4,6,8)mg/ml were selected and used *in vitro* for aqueous and alcoholic extract of plant according to the viability of promastigote.

In vitro effect of *punica granatum* on promastigote of *L. major*:-

1- Different concentration of plant extracts were used.
2- These solutions were sterilized by filtration through a sterile filter paper (0.45 micron) in diameter with sterile filter unit and this was made by using vacum pump.
3- The screw-capped vials were prepared containing 5ml of solid phase medium were prepared.
4- 0.9ml of the liquid phase medium (Lock's solution) which contain different concentration of plant extracts were added to screw-capped vials.
5- Each concentration was done in triplicate.
6- Other vials of diphasic medium were kept as the vehicle control without drug. It was done also in triplicate.
7- On the day of experiment, when the promastigotes were at the logarithmic growth phase. It were adjusted to $1x10^6$ cell / 0.1ml of Lock's solution and added to each vial in step 4.
8- The vials were then incubated at (25-26)°C.
9- The parasites were counted once daily for the following 4 days.
10-A 1:20 dilution in (PBS) together with the trypan blue stain (promastigote permeable to the blue dye are dead while viable ones exclude the dye).
11-The number of a live and death cells was estimated in each of the experimental vials, compared to the number of a live cells of control vials.
12-The chamber of Neubauer haemacytometer is charged and the number of organisms in 16 small corner square are counted. The total number per ml = N x 10 x 1000 x 20

N = No. of cell counted.
10 = No. of cell in lmm^3.
1000= No. of cell in lml.
20= dilution factor.

13-The percentage of growth index GI% was calculated as follow: (Al-Bashir, *et al*., 2000)

N

$$GI = \frac{\text{N}}{\text{No.}} \times 100$$

N = No. of treated promastigote.

No.= No. of untreated (control) promastigote.

14-50% lethal dose LD_{50} values were calculated by linear regression analysis (Healy, 1988).

In vivo effect of *P. granatum* extract:-

Preparation

10gm powder of alcoholic extract of *Punica granatum* dissolved in 100ml of distilled water and then applied locally as losion using dropper.

A- On foot pad thickness of mice:-

were applied locally on the thickness footpad of infected mice started at the 4^{th} weeks post infection and continued for 4 weeks. Footpad thickness was monitored by measuring with Caliper at weekly intervals compare with control infected mice.

B- On the bace mice tail ulcer

Daily topical treatment with alcoholic extract of *P. granatum* as losion were applied locally on the ulcers for 30 consecutive days and their effects on ulcerative lesions was assessed by the followings:

A- Clinically:

The cure for the ulcers was defined as clinical improvement according to the reduction in infiltration cells, erythema and size of the lesions compared with untreated control mice.

B- Parasitology:

Smears were made from cutaneous lesions; stained with leishman stain and examined under oil immersion, the density of the parasite was observed and recorded in 50 fields.

Statistical analysis:

One way analysis of variance and Least Significant Differences were used in the present study (Hill, 1988).

Results:

In vitro effect of aqueous and alcoholic extract of *P. granatum*

The morphology of promastigote as seen by light microscope showed that the treated promastigote became smaller and rounded in size, slow and loss of motility as compared to the normal spindal shaped flagellated promastigote. These changes were more evident at higher concentration of each extract.

A slight difference was found in the sensitivity of *L.major* promastigote to aqueous and alcoholic extract of fruit pell of *P.granatum*.

Tables (1) and (2) show an increase in the number of parasite cells of untreated control group in comparison with a decrease in number of treated group for aqueous and alcoholic extract, during 4 days of plant extract exposure.

Table (1) The effects of various concentrations of aqueous extract of *P. granatum* on the L. major promastigote *in vitro*.

	Total No. of parasite cells / ml (x 10^6)				
	Plant extract concentrations (mg/ml)				
Days after plant extract exposure	0 Control	8	6	4	2
1	5 ± 1.414	2.1 ± 0.141	3.6 ± 0.848	4.2 ± 0.848	4.7 ± 1.838
2	9.8 ± 1.131	3 ±0.565	6 ±0.565	7.9 ± 0.707	8.7 ± 0.141
3	15 ± 0.282	3.8 ± 1.131	8.8 ± 1.414	11±0.565	12 ±0.141
4	19.4 ± 0.848	4.1 ± 0.424	10 ±2.828	13.3 ± 1.555	14 ± 0.282

Data are presented as (mean ± SD) from 3 experiments.

Table (2) The effects of various concentration of alcoholic extract of *P.granatum* on the L. major promastigote in vitro.

	Total No. of parasite cells / ml (x 10^6)				
	Plant extract concentrations (mg/ml)				
Days after plant extract exposure	0 Control	8	6	4	2
1	4.9± 0.989	2.4± 0.565	2.9± 1.272	3.8± 0.282	4.6± 0.848
2	7.8± 0.282	3 ±0.848	3.5± 0.989	5 ±1.131	6.7± 0.989
3	11 ±1.697	3.9± 0.707	4.5± 0.707	6.2± 0.565	9 ±1.979
4	18 ±2.262	5 ±0.848	6.5± 2.121	9 ±1.697	13 ±1.414

Data are presented as (mean ± SD) from 3 experiments.

It was found the LD_{50} of aqueous extract was (5.956) mg/ml while for alcoholic extract on day 4 was (4) mg/ml.

The results shown in Figures (1) and (2) demonstrated that the (GI)% was decreased with increased concentration of this extract from 100% at zero time and zero concentration to (21.1) for aqueous extract and (27.7) for alcoholic extract on day 4 of experiment.

There is no significant differences among different concentration of each extract (aqueous and alcoholic). The plant concentrations can be statistically arranged according to a highly antileishmanial activity as follow ($P<0.05$):-

8 mg/ml ≥ 6 mg/ml ≥ 4 mg/ml ≥ 2 mg/ml for aqueous extract

8 mg/ml ≥ 6 mg/ml ≥ 4 mg/ml ≥ 2 mg/ml for alcoholic extract

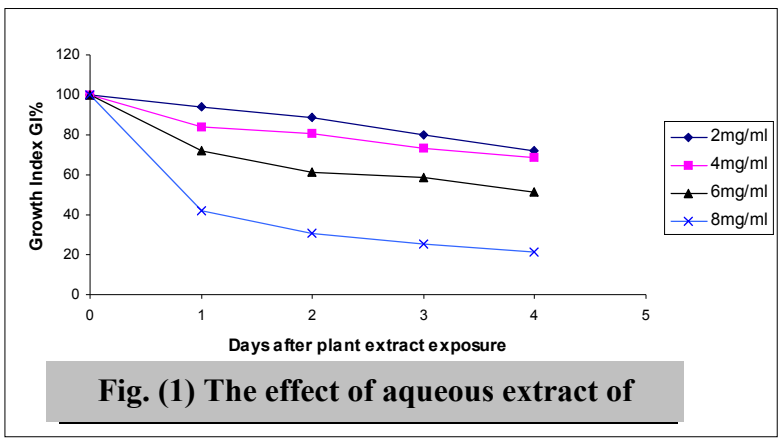


Fig. (1) The effect of aqueous extract of

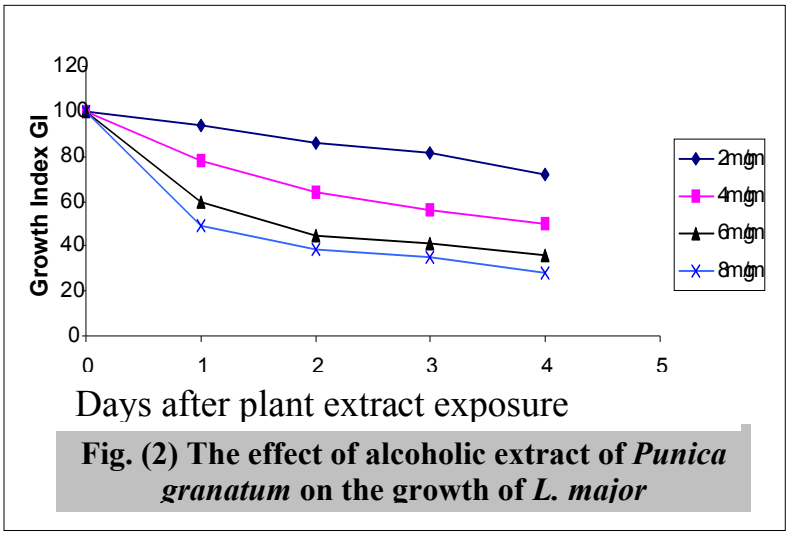


Fig. (2) The effect of alcoholic extract of *Punica granatum* on the growth of *L. major*

In vivo effect of *P. granatum*

A- on footpad thickness:

Table (3) summarized the effects of alcoholic extract of *P. granatum* on footpad thickness during 4 weeks .

The mean thickness of the footpad of the infected treated mice with alcoholic extract of *P. granatum* was significantly lower than infected untreated control.

Table (3) The effects of plant extracts on mice footpad thickness.

Weeks after infection	*P.granatum* (alcoholic)	Control
1	3.9± 0.1	4.175± 0.095
2	3.55± 0.212	4.3± 0.115
3	3.1± 0.141	4.575± 0.170
4	2.9± 0.141	4.75± 0.129

Data are presented as (mean±SD)

Statistical analysis data of mean thickness of the footpad on week 4 of experiment revealed highly significant differences ($P<0.01$) between various treated and control groups.

B- on the base mice tail ulcer

1- Clinically

After topical treatment with previous plant extracts, lesions usually showed a good response to these extracts and improvement signs such a response was usually noticed after 30 days post treatment with different levels according to the type of plant extract.

The swelling, redness and erythema started to decrease at the end of experiment. Flattening of the plaques and decrease in the inflammation, drying up of lesions, reduction and decrease in size of the treated ulcer. In untreated control mice, the ulcer was worsening, more in the cellular infiltration and increased in ulcer size. Table (4) .

Figure (3) showed the picture of the mice treated with *P. granatum* (alcoholic extract)

2- Parasitology:

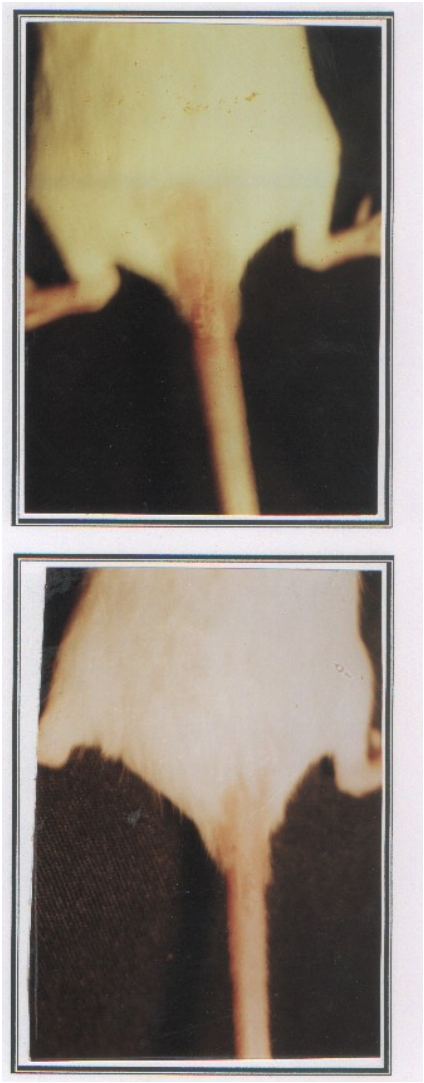
Parasites could be detected in stained impression smear from cutaneous lesions of both infected (control) and infected treated animals. The density and grading of *L.major* amastigote of infected control and infected treated animals before and after treatment are shown in table (5).

Table (4) Ulcer's diameter in control and infected treated groups before and after treatment for 30 days with *P. granatum.*

	Ulcer diameter (mm)			
	Infected treated group		Control group	
P. granatum (alcoholic)	Two months post-infection	One month post-treatment	Two months post-infection	One month without treatment
	5 x 10	3.5 x 9	5 x 10	9 x 14

Table (5) The density of L.major amastigotes in stained smears from the margin of ulcers before and after treatment with P. granatum.

	Before treatment	After treatment
P. granatum (alcoholic)	(20 fields were scanned)	(50 fields were scanned)
	100-200/ field	10



Fig(3)Mouse with CL lesion befor tretment (above)and after 30 days of tretment with ointment of *Punica granatum* extract (below)

Discussion:

The results of the present study showed the effect of *p.granatum* extracts on *L.major* promastigote at various concentration *in vitro*. It was found that alcoholic extract has a strong effect on the growth and cell division of the parasite than the aqueous extract. The results demonstrated a decrease of density of promastigotes with the increase of the concentration of the extracts. The LD_{50} value for the

aqueous and alcoholic extract was (5.956)mg/ml and (4)mg/ml respectively. The effects of gallotanic and gallic acid (phenolic substance) which were isolated from fruit pell of *p.granatum* are quite indicated *in vitro* against the exanic amastigote and promastigote of 3 strains of *Leishmania*: (*L.major, L.tropica, L.donovani*) (Al-Bashir, *et al*., 2000) . It was found from the present study that alcoholic extract of *p.granatum* has more effect than aqueous extract against *L.major* promastigotes. Other study showed that alcoholic extract was more active against growth of dermatophytes and several microorganisms *in vitro* trials (Al-Kinani, 2001).

It was found that the condensed tannis is not easily to dissolve in water when compared with hydrolysable tannins (Reed, 1995) thus it is concluded that the amount of tannins substances in aqueous extract of *p.granatum* is less than its amount in alcoholic extract. So the later has a stronger action.

The investigation trials in BALB/c mice of the present study demonstrated that *p.granatum* has great effect against amastigote, there is significant decrease in the mean footpad thickness of infected treated mice when compared with control mice. Signs of ulcer healing and clinical response were more clear in infected treated mice than control, few number of amastigote were detected by cutaneous smear. The activity of aqueous extract of *p.granatum* against *Entemoba histolytica* was proved *in vitro* and *in vivo* trials in Basrah (Abdul Hussein, 2001).

The effect of fruit pell extract of *P.granatum* due to tannins and some phenolic substance have effectiveness against growth of some microorganisms, the tannins reached to 2.66% in extract (Bakir, 1997).

The mechanism action of *P.granatum* due to tannins and phenolic substances may be related to its ability to precipitate cell membrane proteins during its penetration. These compounds form a hydrogen bounds with nitrogen free and multi hydroxyl-groups that inhibit some enzymes which is very essential to the organisms (Reed, 1995).

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