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Original article

Study on effects of cantharidin on cutaneous leishmaniasis, its mechanism and optimization of the therapeutic modes

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ABSTRACT

Leishmaniasis is one of the major problems in many countries. Leishmania is flagellated protozoa and causative agent of leishmaniasis which is the most important health problem in many countries especially in developing country. Leishmania major causes cutaneous leishmaniasis (CL). CL is endemic in some part of Iran. Pentavalent antimony compounds are main therapy of CL, they have some side effects due to their toxicity, and also relapse is possible. Cantharidin is terpenoid and vesicant compound that can be found in Meloidae and Oedomeridae family beetles. It was used as treatment to cancer and Wart. It is also apoptosis inducer in various cancer cells. In this study, the effect of 0.5, 1, 2, 5, 10, 20 and 50 µg/ml cantharidin on the L. major promastigotes, non-infected macrophages and infected macrophages with parasite amastigotes was studied by (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyle tetrazolium bromide) MTT assay and flow cytometer in vitro. The Effect of cantharidin as 0.5, 0.05 and 0.1% ointment surveyed on the Leishmania lesions in BALB/C as well as. Parasite load as determined by Real Time PCR, and IFN-γ and IL-4 was involved by ELISA. Results showed that the highest cytotoxicity (56.14%) in promastigotes was in a group that treated with 50 µg/ml cantharidin after 48h. The rate **in** non-infected macrophages and infected macrophages was 13.05 % and 30.17% respectively. Maximum cytotoxicity rate in promastigotes treated with 50 µg/ml cantharidin after 72 h was

determined 66.48%, 48.52% in non- infected macrophages and 62.24% in infected macrophages after 48h by flow cytometry. Group treated with 0.05% cantharidin had lowest rate of ulcer growth. Ulcer size was increased in group treated with 0.5% cantharidin. IFN-γ value in group treated with cantharidin was less than it in untreated (control) group, but IL -4 didn't change. Cantharidin through blister formation induces inflammatory reaction and neutrophils and macrophages infiltration in blister site .It can also destroy tissue by cytokines production stimulating such as myeloperoxidase. However, it can destroy parasite and infected macrophage through apoptosis inducing. Following more investigation, cantharidin can be introduced as cutaneous leishmaniasis treatment.

1. Introduction

Leishmaniasis is one of the most important tropical parasitic diseases that is caused by various species of Leishmania protozoa and depending on the species, disease and host response appear the wide range of clinical protests.Because of the importance of this disease, the World Health Organization has focused on that over time (Nylen and Gautam, 2010; Kathy et al., 2006).

Leishmaniasis in many countries, particularly developing countries is a major problem of public health. Flagellated protozoan of leishmaniasis belongs to Trypanosomatidae group. Leishmaniasis divides in three forms: Cutaneous, mucocutaneous, and visceral. Most of the volume is clinical (Ehrchen et al., 2010; Mougneau et al., 2011). Cutaneous Leishmaniasis is more than Leishmania major. Parasite is transferred through the bite of sandflies,Phlebotemus.mosquito bite, leaves a wound that remains for a few months to a year. Leishmaniasis threatens about 12 million people in 88 countries around the world (Paniz, 2011). The annual rate of its occurrence is 2 million that 500 thousand relate to visceral leishmaniasis and the rest relates to cutaneous leishmaniasis. Annually, 90% of cases of cutaneous leishmaniasisis reported from Afghanistan, Brazil, Iran, Peru, Saudi Arabia, Syria, Algeria, Sudan and 90% of visceral leishmaniasis is reported in Bangladesh, India, Nepal and Sudan. According to official reports, 60,000 people died due to visceral leishmaniasis, annually (Charles, 2010; Li et al., 2009). It recalls that the true figure is more than the number of diseases, since in only one third of the countries reporting the disease is compulsory (Huang et al., 2009). Unfortunately, the number of cases of the disease in our country is increasing (Hsieh et al., 2011; Hsieh et al., 2011). As reported by the World Health Organization, quoted by the Center for Disease Control, the number of patients with cutaneous leishmaniasis has increased in Iran from 13,729 in 2002 to 24,092 in 2006.Factors such as the development of agricultural projects, immigration of non-immune persons to the endemic areas, migration to the cities, rapid and unplanned urban expansion, construction of houses near rodent nests, environmental changes such as dams, decrease parasite resistance to some medications and opportunities seeking visceral leishmaniasis parasite in HIV positive patients, involved in the development and increased disease (Simranjeet et al., 2009; Nylen and Gautam, 2010).

Over time, different approaches have been used for the treatment of leishmaniasis (Pal et al., 2010; Shemarova, 2010). Presently, 5 capacity antimony compounds, Pentosodium (sodium Acetibiogloconat), Glocantim, pentamidine and amphotericin B are used for the treatment of cutaneous leishmaniasis . In addition to the side effects of these drugs, the disease may be relapsed (Macey, 2007; Nylen and Gautam, 2010, Simranjeet et al., 2009).

These compounds are essential in treatment of leishmaniasis and are considered as the first drugs for treatment of it (Sagawa et al., 2008; Fan et al., 2004). Using these compounds have some limitations such as toxicity, long term treatment, the expensive drugs, lack of treatment response and in some cases repeating the injection. In other word, the existence of leishmaniasis parasite with HIV virus simultaneuously causes to increase the resistance to drugs. Despite the efforts in producing theVaccine, no vaccinate has been yet produced (Rauh et

al., 2007; Suman, 2011). Therefore, treatment is necessary to control the disease, especially when the person is exposed to the disease (Simranjeet et al., 2009).

The aim of this study is to analyze the effects of Cantharidin on Leishmania major by Invivo and Invitro methods that we use them after accomplishing many studies.

2. Materials and methods

The study is fundamental and applicable. In this study, BALB / C female mice are used that were selected randomly, sampling is done non-randomly in contaminated samples, and the isolated macrophage cells are cultivated. White laboratory mice (BALB / C) between the ages of 8 to 9 weeks were infected with L. major promastigotes. Grouping the mice: mice were randomly selected and were divided into 5 groups of 6.

Infected control group without treatment, the infected group treated with eucerin (as eucerin effect), the next group of contaminated under treatment with Cantharidin 0.05, 0.1, and 0.5% were considered. In order to save money and time, it is used in concentration condition that leads to the best results in Invitro condition.

2.1. Infecting mice method

1 ml solution containing 2×10^{6} Leishmania major promastigotes in the stationary phase by insulin syringe in the tail of mice were injected subcutaneously. It is necessary to mention that to confirm the statistic phase of parasite the number of promastigotes in the N3 has changed and it is counted daily. In the statistic phase the growth of the parasite is decreased. After 4 to 5 weeks from injection of parasite, the small tight nodules were appeared at the injection site that it is wounded after two weeks. After the mice were contaminated with Leishmania major, macrophage cells of skin have been removed. The analysis of the mice started after the parasite is cultivated. Finally, the effect of Carthardin is studied in remarkable volume of the sample.

2.2. Preparing cantharidin

Cantharidin as a 100 mg dried powder with a purity of 98% was purchased from Sigma Company. 20% concentration of Cantharidin solution

(As the stock solution) by dissolving 20 mg Cantharidin powder in 1 ml dimethyl sulfoxide (DMSO) is provided and kept in refrigerator. The concentrations 50 µg/ml, 20, 10, 5, 2, 1, and 0.5 are provided by diluting the stock solution in the cultivation environment RPMI1640. It is necessary to mention that all of the concentrations are provided in 2X.

2.3. Evaluating the survival rate of promastigotes, the contaminated macrophage, and non-contaminated macrophages by (3-(4, 5-dimethyl thiazolyl-2) -2.5- diphenyle tetrazolium bromide) MTT

MTT is a colorimetric method that after entering into the safe cells, it breaks the mitochondrial dehydrogenase enzyme of tetrazolum bromide and changes to the blue insoluble Furmazan, while the dead cells are unable to do that. By taking the absorbance of the sample, the percentage of cell survival can be calculated by the formula.

$$100 \times \frac{\text{Blank absorbance - absorbance of the wells treated with Cantharidin}}{\text{Blank absorbance - absorbance of control wells}} = \text{percentage of cell survival}$$

First, we count 2×10^{6} Leishmania major promastigotes in logramitmic phase of growth in each ml in the cultivation environment and we add 100 µl from the solution with FCS 20% under the hood and sterile conditions to the 96-well plates. Then, we add 100 µl Cantharidin with the specified concentration to the well. We set a plate inIncubator 21^{oC} for 24 hours, the second plate for 48 hours, and the third plate for 72 hours. In each plate, the control well in triplicate has promastigotes in without Cantharidin environment. The other well contains parasite and DMSO 2% is considered to analyze the effect of DMSO on promastigotes.

In order to analyze the mortality effect of Cantharidin on the contaminated macrophages and non-contaminated macrophages, the peritoneal macrophages of BALB / C mice were used. After cultivating the macrophage and contaminating that according to the mentioned method, 100 µl Cantharidin with the specified

concentration was added to the well. Three plates incubated in incubator 37 ^{o}C with CO_2 5% for 24, 48, and 72 hours.

After passing the mentioned time, we add 20 $_{\mu l}$ of the provided dye MTT (with the concentration 0.5 mg/ml). Repeatedly, the plates which contain promastigotes incubated for 3 to 5 hours in 21 ^{o}C as well as the plates which contain macrophage in 37 ^{o}C. Then, the plateswere centrifuged for 10 minutes at 2000 rpm. The cultivated plates containing contaminated macrophages do not need to be centrifuged because the cells stick to the bottom of the plate, and we are able to drain the surface liquid. The surface liquid is removed and we add 100 $_{\mu l}$ of DMSO to each well. After 15 minutes, the light absorption of the well at a wavelength of 570 nm was read by ELISA reader system.

2.4. Method of flow cytometry test

American kits were used for flow cytometry Annexin V-FITC Apoptosis Detection Kit Bio Vision.Promastigotes which exposed to different concentrations of Cantharidin and the cultivated control groups were collected in 24-well plates at 24,48 and 72 hours, and in microtubes ml 1.5 rpm 3000 were centrifuged for 5 minutes.To collect the contaminated macrophages, Trypsin 0.25% should be used.

The surface solution is removed, and according to the instruction of the kit, 500 lμ of Binding butter is added to the deposit. Then, 5 μl Annexin and 5 μl propidium iodide were added to microtubes. The samples were incubated for 5 minutes in the temperature of the room. Color intensity of Annexin-V which is absorbed by the cells were analyzed by FACS Calibur. The results were analyzed by Cell Quest software. The non-contaminated macrophages and the contaminated macrophages were analyzed in 24 and 48 hours after the exposure of 5, 20 and 50 μg/ml, but the promastigotes in 24, 48 and 72 hours after the exposure of the specified Cantharidin.

2.5. The statistical analysis

The statistical analysis of the data is accomplished by SPSS 16 and by the method of monolateral variance analysis. The normality of the data is analyzed by Shapiro-Wilk test. The relaiblity is 95% and the value of P is considered meaningful lower than 0.05. Also, the results of data are presented in mean± S.E.

3. Results

The present study indicates that the mortality rate of Cantharidin with the concentration 0.5 μg/ml was 14.94%, 30.89, and 6.8% after 24, 48, and 72 hours in promastigotes of leishmaniasis, but the mortality rate of the concentration 50 μg/ml was 50.02%, 56.14%, and 32.48% after 24, 48, and 72 hours (fig. 1).

The mortality rate of Cantharidin with the concentration 0.5 μg/ml after 24, 48 and 72 hours in the non-contaminated macrophages was 15.89%, 14.03%, and 0%. The mortality rate of Cantharidin with the concentration 50 μg/ml in the non-contaminated macrophages was 23.05%, 12.98%, and 0% after 24, 48 and 72 hours (fig. 2). The mortality rate of cantharidin with the concentration 0.5 in the contaminated macrophages to Leishmaniamajor was 2.75%, 4.94%, and 0% after 24, 48, 72 hours. The mortality rate of Cantharidin with the concentration of50 μg/ml in the contaminated macrophages was 30.17%, 20.13%, and 11.8% after 24, 48, and 72 hours (fig. 3).

The statistical analysis of the mortality rate in promastigotes indicate that the control group is significantly different with all of the groups after 24, 48, and 72 hours ($p<0.05$). In the non-contaminated macrophage group, Cantharidin hasn't affected on macrophages after 24 hours ($p>0.05$), but it has affected on the other groups comparing to the control group after 48 and 72 hours ($p<0.05$). The control group (contaminated macrophage without Cantharidin) was different with the groups 5, 6, 7, and 8 (contaminated group+ 5 μg/ml Cantharidin, contaminated macrophage+ 10μg/ml Cantharidin, contaminated macrophage+ 20 μg/ml Cantharidin, and contaminated macrophage+ 0.5μg/ml Cantharidin), and the control group was different with the other groups except groups 2 and 3 (contaminated macrophage+ 0.5μg/ml Cantharidin, and contaminated macrophage+ 1 μg/ml Cantharidin) after 72 hours ($p<0.05$).

The results indicate that Cantharidin with the specified concentration has the most mortality effect on promastigotes over 48 hours. The highest mortality rate was observed in promastigotes which were exposed to 50 μg/ml Cantharidin 48 hours. After 72 hours, the mortality rate was reduced in promastigotes. The amount of IC50 was measured to 3.55 μg/ml after 24 hours.

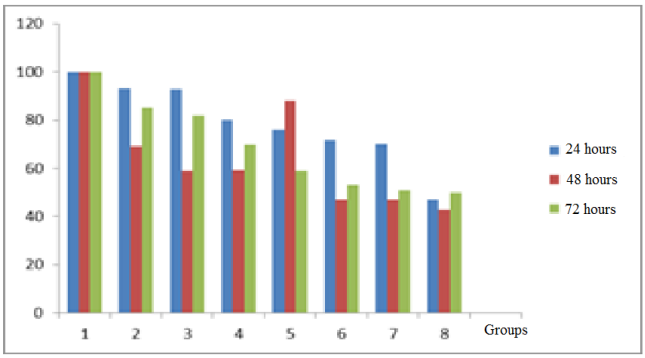


Fig. 1. The percent of survival of promastigotes of Leishmania major after exposing of 0.5 to 50 µg/ml Cantharidin for 24, 48 and 72 hours. Group 1: Control group, 2: Leishmania major+0.5 µg/ml Cantharidin, 3: Leismania major+1 µg/ml Cantharidin, 4: Leishmania major+2 µg/ml Cantharidin, 5: Leishmania major+5 µg/ml Cantharidin, 6: Leishmania major+10µg/ml Cantharidin, 7: Leishmania major+20 µg/ml Cantharidin, 8: Leishmania major+50 µg/ml Cantharidin.

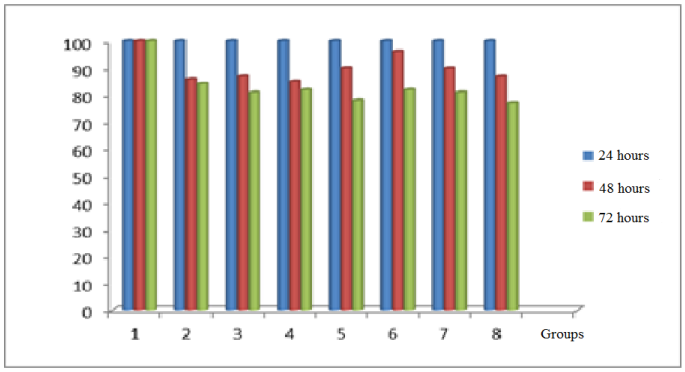


Fig. 2. The survival percent of the non-contaminated macrophage after the exposure of Cantharidin with the concentrations 0.5 to 50 µg/ml in 24, 48, and 72 hours. 1: The control group, 2: non-contaminated macrophages+ 0.5 µg/ml Cantharidin, 3: non-contaminated macrophages+ 1 µg/ml Cantharidin, 4: non-contaminated macrophages +2 µg/ml Cantharidin, 5: non-contaminated macrophages+ 5 µg/ml Cantharidin, 6: non-contaminated macrophages+ 10 µg/ml Cantharidin, 7: non-contaminated macrophages+ 20 µg/ml Cantharidin, 8: non-contaminated macrophages +50 µg/ml Cantharidin.

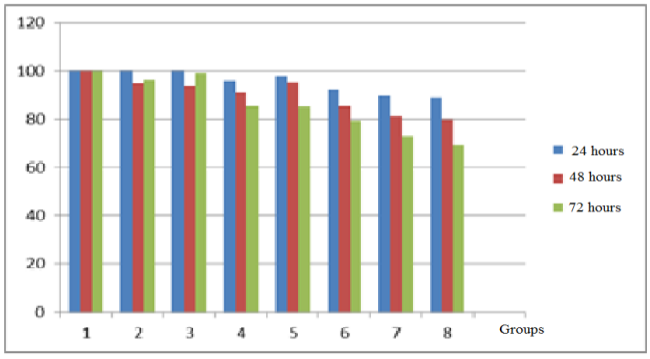
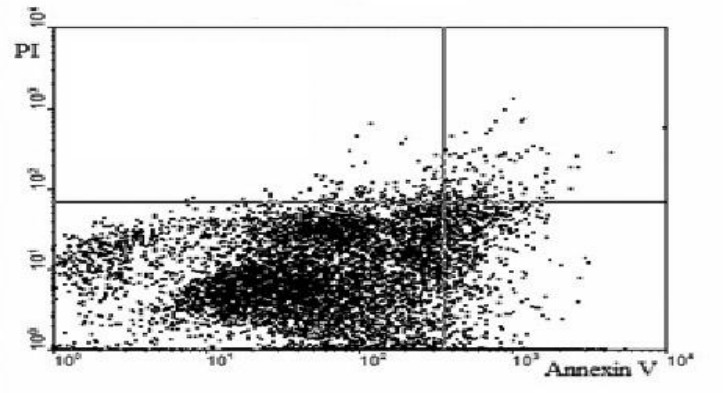
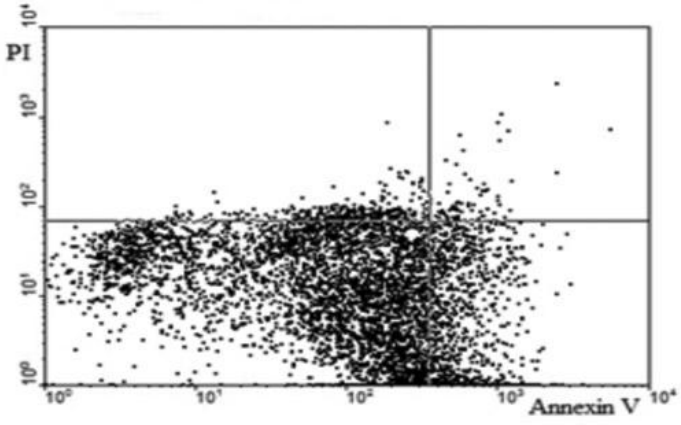


Fig. 3. The percent of survival of the macrophages which are contaminated with Leishmania major amastigotes after exposing to the concentrations of 0.5 to 50 µg/mlCantharidin for 24, 48 and 72 hours. 1: Control group, 2: The contaminated macrophages+ 0.5 µg/mlCantharidin, 3: The contaminated macrophages+ 1 µg/mlCantharidin, 4: The contaminated macrophages+ 2 µg/mlCantharidin, 5: The contaminated macrophages+ 5 µg/mlCantharidin, 6: The contaminated macrophages+ 10 µg/mlCantharidin, 7: The contaminated macrophages+ 20 µg/ml Cantharidin, 8: The contaminated macrophages+ 50 µg/mlCantharidin.

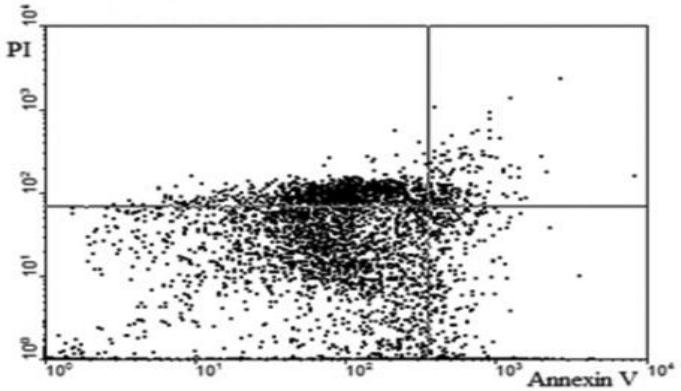
In the study by flow cytometry for non-contaminated macrophages after exposure to Cantharidin 50 and 5 µg/ml over 48 hours, the mortality percent was 42.48 (as 33.80% apoptosis, 6.84% necrosis and 2.2% apoptosis delay) and 89.47% of mortality (12.22% of apoptosis 14.5 % and 63.20% late apoptosis or necrosis) (fig. 4.).



A) Macrophage (control).



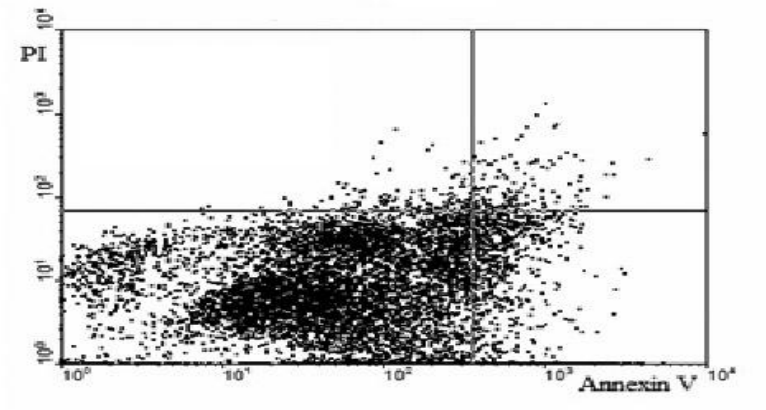
B) Macrophage+ 5 µg/ml Cantharidin.



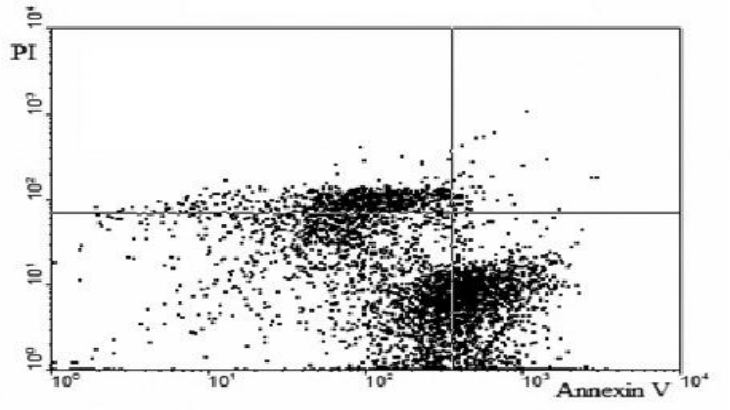
C) Macrophage+ 50 µg/ml Cantharidin.

Fig. 4. The results of flow cytometry in non-contaminated macrophages by Cell Quest software. A) The control sample after 48 hours. B) Macrophages in the exposure of 5 µg/ml Cantharidin after 48 hours. C) The macrophages in the exposure of 50 µg/ml Cantharidin after 48 hours. X axis refers to Annexin, and V axis refers to Propidium iodide. UL (Left and upper (necrosis), UR (Upper Right) right and upper (delayed apoptosis), LL (Low Left) (left and down (living cells), LR (Low Right) (right and down (apoptosis).

The results of flow cytometry in contaminated macrophages in the exposure of 5 and 50 µg/ml Cantharidin after 48 hours indicate 62.14% (as 4.48% apoptosis, 1.5% delayed apoptosis, and 16.80% necrosis), and 45.05% (as 30.59% apoptosis, 11.85% necrosis, and 2.61% delayed apoptosis). (Fig. 5.).



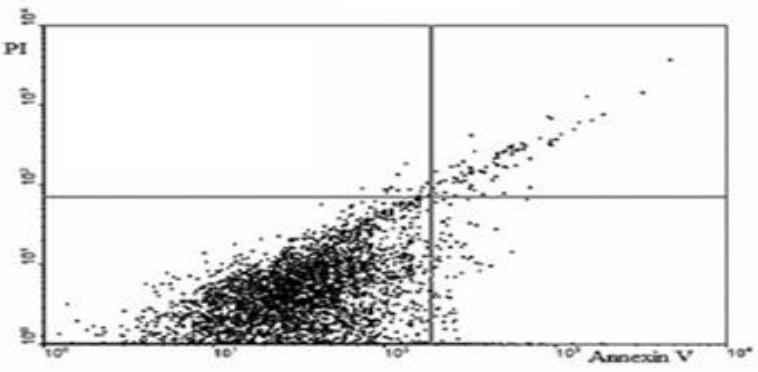
A) Control.



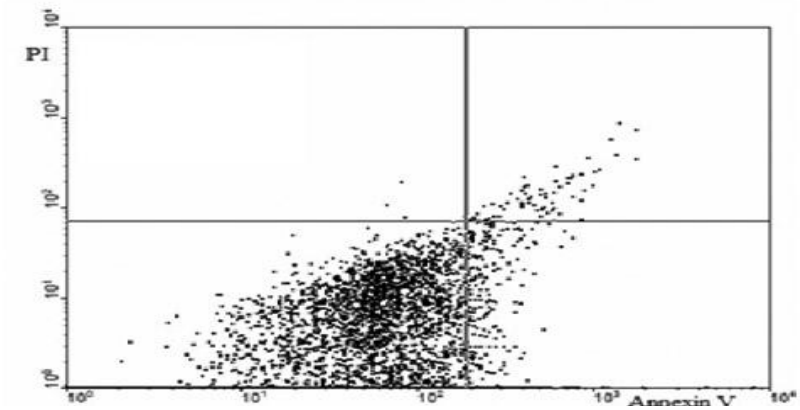
B) The contaminated macrophage+ 50 µg/ml Cantharidin.

Fig. 5. The results of flow cytometry in contaminated macrophages by Cell Quest software. A) Sample after 48 hours. B) The contaminated macrophages in the exposure of 50 µg/ml Cantharidin after 48 hours.

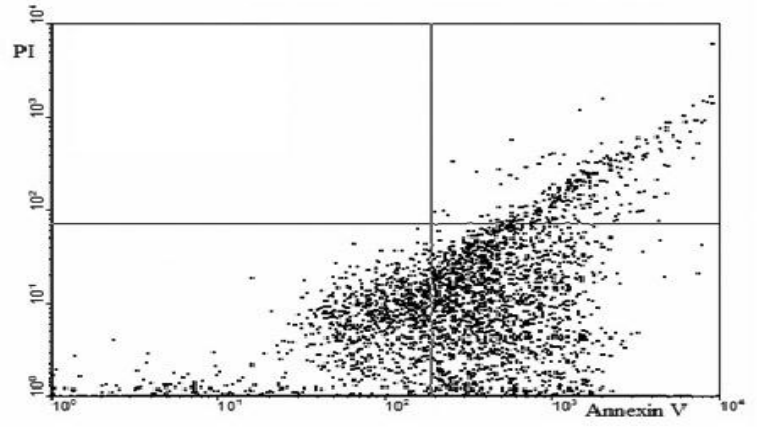
The results of flow cytometry in promastigotes of Leishmania major after exposure of 0.5 and 50 µg/ml after 72 hours indicate that the mortality rate is 66.48% (62.84% apoptosis, 3.64% delayed apoptosis, and 0% necrosis), and 13.94% the mortality rate (as 12.54% mortality rate, 1.1% delayed mortality rate, and 3% necrosis). (Fig. 6.)



A) Leishmania major (control).



B) Leishmania major+ 0.5 µg/ml Cantharidin.



C) Leishmania major+ 50 µg/ml Cantharidin.

Fig. 6. The results of flow cytometry in promastigotes of Leishmania major by Cell Quest software. A) Sample after 72 hours. B) Promastigotes in the exposure of 0.5 µg/ml Cantharidin after 72 hours. C) The treated promastigotes with 50 µg/ml cantharidin after 72 hours.

4. Discussion

In this research, Cantharidin has not affected on macrophages isolated from mice in Invitro. The highest fatality rate (23.5 %) was observed in the group that was exposed to 50 µg/ml Cantharidin after 72 hours. In a study, the highest mortalirty rate in progmatigotes referred to the concentration of 50µg /ml after 72 hours. Also the mortality rate was more than 48 hours after 72 hours. In the mentioned study, determining the mortality rateis done by counting and directly by Neubauer slide; the amount of IC50 is calculated 1.9 µg/ml after 24 hours. In the present study, promastigotes of parasite is counted in logratmic level and cultivated in plate; and immediately, Cantharidin with different concentrations is added to the wells and the mortality rate is counted by MTT after 24, 48, and 72 hours, but in a study, Cantharidin is added to the wells by passing 72 hours after the cultivation of parasite in the plate (Zapata, 2009). Our results indicate that the mortality rate is lower than 48 hours after 72 hours, and in the mentioned study, this rate was more than 48 hours after 72 hours. In our study, the promastigotes were set in the lograrithmic phase and grew by the existence of Cantharidin, but in the mentioned study, the growth of parasite was decreased after 3 days, and adding Cantharidin increased the rate of mortality in the promastigotes. The macrophages have an effective role in creating the immune response against the infections and secretion of cytokines. Leishmania donovani and Leishmania major by removing M -CSF (Macrophage) colony stimulating factor prevents apoptosis in macrophages.Also, Leishmania major prevents the release of cytochrome c from mitochondria and activation of caspase-3 of the contaminated macrophages. Leishmainiasis with this operation helps its survival in the host [23] [24]. Cantharidin causes to increase the Caspase 3, and simultaneously,

it causes to decrease the amount of proteinBcl2 as theinhibitors of Apoptosis, and finally, it causes to induce apoptosis (Wright et al., 2010; Wanderley and Barcinski, 2010). Leishmania doesn't have caspase and apoptosis is done through caspase-like molecules which are called Metacaspase. The precise mechanism of apoptosis induction in leishmaniasis and the effectiveness of Cantharidin is not clear, yet. Miltefosine in Leishmania donovani by reducing the permeability of mitochondrial membrane cause to increase the release of cytochrome c and apoptosis induction (Wanderly and Barcinski, 2010).

The highest concentration of Cantharidin (50 µg/ml) in promastigtes of Leishmania major was 62.48% apoptosis. The value of the apoptosis in the non-contaminated and contaminated macrophages after the exposure of 50 µg/ml Cantharidin was 42.84% and 22.12% after 48 hours. Cantharidin with the concentration 50 µg/ml resulted 20.63% necrosis after 48 hours, while with the concentration 5 µg/ml resulted 6.84% necrosis and 33.80% the apoptosis. The value of necrosis and apoptosis in without drug macrophages (as the control group) was 1.79% and 18.69% after 48 hours. Therefore, the low concentration of Cantharidin in the non-macrophages causes apoptosis, and the high concentration causes the necrosis. In a study, IC50 is measured 2±0.28 µg/ml for promasitgotes, and 7.7±2.6 for macrophage (Macey, 2006; WHO, 2009).

Cantharidin causes to decrease the survival of macrophage in Invitro codition, also, it causes to decrease ATP [24][14]. Most of the studies have been about the effect of Cantharidin on the cancer cells, and these studies resulted that Cantharidin in Hepatoma cells, Colon cells,carcinoma of the oral cavity and leukemia cells cause to apoptosis (Rauh et al., 2007; Wanderly and Barcinski, 2010; Huan et al., 2006).

The macromaphages have an important role in creating immune response against the infections andCytokine secretion. Leishmania donovani and Leishmania major prevents apoptosis of macrophage by removingcolony stimulating factor M-CSF, but it increases the transcription of genes TNF- α (Tumor necrosis factor- alpha) ،GM-CSF(Granulocyte macrophage colony – stimulating factor), and IL- 6 (Interleukin 6). Also, Leishmania major prevents the release of Cytochrome c and induction of apoptosis of cells (Liao et al., 2011). The precise mechanism of induction of apoptosis of cells by Cantharidin is not clear. Different studies indicate that Cantharidin in the cancer cells by increasing theOxidative stress causes to damage DNA of cells, and it induces apoptosis through mitochondria by induction of P53 molecule (Yang et al., 2011; Li et al., 2013). There is no caspase in Leishmania, but there isCaspase-like protein which is called Metacaspase.Apoptosis happens for Leishmania in controlling its population in intestine of the mosquito and in macrophages through displacement of Phosphatidylserine. Some drugs, such as miltefosine induce apoptosis in Leishmania (Liao et al., 2011; WHO, 2007). The occurrence of Phosphatidylserine (PS) in the externalmembrane of promastigotes is a sign of apoptosis, but In Amastigotes not only death but also it attaches the macrophage and enters it through phosphatidylinositol serine (Barati et al., 2009). As leishmaniasis prevents apoptosis in macrophage, and Cantharidin helps to remove the parasite by induction of apoptosis in Promastigotes Leishmania major and contaminated macrophage, further studies about cutaneous leishmaniasis in Invitro should be accomplished, and it should be considered as a drug for treatment of cutaneous leishmaniasis.

5. Conclusion

Cantharidin affects on promastigotes of Leishmania major and the macrophages which are contaminated with parasite. It is recommended that further studies should be accomplished in Invivo conditions for the effect of this chemical compound.

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