



Research Journal of  
**Parasitology**

ISSN 1816-4943



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## Effect of Cantharidin on Apoptosis of the *Leishmania major* and on Parasite Load in BALB/c Mice

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### ABSTRACT

*Leishmania major* is causative agent of cutaneous leishmaniasis. Leishmaniasis is most public health problem in developing countries. Cantharidin is vesicant found in the *Meloidae* beetles hemolymph. Protein phosphatase 1 and 2A is the primary target of cantharidin. Cantharidin induce apoptosis in variant cancer cells. In this study, the effect of various concentrations of cantharidin on the *Leishmania major* viability was investigated *in vitro* by flow cytometry and MTT assay. Cantharidin also was applied as ointment by three doses (0.05, 0.5 and 0.1%) on leishmaniasis lesion in BALB/c for 4 weeks, quantity of parasite assessed by Real-Time PCR. MTT test results indicated that 0.5  $\mu\text{g mL}^{-1}$  and 50  $\mu\text{g mL}^{-1}$  cantharidin had 14.26 and 49.86% cytotoxicity in leishmania promastigotes after 72 h, respectively. But, flow cytometry data showed 14.29% cytotoxicity (as 13.12% apoptosis, 1.12% late apoptosis and 0.05% necrosis) and 68.51% cytotoxicity (as 5.28% apoptosis, 63.23% late apoptosis and 0% necrosis) in promastigotes due to 0.5 and 50  $\mu\text{g mL}^{-1}$  cantharidin after 72 h, respectively. Mean of parasite load in control, 0.05, 0.1 and 0.5% cantharidin-treated groups were 85000, 2400, 2200 and 250 parasite/ $\mu\text{L}$ , respectively. It can be concluded that cantharidin induces apoptosis in the *Leishmania major* promastigotes and infected macrophages in the time and dose-dependent manner; also it can decrease parasite load in infected mice.

**Key words:** *Leishmania major*, cantharidin, apoptosis, parasite load

### INTRODUCTION

*Leishmania major* is causative agent of Cutaneous Leishmaniasis (CL). Leishmaniasis is most health problem in developing countries. There is 12 million people infected with incidence of 1.5 to 2 million new case of CL, annually. There is no effective vaccine. Chemotherapy by antimony components, sodium stibogluconate and meglumine antimoniate is the most way to treatment. Resistance and relapse have been reported (Hepburn, 2000; Simranjeet *et al.*, 2009).

Cantharidin is vesicant, principally found in Meloidae beetles hemolymph. Robiquet (1810) first isolated it from *Lytta vesicatoria* (Bonness *et al.*, 2006). Cantharidin (2,6-Dimethyl-4,10-

dioxatricyclo-decane-3, 5-dione) belongs to terpenoids and soluble in organic solvents. Chinese used it as traditional medicine about 2000 years ago (Moed *et al.*, 2001; Deponte, 2008). About 100 years ago, blister beetles used to cure wart and cutaneous lesions (Moed *et al.*, 2001). Hakim Jorjani described cantharidin to cure wart, hair loss, rabidity and black nails (Yousefi, 2004).

Cantharidin is protein phosphatase 1 and 2A (PP1 and 2A)inhibitor, PP1 and 2A are primary target of cantharidin (Bonness *et al.*, 2006; Li and Casida, 1992; Greenway *et al.*, 1995).

Apoptosis is programmed cell death, involved morphological changes (blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation) and biochemical changes (externalization of phosphatidyl serine in the plasma membrane, oxidative stress, release of proteins from mitochondria e.g., cytochrome c, endonuclease G, apoptosis-inducing factor, activity of proteases e.g., caspases) (Deponte, 2008; Shemarova, 2010). Cantharidin can induce apoptosis by increasing caspase 3, 8 and 9 caspase and block caspase inhibitors simultaneously (Wang *et al.*, 2007; Huan *et al.*, 2006; Sagawa *et al.*, 2008). Sometimes cantharidin induces apoptosis through p53 pathway (Efferth *et al.*, 2005; Huh *et al.*, 2004; Kok *et al.*, 2005; Huh *et al.*, 2003). Several studies show that cantharidin induces apoptosis in various cancer cells (Wang *et al.*, 2007; Sagawa *et al.*, 2008; Fan *et al.*, 2004; Rauh *et al.*, 2007). Apoptosis occur in *Leishmania* by caspase analogs named metacaspase (Shaha, 2006; Barcinski and DosReis, 1999; Wanderley and Barcinski, 2010; Debrabant *et al.*, 2003; Nguewa *et al.*, 2004; Kosec *et al.*, 2006). *Leishmania* inhibit macrophages from apoptosis. Macrophage has critical role in host immune response and cytokines release. *L. donovani* and *L. major* inhibit apoptosis of macrophages by remove macrophage colony-stimulating factor (M-CSF). *L. major* also prevent macrophage caspase 3 activation by inhibiting cytochrome c release from mitochondria (Moore and Matlashewski, 1994; Akarid *et al.*, 2004).Therefore, activation of apoptosis is one of the most mechanisms to eliminate intracellular infections (Donovan *et al.*, 2009; Valdes-Reyes *et al.*, 2009). Anti-*Leishmania* drugs induce apoptosis in *Leishmania*. Miltefosine induces apoptosis in *L. donovani* through increase cytochrome c releasing followed by mitochondrial membrane permeability decrease (Verma *et al.*, 2007).

The aim of present work was investigating the effect of cantharidin in inducing apoptosis in *L. major*.

## MATERIALS AND METHODS

**In vitro study:** The present work is designed by an experimental study. The control groups included of *Leishmania major* promastigotes, macrophages and infected macrophages with *Leishmania major* and experimental groups included *Leishmania* promastigotes, macrophages and infected macrophages with *L. major* treated by various doses of cantharidin.

**Cantharidin preparation:** Cantharidin was purchased from Sigma (Germany). It dissolved in dimethyl sulfoxide (DMSO) and stored as 20% stock solution. Various concentrations 0.5, 1, 2, 5, 10, 20 and 50  $\mu\text{g mL}^{-1}$  prepared by dissolving stock solution in the RPMI 1640 medium, then stored at 4°C.

**Parasite culture:** *L. major* (MRHO/IR/75/ER) was cultured in the RPMI 1640 medium (GibCo) with 10% heat-inactivated fetal bovine serum (FBS) (GibCo) and incubated at 21°C.

**Peritoneal macrophages culture and infecting macrophages with *Leishmania major*:**

Resident peritoneal macrophages were harvested through injecting and aspirating phosphate buffered saline (PBS) to peritoneal cavity of BALB/c mice. Macrophages were cultured in the RPMI 1640 supplemented with 10% heat-inactivated FBS and 100 U mL<sup>-1</sup> penicillin, 100 µg mL<sup>-1</sup> streptomycin, then incubated at 37°C with 5% CO<sub>2</sub> (Tanaka *et al.*, 2007). Promastigotes in stationary phase was added to macrophage (10:1) in 24-well plates. After 6 h, supernatant was discharged to remove dead cells and non-entered promastigotes and fresh medium added to wells. Plate incubated at 37°C with 5% CO<sub>2</sub>.

**MTT assay to promastigotes, macrophage and infected macrophages viability:**

By hemocytometer chamber (Neubauer chamber) 10<sup>6</sup> promastigotes in log phase were counted and cultured in RPMI 1640 medium with 10% FBS in 96 well plate (Nunc, Denmark). Cantharidin with final concentrations 0.5, 1, 2, 5, 10, 20 and 50 µg mL<sup>-1</sup> was added to wells as triplicate. Plate incubated at 21°C. Plate was investigated by MTT [3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (sigma, Germany) after 24, 48 and 72 h. MTT was dissolved in Phosphate Buffer Saline (PBS) by 5 mg mL<sup>-1</sup> and was filtered by 0.2 µm filter. Then 20 µL of prepared MTT was added to each well (0.5 mg mL<sup>-1</sup>) and plate was incubated 3-5 h at 21°C in dark. Plate was centrifuged in 2500 rpm, 10 min, supernatant discharged and 100 µL DMSO added to wells. After 10 min Optical Density (OD) of plate was read by ELISA reader at 540 nm. Cytotoxicity percentage calculate by  $[1-(AT-AB)/(AC-AB)] \times 100$ . AB is OD of blank well, AC is OD of control and AT is OD of treated cells.

**Flow cytometry:**

Annexin V-FITC Apoptosis Detection Kit (BioVision; USA) was used to flow cytometry assay. *Leishmania*, macrophage and infected macrophages were cultured in the 24-well plates with cantharidin (0.5, 1, 2, 5, 10, 20 and 50 µg mL<sup>-1</sup> for *Leishmania* and 5, 20 and 50 µg mL<sup>-1</sup> for macrophages and infected macrophages) as mentioned above. Promastigotes were collected after 24, 48 and 72 h, but macrophages and infected macrophages harvested by adding 0.1% trypsin and 0.2% EDTA as described (Macey, 2007). Samples centrifuged in 3000 rpm 5 min, supernatant was discharged and 500 µL binding buffer, 5 µL annexin and 5 µL Propidium Iodide (PI) added to residue. Samples were incubated at room temperature and dark for 5 min. Then the samples were analyzed with FACSCalibur flow cytometer (Becton Dickinson) and Cell Quest software.

**In vivo study:**

Ointments doses (0.05, 0.1 and 0.5%) were made by dissolving cantharidin in eucerin as ointment base. Thirty BALB/c female mice (6-8 week) were infected by injection 2×10<sup>8</sup> mL<sup>-1</sup> promastigotes in base of tail and divided them into 5 groups as control, eucerin, 0.05, 0.1 and 0.5% cantharidin ointment groups. Ointment applied daily once for 4 weeks.

**Quantitative real-time PCR:**

Briefly, 100 mg of leishmaniasis lesion and spleen of some mice in our control and experimental groups cut off using scalpel. Samples maintained in -80°C freezer until use. RNA of samples was extracted by RNX -plus solution kit (cinnagen) and RT-PCR carried out by First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacture's instruction. Real-Time PCR was performed by LightCycler 1.5 system and SYBR green I kit (Roche, Germany). To normalize the parasite load a 224 bp fragment of glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) gene was amplified with the primer forward: 5'-TCAAGGGTGGTGC GAAGAAG-3' and reverse: 5'-TCGCCGTGTAGGAGTGGATG-3' designed previously (Pal *et al.*, 2010). The reactions mixture included a 5  $\mu$ L sample of DNA, 0.5  $\mu$ m of each primer; 9  $\mu$ L of PCR graded H<sub>2</sub>O and 4  $\mu$ L of LightCycler® FastStart DNA Master SYBR Green I. The reaction was performed with an initial denaturation step at 95°C for 10 min, followed by 45 cycles of amplification (95°C/10 sec, 61°C/10 sec, 72°C/25 sec), melting curve (95°C/0 sec, 65°C/60 sec, 95°C Ramp Rate = 0.1°C/sec, 0 sec) and cooling (40°C/30 sec).

**Standard preparation:** PCR product was cloned in PTZ577T by InsTAclone™ PCR Cloning Kit according to manufacture's protocol (Fermentas, USA) and then transformed into the *Escherichia coli* strain TG1. The plasmids were purified by commercial kit (Roche, Germany), linearized and DNA concentrations were determined by UV spectrophotometry. Five 10-fold dilutions of DNA solution (45 ng mL<sup>-1</sup> to 4.5 pg mL<sup>-1</sup>, containing about 183300000-1833 copies of the target/ $\mu$ L) were prepared triplicate to design standard curve. According to standards, samples were quantified.

**Data analysis:** Each experiment in vitro was performed in triplicate. Result showed as mean and standard deviation values. All statistical analysis of cytotoxicity in control and experimental group were performed using SPSS 16 for Windows. The non-parametric independent groups were compared with Mann-Whitney test.

## RESULTS

### *In vitro* Cytotoxic effect of cantharidin on *L. major* and macrophages by MTT assay:

Cytotoxic effect of cantharidin with various doses on the *L. major* promastigotes, macrophages and macrophages infected with *L. major* was estimated by MTT assay 24, 48 and 72 h after incubation. Data showed in Table 1 and 2 present cell viability {Cytotoxicity % = 100 - cell viability %}. Cytotoxicity of *Leishmania* promastigotes due to minimum dose of cantharidin (0.5  $\mu$ g mL<sup>-1</sup>) at 24, 48 and 72 h was 8.20, 31.47 and 14.26%, respectively. But cytotoxicity in *Leishmania* with maximum dose of cantharidin (50  $\mu$ g mL<sup>-1</sup>) after 24, 48 and 72 h was 32.08, 55.54 and 49.86%, respectively. Data showed in Table 1 present cell viability % of *Leishmania* major promastigotes.

Minimum dose of cantharidin (0.5  $\mu$ g mL<sup>-1</sup>) caused 0, 13.83 and 15.23% cytotoxicity in macrophages after 24, 48 and 72 h, respectively. Cytotoxicity in macrophages with maximum dose of cantharidin (50  $\mu$ g mL<sup>-1</sup>) after 24, 48 and 72 h was 0, 13.01 and 22.86%, respectively. Data showed in Table 2 present cell viability % of macrophages.

Table 1: Percentage of cell viability of promastigotes after treatment with different dose of cantharidin by MTT assay

Groups	After 24 h (%)	After 48 h (%)	After 72h (%)
(control)	100	100	100
L. m <sup>+</sup> +cantharidin 0.5 $\mu$ g mL <sup>-1</sup>	93.80	68.53	85.74
L. m+cantharidin 1 $\mu$ g mL <sup>-1</sup>	93.60	59.01	81.88
L. m+cantharidin 2 $\mu$ g mL <sup>-1</sup>	89.51	58.78	70.31
L. m+cantharidin 5 $\mu$ g mL <sup>-1</sup>	76.16	47.44	59.42
L. m+cantharidin 10 $\mu$ g mL <sup>-1</sup>	72.42	46.72	53.45
L. m+cantharidin 20 $\mu$ g mL <sup>-1</sup>	70.20	46.11	51.47
L. m+cantharidin 50 $\mu$ g mL <sup>-1</sup>	67.92	44.46	50.14

<sup>a</sup>*Leishmania major* (L. m)

Table 2: Percentage of macrophages viability after treatment with different dose of cantharidin by MTT assay

Groups	After 24 h (%)	After 48 h (%)	After 72 h (%)
Macrophage (control)	100	100	100
Macrophage+cantharidin 0.5 $\mu\text{g mL}^{-1}$	100	89.43	84.77
Macrophage+cantharidin 1 $\mu\text{g mL}^{-1}$	100	89.12	82.44
Macrophage+cantharidin 2 $\mu\text{g mL}^{-1}$	100	89.05	82.04
Macrophage+cantharidin 5 $\mu\text{g mL}^{-1}$	100	86.99	81.63
Macrophage+cantharidin 10 $\mu\text{g mL}^{-1}$	100	86.58	81.22
Macrophage+cantharidin 20 $\mu\text{g mL}^{-1}$	100	86.17	78.36
Macrophage+cantharidin 50 $\mu\text{g mL}^{-1}$	100	84.95	77.14

Table 3: Infected macrophages viability after treatment with different dose of cantharidin on macrophages infected with *Leishmania major* by MTT test

Groups	After 24 h (%)	After 48 h (%)	After 72 h (%)
Macrophage (control)	100	100	100
Macrophage+L. m <sup>a</sup> +cantharidin 0.5 $\mu\text{g mL}^{-1}$	100	96.44	94.73
Macrophage+L. m+cantharidin 1 $\mu\text{g mL}^{-1}$	100	94.96	91.67
Macrophage+L. m+cantharidin 2 $\mu\text{g mL}^{-1}$	98.65	94.76	89.97
Macrophage+L. m+cantharidin 5 $\mu\text{g mL}^{-1}$	97.89	91.27	87.35
Macrophage+L. m+cantharidin 10 $\mu\text{g mL}^{-1}$	93.08	84.27	80.81
Macrophage+L. m+cantharidin 20 $\mu\text{g mL}^{-1}$	90.35	82.75	73.27
Macrophage+L. m+cantharidin 50 $\mu\text{g mL}^{-1}$	89.92	80.05	70.03

<sup>a</sup>*Leishmania major* (L. m)

Minimum dose of cantharidin (0.5  $\mu\text{g mL}^{-1}$ ) caused 0, 5.04 and 2.33% cytotoxicity in infected macrophages after 24, 48 and 72 h, respectively. Cytotoxicity in infected macrophages with maximum dose of cantharidin (50  $\mu\text{g mL}^{-1}$ ) after 24, 48 and 72 h was 10.08, 19.95 and 29.97%, respectively. Data showed in Table 3 present cell viability % of infected macrophages.

**Flow cytometry results:** A dot plot of PI on the y-axis against annexin V on the x-axis can be used to distinguish viable cells which are negative for both PI and annexin V, apoptotic cells (annexin V-positive but PI-negative) and late apoptotic cells (double-positive for PI uptake and annexin V staining) or necrotic cells (annexin V-negative but PI-positive).

Apoptosis in macrophages caused by 5, 20 and 50  $\mu\text{g mL}^{-1}$  cantharidin after 48h was 34.90, 34.99 and 21.35%, respectively and necrosis was 7.27, 15.36 and 23.76%, respectively. But, apoptosis in infected macrophages caused by 5, 20 and 50  $\mu\text{g mL}^{-1}$  cantharidin after 48h was 31.05, 43.50 and 43.42%, respectively and necrosis was 10.08, 11.98 and 17.11%, respectively. Apoptosis and necrosis in control group (macrophages without cantharidin) was 19.06% and 1.97% after 48 h, respectively (Fig. 1).

Apoptosis in *L. major* promastigotes due to minimum dose of cantharidin (0.5  $\mu\text{g mL}^{-1}$ ) and maximum dose of cantharidin (50  $\mu\text{g mL}^{-1}$ ) after 72 h was 13.12 and 63.23% respectively, but necrosis was 0.05 and 0%. Apoptosis and necrosis in control group (promastigotes without cantharidin) was 2.44 and 0.29 after 72 h, respectively. Other data of flow cytometry are shown in Fig. 2 and not mentioned in the context.

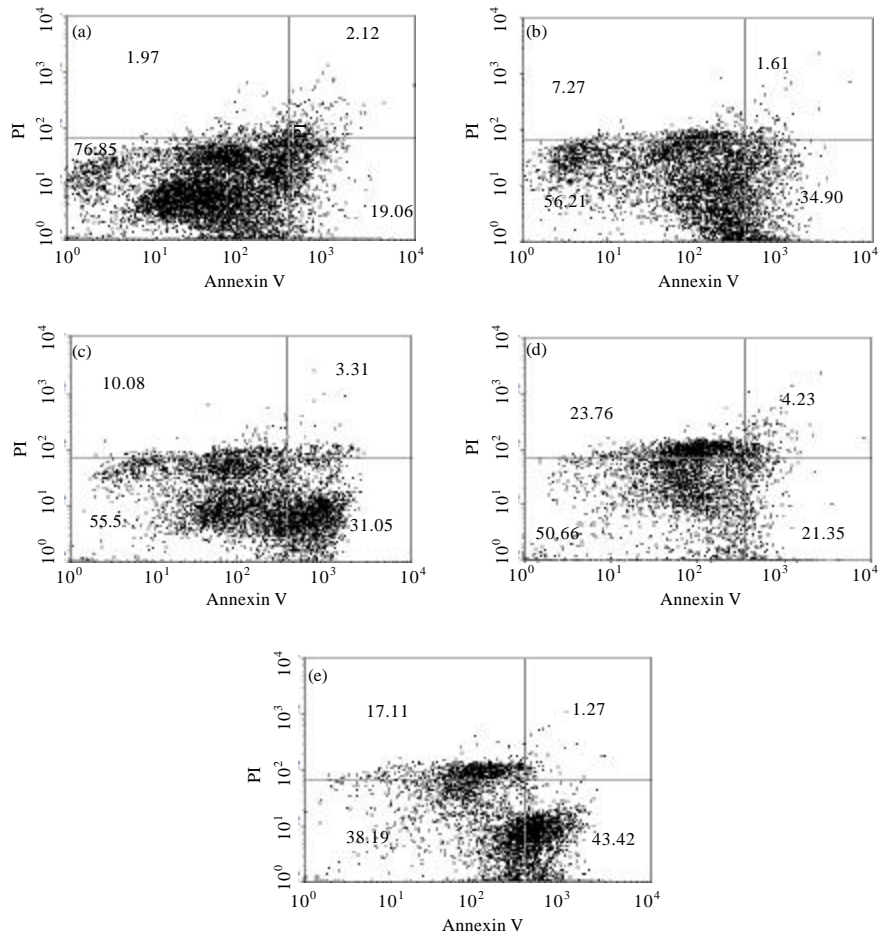


Fig. 1(a-e): Flow cytometry results show the effect of cantharidin with different concentrations on the non-infected macrophages and infected macrophages viability, (a) Control sample, (b) Macrophage with  $5 \mu\text{g mL}^{-1}$  cantharidin after 48 h, (c) Macrophage with  $50 \mu\text{g mL}^{-1}$  cantharidin after 48 h, (d) Infected macrophage with  $5 \mu\text{g mL}^{-1}$  cantharidin after 48 h, (e) Infected macrophage with  $50 \mu\text{g mL}^{-1}$  cantharidin after 48 h. Regions of quadrates show necrosis in upper left, late apoptosis in upper right, apoptosis in low right and live cells in low left

**In vivo Real-time PCR results:** Amplification curve of samples was shown in Fig. 3a. Samples with high concentration amplified in low cycle show. Non-detected samples were amplified in more than 38 cycles. Standard curve was shown in Fig. 3b, according to standard curve Parasite load of samples was quantified.

Load of parasite in cutaneous lesion and in spleen in control, 0.05, 0.1 and 0.5% of cantharidin-treated groups is shown in Table 4. Parasites load mean in lesions samples of control and Eucerin-treated groups was 85000; it was 34000 and 136000 in spleen samples, respectively. Parasite load in lesion samples of 0.05% cantharidin-treated group was not detected exception one sample that was 11000; parasite load mean in spleen samples was about 5000. Parasite load in lesion samples of 0.1% cantharidin-treated group was not detected exception one sample that was

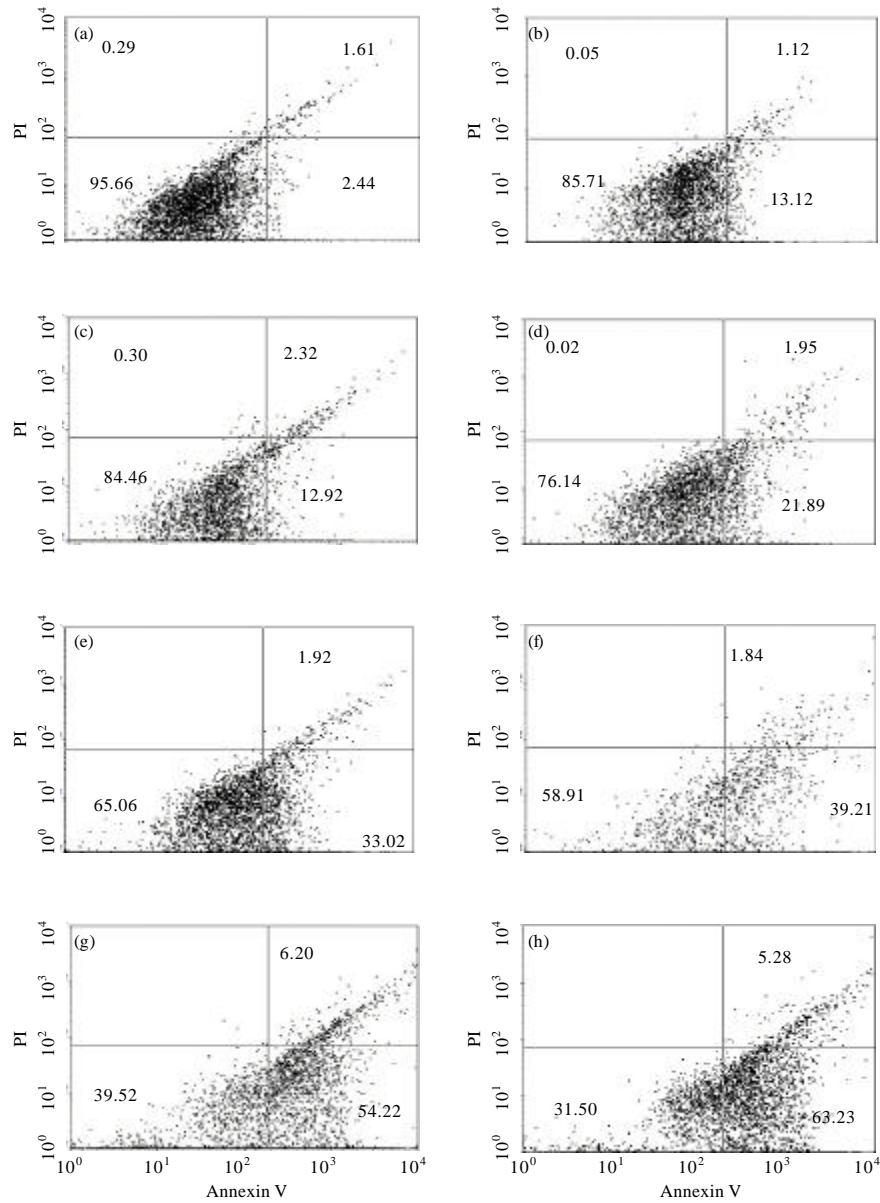


Fig. 2(a-h): Flow cytometry results shown the effect of cantharidin with different concentrations on the promastigotes viability, (a) Control sample, (b) Promastigotes with 0.5 µg mL<sup>-1</sup> cantharidin after 72 h, (c) Promastigotes with 1 µg mL<sup>-1</sup> cantharidin after 72 h, (d) Promastigotes with 2 µg mL<sup>-1</sup> cantharidin after 72 h, (e) Promastigotes with 5 µg mL<sup>-1</sup> cantharidin after 72 h, (f) Promastigotes with 10 µg mL<sup>-1</sup> cantharidin after 72 h, (g) Promastigotes with 20 µg mL<sup>-1</sup> cantharidin after 72 h, (h) Promastigotes with 50 µg mL<sup>-1</sup> cantharidin after 72 h. Regions of quadrate show necrosis in upper left, late apoptosis in upper right, apoptosis in low right and live cells in low left

10000; parasite load mean in spleen samples was about 4500. In 0.5% cantharidin-treated group lesion and spleen samples parasite was not detected.



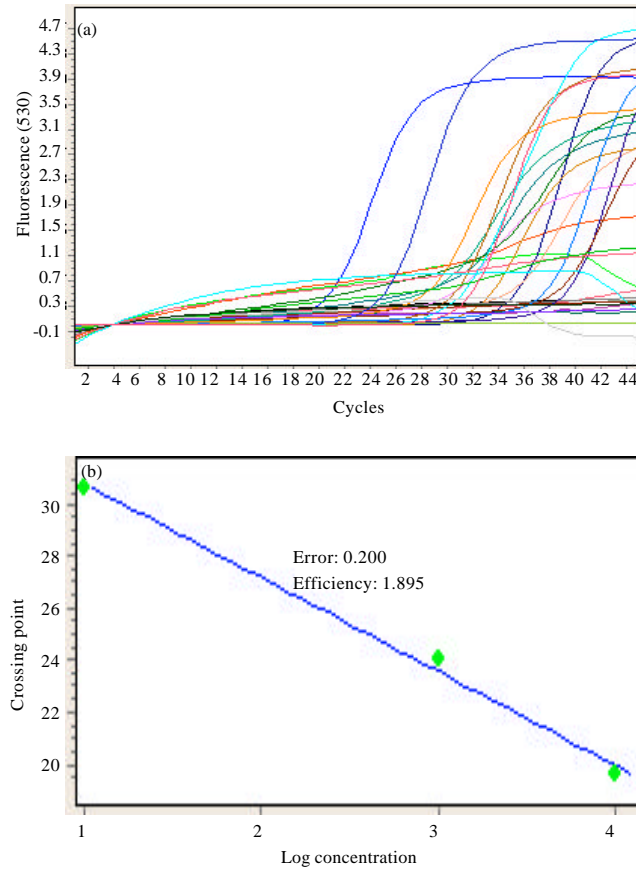


Fig. 3(a-b): (a) Amplification curve of samples by Light Cycler software and (b) Standard curve with efficiency 1.89

## DISCUSSION

MTT results indicate that cantharidin with 0.5 and 50  $\mu\text{g mL}^{-1}$  has 14.26 and 49.86% cytotoxicity in the promastigotes after 72 h. Cytotoxicity in control group was 0% by MTT assay. Flow cytometry results showed 14.29% cytotoxicity (as 13.12% apoptosis, 1.12% late apoptosis and 0.05% necrosis) and 68.51% cytotoxicity (as 5.28% apoptosis, 63.23% late apoptosis and 0% necrosis) in promastigotes due to 0.5 and 50  $\mu\text{g mL}^{-1}$  cantharidin after 72 h respectively. Cytotoxicity in control group was 4.34% (as 2.44% apoptosis, 1.61% late apoptosis and 0.29% necrosis) by flow cytometry. Cytotoxicity of macrophages caused by 5 and 50  $\mu\text{g mL}^{-1}$  cantharidin in MTT assay was 10.57 and 13.01% after 48 h, respectively. Cytotoxicity in control group was 0% by MTT assay. Flow cytometry results showed 43.78% (as 34.90% apoptosis, 1.61% late apoptosis and 7.27% necrosis) and 49.34% (as 21.35% apoptosis, 4.23% late apoptosis and 23.76% necrosis) cytotoxicity of macrophages caused by 5  $\mu\text{g mL}^{-1}$  and 50  $\mu\text{g mL}^{-1}$  cantharidin after 48 h respectively. Cytotoxicity in control group was 23.15% (as 19.06% apoptosis, 2.12% late apoptosis and 1.97% necrosis) by flow cytometry. Harvesting the macrophages using trypsin may increase apoptosis rate. By MTT assay, cytotoxicity of infected macrophages caused by 5  $\mu\text{g mL}^{-1}$  and 50  $\mu\text{g mL}^{-1}$  cantharidin was 3.56 and 19.95% after 48 h, respectively. Cytotoxicity in control group was 0% by MTT assay. Flow cytometry results showed 44.44% (as 31.95% apoptosis, 3.31%

Table 4: Parasite load of samples quantified by Real-Time PCR

Samples	Parasite $\mu\text{L}^{-1}$
Cutaneous lesion of Control group	70000
Cutaneous lesion of Control group	100000
Spleen of Control group	54000
Spleen of Control group	14400
Spleen of Control group	34000
Cutaneous lesion of Eucerin group	95000
Cutaneous lesion of Eucerin group	75000
Spleen of Eucerin group	110000
Spleen of Eucerin group	162000
Cutaneous lesion of 0.05% group	11000
Cutaneous lesion of 0.05% group	N <sup>a</sup>
Cutaneous lesion of 0.05% group	N
Cutaneous lesion of 0.05% group	N
Cutaneous lesion of 0.05% group	N
Spleen of 0.05% group	N
Spleen of 0.5% group	7000
Spleen of 0.05% group	9000
Cutaneous lesion of 0.1% group	N
Cutaneous lesion of 0.1% group 0	N
Cutaneous lesion of 0.1% group 0	N
Cutaneous lesion of 0.1% group	10000
Cutaneous lesion of 0.1% group 0	N
Spleen of 0.1% group 0	N
spleen of 0.1% group	9000
Cutaneous lesion of 0.5% group 0	N
Cutaneous lesion of 0.5% group 0	N
Cutaneous lesion of 0.5% group 0	N
Cutaneous lesion of 0.5% group	N
Spleen of 0.5% group	N

<sup>a</sup>Not detected

late apoptosis and 10.08% necrosis) and 61.81% (as 43.42% apoptosis, 1.27 late apoptosis and 17.11% necrosis) cytotoxicity in infected macrophages caused by 5 and 50  $\mu\text{g mL}^{-1}$  cantharidin after 48 h, respectively. Cytotoxicity in control group was 23.15% (as 19.06% apoptosis, 2.12% late apoptosis and 1.97% necrosis) by flow cytometry. Data of Real-Time PCR indicated decreasing parasite load in cantharidin-treated group. High parasite load in spleen of some sample indicated visceral leishmaniasis in these cases.

MTT results show that cytotoxicity at 72 h was less than it at 48 h. MTT test can assess only total cytotoxicity but can't distinguish necrosis from apoptosis. Flow cytometry is an accurate test with ability to determine apoptosis, late apoptosis and necrosis.

Macrophages remove *Leishmania guyanensis* through apoptotic process include reactive oxygen intermediated (Sousa-Franco *et al.*, 2006). It was found that size of lesions in treated groups was larger than it in control group. Cantharidin induces neutrophil inflammation by mediators including IL-8, TNF- $\alpha$ , IL-1 $\beta$  and C3a (Day *et al.*, 2001). Neutrophils secrete variety cytokines that activate T cells. Moreover, neutrophils damage tissues via cytokine, leukotrienes and prostaglandins secretion (Wright *et al.*, 2010).

It was concluded that cantharidin induces apoptosis in promastigotes in dose- and time-dependent manner. Apoptosis in macrophages just induces by low concentrations ( $<10 \mu\text{g mL}^{-1}$ ) of cantharidin, high concentration ( $>10 \mu\text{g mL}^{-1}$ ) of cantharidin cause necrosis.

So far, there is no study about cantharidin to induce *Leishmania* apoptosis. In one study, cantharidin with concentrations of 0.5, 1, 2, 5, 10, 20 and  $50 \mu\text{g mL}^{-1}$  inhibited the growth of *Leishmania major* promastigotes after 24 h and the resultant inhibition levels were 39.22, 41.95, 49.88, 54.78, 58.01, 68.30 and 80.04%, respectively. After 72 h, the mean number of amastigotes per macrophage in the culture using  $2 \mu\text{g mL}^{-1}$  of cantharidin, (the 50% inhibitory concentration dose ( $\text{IC}_{50}$ )), was 1.2 while it was 2.7 in the control group. This results obtained through direct counting by Neubauer chamber with a light microscope (Ghaffarifar, 2010).

It isn't known how cantharidin induces apoptosis in *Leishmania*. Expression of PS on the outer layer of cell membrane of promastigotes is sign of apoptosis. Amastigotes through PS on outer layer of membrane don't undergo apoptosis, but they use PS to enter into macrophages (Wanderley and Barcinski 2010).

## CONCLUSION

Cantharidin with apoptosis inducer ability can be considered as drug to cutaneous leishmaniasis. It needs more investigation in vivo to determine cantharidin as cutaneous leishmaniasis drug.

## ACKNOWLEDGMENTS

This study was performed as a PhD thesis and supported by the Tarbiat Modares University. The project was authorized by the Ethic Committee of Tarbiat Modares University. The authors declare no conflict of interest.

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