Immunomodulation of Visceral leishmaniasis with *Peganum harmala* Seeds Extract in experimental Animals

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ABSTRACT :

BACKGROUND :

Visceral leishmaniasis is an endemic disease in Iraq with high incidence among children below two years. Some recent reports indicated a immunopotentiation role of peganum harmala seed extract against some intra cellular pathogen.

OBJECTIVES :

To investigate the immunostimulation effect of peganum harmala seeds extract before infection with leishmania donovani in BALB/C mice through various parameter's .

METHODS :

BALB/C mice were divided into experimental groups with each group consisting of six animals . The experimental groups injected introperitoneally with $5x10^6$ promastigotes. Experimental groups were injected intraperitoneally by peganum harmala seed extract at doses of (125 , 250 , 500) µg/20gm b. wt. On day 5,10 and successive six days before infection with L. donovani . These animal were sacrificed after (8) weeks. Total parasite burden in spleen , liver percent of infected cells, leishmancidal index in peritoneal macrophage, number of macrophage forming formazan.

RESULT :

Decrease in number of total parasite burden in liver and spleen in all mice groups treated with P. harmala seed extracts also, decrease in percent of infected cells, increase in leishmanial index in peritoneal macrophage, increase in macrophage forming formazan .

CONCLUSION :

Peganum harmala seeds extract was found to have anti leishmanial had anti leishmanial activity through killing of leishmania donovani amastigotes by activated macrophages .

KEYWORDS: Immunomodulation , Peganum harmala , Leishmania donovani , Activated Macrophage .

INTRODUCTION :

Leishmania donovani is an obligate intracellar protozoan whcih parasitizes tissue macrophages . The sand fly vector introduce the promastigotes during blood meal thereby intiating the infection recognized in man as disseminated visceral Leishmaniasis [1] . The pathological consequence of this severe infection are due to non flagellated amastigotes tissues forms as they multiply within phagocytic elements after conversion from promastigotes form [2] . The disease intiated by fever tends to be intermittent , chills and sweating present . The spleen is greatly enlarged due to increase of reticuloendothelial cells , many of which are heavily parasitized, the liver is also enlarged due to proliferation of kupffer cells which contain parasities. Thrombocytopenia , markedly leukopenia and anemia develop together with this disease [3] .

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Leishmania replicated only within macrophages of an infected host , elimination of the parasites and resolution of the disease must evoke extraordinary changes in infected macrophage [4]. These changes modified the intracellular environment of the parasites from one that supportive of replication into another hostile to survival [5] . Activated macrophages which were obtained from mice inoculated with rice starch and Rhizobium meliloti polysaccharides were noticed to be very resistant to infection with amastigotes of L. donovani [6] . Also activated macrophage which were obtained from mice inoculated with Esculetin posses a good protection against leishmanisis due to its immunological properties[7] some studies in Iraq were demonstrated the ability of P. harmala seed extract to induce immune response against bacteria, fungi , and parasites [8] in this study P. harmala seed extract was attempted as immunomodulator against L. donovani in vivo and in vitro.

MATERIAL AND METHODS :

Materials :

Preparation of extract : Aqueous extract of harmala seeds prepared as described by Adday et.al.1989 [11].

Parasites and their maintenance : A stock of L. donovani (MHOM/IQ/BRC1(AA3)) was obtained from Department of Biology , College of science , University of Baghdad .

Animals : Males of Balblc mice , 6-8 weeks of age and 18-20 gm body weight , these animal were obtained from Al-Nahrain medical college .

Experimental protocol :

Note : each group contain 6 animals .

1- control group .

1-1- group injected intra peritoneally with (125µg / 20 gm b.wt of P. harmala).

1-2- Group injected intraperiotoneally with 250µg of 20gm b.wt of p. harmala . 1-3- Group injected intraperitoneally with 500 µg /20gm b.wt of p. harmala .

1-4- Group injected intraperitoneally with $5x10^5$ promastigotes of L. donavoni .

1-5- Group injected with 0.2ml sterile PBS and serves as negative control .

2- Experiment groups :

2.1.1. Group was treated with 125µg/20 gm b.wt. I.P. of *P. harmala* (I.P.)and infected with $5x10^6$ promastigotes after (5) days .

2.1.2. Group was treated with 250µ/20gm b. Wt. Of *P. harmala* (I.P.) and infected with $5x10^6$ promastigotes afer (5) days .

2.1.3. Group was treated with 500µg/20gm b.wt. of P. harmala I.P. and infected with 5x106 promastigotes after (5) days .

2.1.4. Group was treated with 125µg/20gm b.wt. P. harmala I.P. and infected with 5x106 promastigotes after (10) days .

2.1.5. Group was treated with 250µg/20 gm b.wt. of P. harmala I.P. and infected with 5x106 promastigotes after (10) days .

2.1.6. Groups was treated with 500µg/20gm b.wt. of P. harmala I.P. infected with 5x106 promastigotes after (10)days .

2.1.7 Groups was treated with 125µg /20gm b.wt. of P. harmala I.P. for successive six days then infected with 5x106 promastigotes .

2.1.8 Groups was treated with 250 µg / 20 gm b.wt. of P. harmala I.P. for successive six days and infected with 5x106 promastigotes .

2.1.9 Groups was treated with 500 µg / 20 gm b.wt. of P. harmala extract for successive six days and infected with 5x106 promastigotes .

All these groups of mice are sarcified after (8) week and concern on the following parameter .

1.Total of leukocytes and differential count .
2.Percent of leukocyte forming formazan .
3.Percent of infected cells and phagocytic index .
4.The changes of spleen and liver weight and Organ index .
5.Total parasite burden (TPB) and prophylactic index .Calculate : infected cells percent, leishmancidal index, macrophage forming formazan .

A. Preparation of peritoneal macrophages: five ml of HBSS contain 50 I.U/heparin were injected into the peritoneal cavity a septically, after gentle massage of the abdomen then the mouse was sacrificed .The skin from the ventral body was removed peritoneal fluid was pooled with pasteur pipette from small pores on the peritoneal membrane into the poethylene tube . 0.5 ml of peritoneal fluid was placed at tissue culture plate for 1 hour at 37°C after incubation three times washed with PBS , fixed with methanol for 3-5min , washed with PBS , stained for 20min with Geimsa stain , washed with PBS , examined under oil immersion , then leishmanial index was Calculated according to Al-Jorany et.al., 1992(6) .

Leishmanial index =

$$\frac{\text{Infected cells – infected and treated cell}}{\text{Infected cells}} \times 100$$

B. Calculate of Macrophages number which forming formazan .

This parameter depends on superoxide production by macrophages , then the superoxide reduced the undissolved yellow stain into dark blue indissoluble formazan salt which sediment in macrophage cytoplasm casper et. al., 1992 [12] .

Methods :

1.Peritoned macrophage prepared as in A .
2.0.5ml of NBT was added to macrophages and incubated at 3°C for 25min.
3.Wash macrophages by PBS, then calculate the macrophage which forming formazan among (200) macrophages .

Total parasite burden in spleen and liver :

A cut section of liver and spleen was plotted on filter paper and impression was made on glass slides , air dried smear were fixed in methanal for 3-5 min and stained with Geimsa for 20min . Slides were examined under oil immersion and the ratio of amastigotes to organ cells nuclei was determined . Total parasite burden were quantified according to stauber – 1956 [13] .

Leishmain donavani = liver or spleen wt mg x ratio of amastigoter x 200.000

Unit per liver or spleen While the prophylactic index was calculated according to Riffat et.al.,

1989 [14].Counting of monuclear leukocyte was carried out by using a haemocytometer .

Prophylactic index :

$$\frac{\text{No. Of amastigotes in spleen or liver in infected and treated animal}}{\text{No. Of amastigotes in spleen or liver in infected and non treated animal}} \times 100$$

Statistical analysis : ANOVA testy was used to compare the result .

RESULTS :

1- Quantified of Total white blood cells and differential count . Table (1) shows the changes in W.B.C. number in mice treated with (125, 250, 500) µg/20gm for (5,10) days and successive six days before the infection . The number of WBC for all treated group were ($P<0.05$) it reaching (8725, 9575, 10283) cell / cumm respectively comparing with the untreated control which was (7853.3) cell / Cumm in contrast with group infected with parasite alone which reach (1791-6) cell / Cumm . Also , the number of W.B.C. decreasing in groups treated with P. harmala seed extract and infected with L. donovani promastigates comparing with control ($P>0.05$) . In addition to that the increasing number was in the number of lymphocytes . Calculation of infected cells percent , lishmanial index in peritoneal macrophages and number of macrophages forming formazan . Table (2) shows the changes of leishmanial index and infected cells percent in peritoneal macrophages for treated mice with (125, 250 , 500)µg/20 gm b.w.t. On (5, 10 daily for six days) before infection with L. donovani promastigotes , also the same change for the infected mice . The result shows a decrease in infected cells percent for all treated groups . It reach to (32.4) cells in mice treated with 125µg for ten days , which were significant ($P<0.05$), while the leishmanial index increase for treated group , the higher level for it were obtained in the group activated with 250µg for successive six days it was (85.27%) .Table (3) shows the changes in mean of macrophage forming formazan , they reach (60.6) macrophage in group treated with 250µg which was significant($P<0.05$) in contrast to infected alone , the mean number of macrophages forming formazan in infected mice reached (8.83) .

3- Quantified of Total parasites burden and prophylactic index in spleen and liver. Table (4) shows the changes in amastigotes loads , and prophylactic index in spleen and liver of mice treated with (125, 250 , 500) µg of P. harmala extract on days (5, 10 daily for six days) before infection and the same change in mice infected alone . The number of Total parasite burden in spleen and liver for all treated groups were quite low , caused significant suppression of parasite burden ($P<0.05$) . Higher level of prophylactic index were obtained in the group treated with (500) µg for successive six days . The total parasite burden was $0.471x10^6$ amastigotes in spleen and $7.60x10^6$ in liver, while the prophylactic index reach (99%) in spleen and (97.8) in liver .

4- Estimation of spleen and liver weight and organ index .Table (5) shows the changes in spleen and liver weight and organ index of mice treated with (125 , 250 , 500) µg of P. harmala seed extract on day (5, 10 successive six days) before infection and same change of infected mice alone . The weight of spleen and liver for all treated group were quite low ($P<0.05$) comparing with the weight of infected mice which reach (0.22 , 1.78) gm for spleen and liver respectively . Higher level of organ index were obtained in the group of mice infected alone which reach (9.64 , 78.8) for spleen and liver respectively .

DISCUSSION :

The activation of macrophages by P. harmala leads to changes in macrophages surface receptors which used by leishmanial promastigotes for invading macrophages , among these receptors the mannose – fucose receptors (16) . The result were consistent with Mahmoud and Tuwajri (1991) (17) . When demonstrated reduction the proliferation of L. donovani amastigotes in both spleen and liver of glucan pre-treatment mice . Also , the reduce of parasites burden in spleen and liver were observed when treating infected mice with Esculetin (6,7 dihydroxy coumarin) (7) and polysacchorides of Rhizobium meliloti (6) . Murray, 1988 reported the same result when treated visceral leishmaniasis with interferon - γ . Also Rachamin and Jaffe (1993) (19) demonstrated that immumized mice with a protein purified from L. donovani promastigotes dp 72 before infected I.V. with L. donovani showed 78% reduction in liver parasites burden compared with control . Also , the result of this study demonstrated that the immunopotentiation with P. harmala induced significant prophylactic index in mice against infection with L. donovani *in vivo*. The mechanism(s) that mediated this effect are unknown at present time . The possible mechanism(s) responsible for this effect might involved an increasing the released of reactive oxygen , so this lead to augment that the capacity of macrophages to eliminate the infections especially the parasites very sensitive to hydrogen peroxide , generally , macrophages could be activated for engulfed and killing leishmanial parasites by Esculetin (7) . hydroxyethylstarch (20)

and thymic ectract (14) . All treated groups showed an increased number of macrophages forming formazan, this agreed with Gasper *et al.,* (1992) (12) study which measured the estimation of respiratory burst in non-infected macrophages by nitroblue Tetrazolium (NBT) because activated macrophages by non-particles of polyalkey anoaerylate (PACA) . While the decrease in number of macrophage forming formazan in infected mice were consistent with Mallinson and Coombs (1984) (21) when improved that the infected macrophages with leishmania amastigotes didn't reduce NBT , become the macrophages couldn't produce superoxide , due to ability of parasites to enhance lipoxygenation and cycloxygenation then enhance prostoglandins, leukotreins and thromboxanes which suppress the production of super oxide (22) .

Table (1) Total leukocyte and differential count in groups of mice treated with peganum harmala seeds extract and infected with L. donovani .

Differential count								Total W.B.C. count		Activation periods	Concentr-ation µg
Eosinophil		Neutrophil		Monocyte		Lymphocyte					
Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD		
	-	5.2717	± 0.1746	*4.1517	±0.4951	*90.576	* 0.435	*5100	±126.49	5	125
		6.3467	±0.333	*2.761	±3.0720	*92.208	±0.291	*6491.6	±66.45	5	250
		7.5050	±1.9265	*1.669	±0.582	*90.873	±1.531	*6708.3	±120.0	5	500
	-	13.3467	±0.1527	*4.223	±1.3495	*81.930	±0.2248	*5991.6	±149.72	daily six days	125
		12.1850	±0.3364	*0.2717	±0.099	*87.543	±0.2835	*7658.3	±73.59	daily six days	250
		10.7717	±0.9036	*1.6233	±0.9010	*88.2050	±0.149	*7783.3	±103.27	daily six days	500
	-	10.446	±0.221	*6.1417	±0.516	*83.4117	±0.510	*4350	±89.44	10	125
		9.825	±0.194	*3.2517	±0.4225	*87.690	±0.584	*5433	±136.62	10	250
		9.883	±0.488	*2.8417	±0.585	*87.275	±0.137	*5791	±97.032	10	500
	-	18.3650	±0.0909	*1.4317	±0.1059	*80.203	±0.033	*8725	±121.44	Treated alone	125
		15.4917	±0.8717	*1.295	±0.477	*83.213	±0.585	*9575	±154.11	Treated alone	250
		14.1	±0.1302	*1.4717	±0.2081	*84.928	±1.243	*10283	±112.54	Treated alone	500
0.360	± 0.2925	11.25	±0.6546	15.90	±0.634	*72.480	±0.4671	*1791.6	±58.45	-	C+
		14.3467	±0.257	0.3117	±0.1125	85.3417	±0.295	7858	±257.71	-	C-

* P< 0.005 .
Infected control : C+
Un infected : C-

Table (2) Percent of infected periotoneal macrophages and leishmanicidal index in groups of mice treated with P. harmala seeds extracts and infected with L. donovani .

Leishmanicidal index	Percent of infected cells Mean ± SD	Activation periods	Concentration µg
72.52	* 25.11 ± 0.072	5	125
75.1	* 21.77± 0.314	5	250
75.21	* 21.66 ± 0.268	5	500
79.98	* 17.051 ± 0.097	Daily six days	126
85.27	* 12.87 ± 0.185	Daily six days	250
85.07	* 12.13 ± 0.115	Daily six days	500
62.25	* 32.42 ± 0.301	10	125
70.22	* 27.68 ± 0.326	10	250
70.31	* 27.198 ± 50	10	500
-	91.6 ± 0.458	-	C+

C+ : infected control .
C- : un infected control .
* P < 0.05 .

Table (3) Total of isolated periotoneal macrophages forming formazan from groups of mice treated with P. harmala seed extract and infected with L. donovani .

Macrophage forming formazan		Activation periods	Concentration (µg)
Mean	±SD		
* 25.66	± 0.516	5	125
* 30.63	± 0.983	5	250
* 32.0	± 0.0	5	500
* 40.44	± 0.516	Daily six days	125
* 52.166	± 0.408	Daily six days	250
* 53.0	±0.0	Daily six days	500
* 22.0	± 0.0	10	125
*28.33	± 0.516	10	250
* 29.0	± 0.0	10	500
* 52.5	± 0.516	Treated alone	125
* 60.6	± 0.408	Treated alone	250
* 61.5	± 0.0	Treated alone	500
25.166	± 0.4082	-	C-
8.833	± 0.4082	-	C+

Infected control : C+
Un infected control : C –
* P <0.05 .

Table (4) Total parasites burden in spleen and liver in groups of mice treated with P. harmala seeds extracts and infected with L. donovani

Prophylaction index	Total parasite in liver		Prophylactic Index	Total parasite in spleen		Activation periods	Concentr-ation µg
	Mean x 10^6	±SD		Mean x 10^6	±SD		
93.1	*24.45	±0.413	93.1	*3.326	±0.150	5	125
95.8	*14.75	±0.187	94.8	*2.528	±0.040	5	250
96.0	*14.0	±0.1414	94.9	*2.475	±0.028	5	500
95.7	*15.250	±0.1871	95.3	*2.27	±0.054	Daily six day	125
97.6	*8.33	±0.307	98.9	*0.496	±0.021	Daily six day	250
97.8	*7.60	±0.322	99.0	*0.471	±0.024	Daily six day	500
93.6	*22.71	±2.155	93.0	*3.40	±0.0216	10	125
95.01	*17.67	±0.402	94.1	*2.88	±0.080	10	250
95.21	*16.96	±0.103	94.1	*2.855	±0.0394	10	500
-	358.6	±2.155	-	49.27	±0.255	-	C+

C+ : infected control .
* p<0.05 .

Table (5) The changes in spleen and liver weight / (gm) in mice treated P. harmal seeds extract and with L. donovani.

Differential count								Activation periods	Concentr-ation µg
Liver index		Spleen index		Liver weight		Spleen weight			
Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD		
*51.01	±	*5.82	±1.85	*1.1581	±0.029	*0.132	±0.035	5	125
*49.67	±	*5.66	±1.33	*1.27	±0.035	*0.128	±0.027	5	250
*47.93	±	*4.97	±1.2	*1.08	±0.043	*0.115	±0.010	5	500
*50.19	±	*5.39	±1.76	*1.155	±0.024	*0.124	±0.002	daily six days	125
*44.4	±	*4.77	±0.33	*1.019	±0.006	*0.106	±0.007	daily six days	250
*44.03	±	*4.44	±0.31	*1.012	±0.001	*0.102	±0.002	daily six days	500
*50.56	±	*6.08	±1.23	*1.163	±0.027	*0.140	±0.022	10	125
*49.65	±	*5.85	±1.6	*1.1421	±0.018	*0.134	±0.031	10	250
*49.98	±	*5.57	±1.7	*1.150	±0.020	*0.128	±0.0194	10	500
*55.0	±9.46	*6.57	±0.63	*1.154	±0.011	*0.144	±0.017	Treated alone	125
*56.11	±14.74	*7.16	±1.2	*1.793	±0.02	*0.145	±0.001	Treated alone	250
*13.1	±56.1	*7.19	±1.2	*1.795	±0.01	*0.146	±0.003	Treated alone	500
*78.8	±	*9.64	±1.21	*1.786	±0.087	*0.22	±0.046	-	C+
*43.78	±	4.3	±1.14	1.0	±0.007	*0.098	0.015	-	C-

* P< 0.005 .
Infected control : C+
Un infected : C-

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