Miltefosine induces metacaspase and PARP genes expression in *Leishmania infantum*

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ABSTRACT

Objectives: Apoptosis is the process of programmed cell death (PCD) that occurs in both animal and plant cells. Protozoan parasites possess metacaspase and these caspase-related proteases could be involved in the PCD pathways in these organisms. Therefore we analyzed the activities of metacaspase and PARP genes in *Leishmania infantum* (MCAN/IR/96/LON49) treated with miltefosine. **Materials and Methods:** Anti-leishmania activity of miltefosine was studied by treatment of cultured promastigotes with various concentration of miltefosine. MTT assay and Annexin-V FLUOS staining by using FACS flow cytometry methods were used. Cytotoxic potential of HePC on the amastigots of *L. infantum* was evaluated in J774 cell line. In addition, metacaspase and PARP genes expression of treated *L. infantum* were studied. **Results:** Miltefosine led to dose-dependent death of *L. infantum* with features compatible with apoptosis. Over expression of metacaspase and PARP was seen 6 hr after treatment. **Conclusions:** Our study showed that miltefosine exerts cytotoxic effect on *L. infantum* via an apoptotic-related mechanism.

Keywords: *Leishmania infantum*; apoptosis; gene expression.

INTRODUCTION

Apoptosis is the process of programmed cell death (PCD) that occurs in both animal and plant cells.[1] Integrity maintenance, cell population and differentiation are controlled by apoptosis. In addition to multicellular organisms, PCD was also studied in unicellular systems. Several studies have shown that different drugs induce the *Leishmania* spp. to die, an apoptosis-like death.[2-5] Anti-parasitic drugs kill the parasites by three distinct mechanisms: autophagy, necrosis and apoptosis.[6,7] In protozoology, improvement of new drugs able to kill parasitic protozoa without interference in host cells is the main goal of the researchers.

In animal cells, activation of caspases (cysteine aspartate proteases) is a central foundation execution switch for apoptosis. In this route caspase induce cascades of reactions eventually leading to cell death. Caspases cleave their substrates after aspartate residues and the different caspases have different peptide recognition sites.[8]

In organisms such as plants, yeasts, or protozoan parasites, there is a metacaspase instead of caspase gene. Therefore these caspase-related proteases could be playing the same role in the PCD pathways in these organisms. Human protozoan parasites of the genera *Plasmodium*, *Leishmania* and *Trypanosoma* also possess genes encoding metacaspase that may be present redundantly in their genomes.[1,9]

Metacaspase has folds similar to caspase-3 and caspase-1 in its secondary structure.[9] However, it is functionally different from caspases. Thus, while caspases have an aspartic acid-directed substrate specificity at P1 position, some plant metacaspases have been found to possess a strict arginine/lysine substrate specificity.[10] Also an arginine-directed specificity has also been shown for the *Leishmania* major metacaspase expressed in yeast.[11]

The participation of metacaspase in PCD in yeast and plants has been studied and revealed that metacaspase play a crucial role in the induction of PCD in response to various stress agents, ageing and impairment of some biological functions.[10,12-15] The possible role of metacaspase in the cell death pathway in protozoa lead researchers to study this attractive issue. Study on *Trypanosoma cruzi*, *Trypano-*

soma brucei and *Leishmania* sp., showed that apoptosis-like death occur in these trypanosomatids.[16-18]

During apoptosis in metazoans, caspases cleave PARP [Poly (ADP-ribose) polymerase], a DNA repair enzyme. Treatment of *Leishmania* with hydrogen peroxide resulted in a similar process involving the cleavage of a PARP-like protein.[19]

Miltefosine (HePc), originally developed as an anticancer drug, has been introduced with success for oral treatment of visceral leishmaniasis caused by *Leishmania donovani* in India. This drug has a low toxicity profile.[20] Different investigations on miltefosine showed induction of apoptosis-like cell death in *Leishmania* sp.[21] In this study, we analyzed the activities of metacaspase and PARP genes in *Leishmania infantum* (MCAN/IR/96/LON49). This is the first report of the PARP and metacaspase activity in *L. infantum* treated by miltefosine.

MATERIALS AND METHODS

Materials

Annexin-V FLUOS staining kit, primers and Taq DNA polymerase were purchased from Roche-applied-science, Germany. RNX™ isolation reagent was purchased from Cinnagen Co., Tehran, IRAN, and cDNA synthesis kit was obtained from Fermentas, Vilnius, Lithuania. Miltefosine (1-O-hexadecylphosphocholine) with structural formula $C_{21}H_{46}NO_4P$ was prepared by Zentaris GmbH (Zentaris, GmbH, and Frankfurt,Germany). All other chemicals were obtained from Sigma (Sigma, Chemical Co., St. Louis, MO, USA).

L. infantum promastigotes culture

Briefly, 5×10^5 cells/mL *L. infantum* promastigotes (MCAN/IR/96/LON49), were cultured in $RPMI_{1640}$ medium (pH 7.2, containing 25 mM HEPES) (Sigma, Chemical Co., St. Louis, MO, USA) supplemented with 10% heat inactivated fetal bovine serum and antibiotics at 24°C for 96 hr and subcultured at cell densities of 2×10^7 to 2.5×10^7 cells/mL. After subculturing, promastigotes were seeded in 96-well culture plates at a density of 2×10^6 cells/mL and treated with HePC in final concentrations ranging from 1-100 µM. The plates were incubated at 25°C for 48h before MTT assay.

In vitro infection of cell line macrophages by *L. infantum*

Cytotoxic potential of HePC on the amastigots of *L. infantum* was evaluated. J774 cell line was cultured in RPMI medium (containing 10% FCS, 2 mM L-glutamine and 100 µg/mL penicillin streptomycin) at 37°C with 5% CO_2. Monolayer J774 cells were inoculated with *L. infantum* (MCAN/IR/96/LON49) in a ratio of five parasites per macrophage. After four hours incubation at 32°C, flasks were washed two times to remove free promastigotes. Miltefosine was added in different concentrations (1, 2.5, 5, 10, 20, 30 µM) and then flasks were incubated for 48 hr in 32°C with 5% CO_2. Microscopic slides were prepared from each cell suspension and stained by Giemsa (100 macrophages per treatment) to find the percentage of infected cells and the number of parasites per infected macrophage. The EC_{50} was defined for each strain as the effective dose of miltefosine that reduced the survival of *leishmania* parasites by 50%. Each test was done in triplicate.

In vitro cell cytotoxicity by colorimetric assay (MTT)

Colorimetric assay MTT [3-(4,5-methylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] performed to determination of relative numbers of live and death cells based on the optical absorbance of the treated and untreated samples.

The basis of this test is measurement of MTT dye (tetrazolium) reduction into formazan by mitochondrial enzymes in viable cells.

Anti-*leishmania* activity of miltefosine was measured using the following formula:[22] Viable cells (%) = $(AT-AB) / (AC-AB) \times 100$. Where, AC is the absorbance of the untreated samples, AT is the absorbance of the treated samples, and AB is the absorbance of the blank. All values are means of triplicate wells. Results were expressed as the concentration that inhibited parasite growth by 50% (IC_{50}).

Primer design, isolation of total RNA and cDNA synthesis

The sequences of the primers were designed for *L. infantum*: Metacaspase (610bp): forward primer 5-TGC CGG AAG GCG GCT CAT TC-3, reverse primer 5-CGC AGT GCG TTG CGC ATA CC-3; PARP (350bp): forward 5-TGC CGG AAG GCG GCT CAT TC-3, reverse primer 5-CGC AGT GCG TTG CGC ATA CC-3; and GAPDH (glyceraldehyde-3-phosphate dehydrogenase.) primers were: forward primer 5-GTC TTC ACC ACC ATG GAG-3 and reverse 5-CCA AAG TTG TCA TGG ATG ACC-3. Total RNA was isolated from 1 x 106 promastigotes in post logarithmic phase using RNX™ isolation reagent according to the manufacturer's instruction.

Complementary DNA was prepared from total RNA using a reverse transcription system. Briefly, 1 µg of extracted RNA was added to 10 U RNAse inhibitor, 500 mM each of dNTP, 20 unit of M-MuLV reverse transcriptase, 160 pM of oligo (dT) primer, and 5 mM $MgCl_2$ in a total volume of 20 µL. The reaction tube was incubated at 37°C for 1 hr, and followed by 10 min at 95°C for inactivate the enzyme.

Polymerase chain reaction (PCR) and quantification mRNA expression

The cDNAs based on the expression of GAPDH products were normalized, depending on whether the genes were to be detected. Of the cDNA, 2 µL were amplified with each primer

pair, separately. Each reaction contained, in a total of 20 µL, 2 µL cDNA, 2 µL 10 x PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$,), 0.4 µL dNTP (10 mM), 0.5 µL of each primer (50 pm/µL), 0.5 µL Taq DNA polymerase (1 U/mL). Cycling parameters for GAPDH mRNA amplification was 94°C/30s, 65°C/45s and 72°C/30s for 30 cycles and for the amplification of metacaspase were 32 cycles and for the amplification of PARP were 30 cycles in a DNA Eppendorf Mastercycler gradient thermal cycler (Eppendorf-Netherland, Hinz, Hamburg, Germany). After amplification, the PCR products (8 µL with 2 µL of a tracking dye) were run on a 1.5% agarose gel containing 1 mg/mL ethidium bromide. The products were scanned (Uvidoc, Gel Documentation System, Cambridge, UK) and the amount of PCR products present in each lane was determined using the Molecular Analyst software (Bio-Rad, Philadelphia, PA, USA) version 1.4. The intensity of bands was measured by densitometry and normalized based on the GAPDH expression.

FACS analysis for determination of phosphatidylserine (PS) externalization

For the detection of apoptotic and necrotic cells death, the Annexin-V FLUOS Staining Kit (Roche, Germany) was used according to the manufacturer's protocol. Briefly, promastigotes were washed in cold phosphate-buffered saline (PBS) (x 2) and centrifuged at 1,400 g for 10 min. Then, they were incubated for 15 minutes in dark and at room temperature in 100 µL of Annexin-V FLUOS in the presence of PI. FACS analysis was performed with a Becton Dickinson FACSCalibur using the FL2-A (detecting fluorescence emission between 585 and 642 nm), the forward scatter (FSC, cell size) and the side scatter detectors (SSC, cell granulometry or internal complexity). Data were analyzed using the CellQuest software and the percentage of positive cells was determined for each sample.

Effect of miltefosine on the cell cycle

Parasites (1 x 10^6 cells) were treated with an IC_{50} dose of for 24, 36 and 48 h at 24°C; at each time point, cells were fixed in chilled 70% ethanol and kept at -20°C until analysis. After washing the cells in PBS, the resultant pellet was resuspended in 500 mL DNase-free RNase (200 µg) and incubated for 1 hr at 37°C. Cells were then stained with PI (40 µg) and incubated in the dark for 20 min at 20-25°C. Data acquisition was carried out using a FACSCalibur and analyzed using CELLQUEST PRO software.

RESULTS

Determination of the IC_{50} of miltefosine-mediated death in *L. infantum* promastigotes

MTT assay was used for evaluation of the viability of *L. infantum* promastigotes.

Changing of MTT to formazan by mitochondrial enzymes indicates the cell viability. Therefore low production of formazan predicts decreasing cell viability. Using HePC for treating *Leishmania* promastigotes demonstrated inhibition of parasite growth and the IC_{50} was 7 µmol for (MCAN/IR/96/LONDON49) promastigotes. Miltefosine showed a dose-dependent cytotoxic effect with almost 100% death at a concentration of about 22 µM (Figure 1).

In vitro effect of miltefosine in *L. infantum* amastigotes

ED50 (half maximal effective dose) for *L. infantum* amastigotes was determined after 48 hours exposure to different concentration of miltefosine.

The percentage of infected J774 cell lines was evaluated by microscopic examination of at least 100 cells. The data represent the means ± standard deviations (SDs) of three independent experiments. ED50 of miltefosine was 12.5 µM for *L. infantum*. In amastigote-infected macrophages [more than 93% of cells (parasite and macrophage)] were killed after 48 hours of incubation with 25 µM of miltefosine (Figure 2).

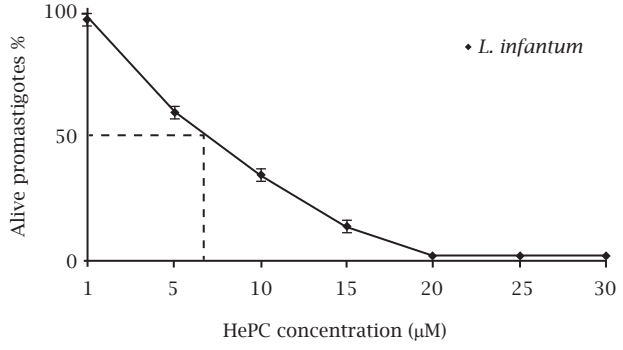

Figure 1: The viability of *L. infantum* promastigotes (MCAN/IR/96/LONDON49) at various concentrations of HePC were assessed by MTT. Each point represents the mean of 3 independent determinations.

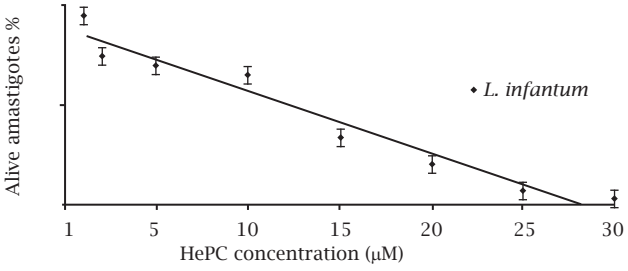

Figure 2: Effect of different concentrations of miltefosine on the proliferation of *L. infantum* amastigotes. Microscopic examination of at least 100 infected J774 was evaluated. Each value represents the mean ± standard deviation (SDs) of three independent experiments.

Expression of metacaspase and PARP during treatment with miltefosine

Metacaspases are caspase-related cysteine-proteases that are present in organisms without caspases such as plants, yeast, and protozoan parasites. Since caspases are important effector molecules in mammalian apoptosis, the possible role of metacaspases in PCD was evaluated in the *L. infantum* promastigotes (MCAN/IR/96/LONDON 49). After treatment of promastigotes with IC_{50} (7 µmol) of miltefosine, metacaspase gene expression was analyzed with RT-PCR in various time periods. Expression of metacaspase detected in 6, 18, 24, 36 and 48 hours post treatment with miltefosine. Over expression of metacaspase was seen 6 to 24 hours after treatment. Untreated cells expressed metacaspase gene steadily in several time points. In addition, *L. infantum* promastigotes (MCAN/IR/96/LONDON 49) were treated with 7 µmol miltefosine for various time periods and the expression of PARP was studied by RT-PCR. The expression of PARP could be detected in all cells at 6, 18 and 24 hours post-treatment. However, PARP expression could not be detected in cells treated with miltefosine for 36 hr and the effect continued even 48 hr after treatment. This was observed when the *L. infantum* promastigotes were treated with miltefosine (Figure 3).

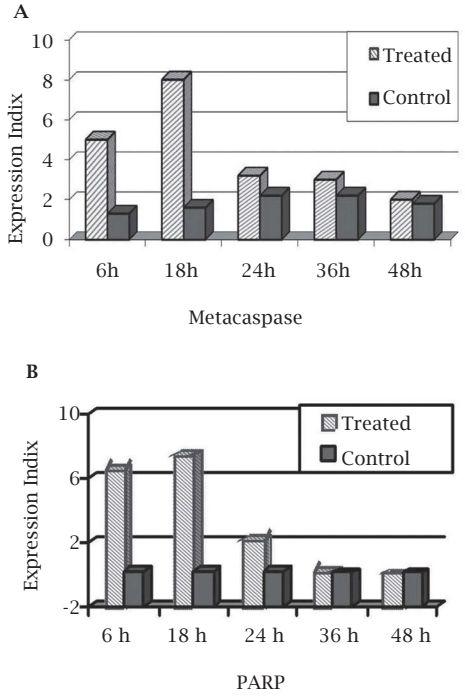

Figure 3: (A and **B)** Relative gene expression of *L. infantum.* **(A)** Metacaspase gene expression in treated and control group. **(B)** PARP gene expression in treated and control group in different time periods. The bars indicate the mean value of metacaspase and PARP gene expression for each group. The density of each band in PCR products was digitized using molecular analyst software for densitometry. This represents a level of expression for each gene in different time periods. The PARP and metacaspase gene expression responses to HePC (7 µM) were also expressed as the expression index defined as the ratio. Results are shown in relation to GAPDH, defined as 100%. The values (mean ± SEM) are derived from three independent experiments.

Detection of phosphatidylserine externalization and mode of cell death

During apoptoic cell death process, phosphatidylserine is translocated from the inner side to the outer layer of the plasma membrane in metazoan and unicellular cells. Annexin V is routinely used to label externalization of phosphatidylserine. Staining by Annexin V and PI simultaneously can differentiate apoptotic, necrotic and living cells. After 36 and 48 hours of treatment, the percent of annexin-positive cells were 58% and 80%, respectively, whereas the corresponding figures in the control group were just 4% for both time points (Figure 4). The negative results of PI staining in several time points showed that miltefosine did not induce necrosis even after prolonged incubation.

Miltefosine induces sub-G0/G1 phase cell-division arrest

To evaluate the ratio of pseudohypodiploid cells, flow cytometric analysis after cell permeabilization and labeling with PI was used. In a given cell, the amount of bound

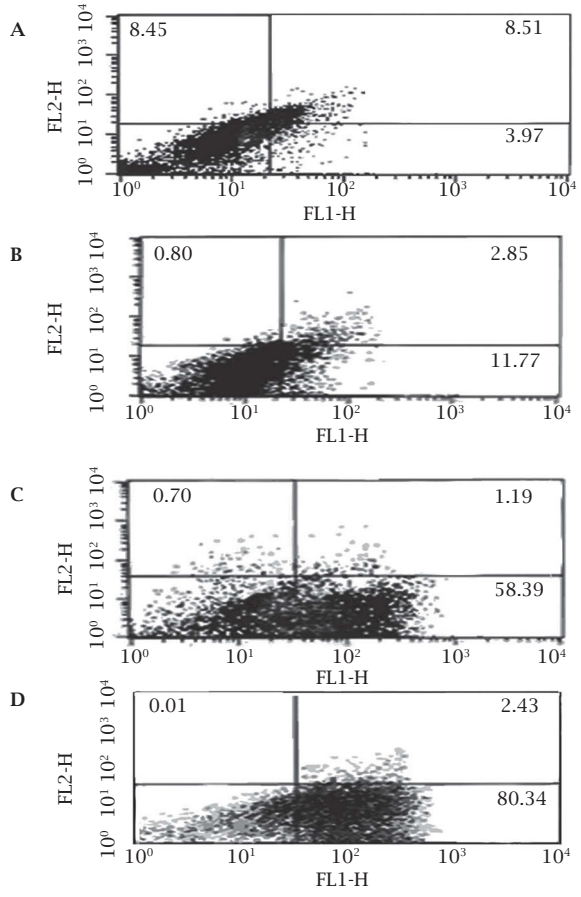

Figure 4: Flow cytometry analysis of promastigotes following treatment with 7 µM HePC and after labeling with annexin-V and PI. Miltefosine did not lead to necrosis even after prolonged incubation, as the entire cells remained negative for PI. Lower right region (LR) belongs to apoptotic cells (annexin positive) and upper left region (UL) belongs to necrotic cells (PI positive). **A, B, C** and **D** flow cytometry analysis in 12, 18, 36 and 48 hours respectively. Values are percentages.

dye correlates with the DNA content and thus DNA fragmentation in apoptotic cells is translated by fluorescence intensity lower than that of G0/G1 cells, i.e. a sub-G0/G1 peak. Incubation of *L. infantum* promastigotes with 22 µM miltefosine for 24 hr indicated an increased proportion of cells in the sub-G0 G1 phase to 16.5% compared with 3.22% of controls (Table 1). In the HePc treatment, in 48 hr, the percentage of promastigotes in the sub-G0/G1 phase was increased to 49% compared to 4% in the control cells. In contrast, an increase in the cell number in sub-G0/G1 phase led to a decrease in the number of cells in the G2/M phase compared with untreated cells.

DISCUSSION

Leishmaniasis is one of the most significant causes of morbidity and mortality in several countries. The most severe form of leishmaniasis is visceral leishmaniasis (VL) or kala-azar, which is fatal in 90% of untreated patients. VL is more frequently observed in developing countries with an estimated incidence of 500,000 per year. *L. infantum* is widespread in the Mediterranean areas and causes lethal VL in children under 9 years old.[23]

The current medications for treatment of VL are pentavalent antimony, pentamidine and amphotericin B. However, their use is limited due to their high toxicity and treatment failure in endemic areas.[23] Miltefosine (HePC) has been proved to be an effective oral treatment for VL with fewer side effects and a cure rate of about 98%.[20] Induction of PCD is one of the advantages of miltefosine against other currently used drugs, including antimony.[22,24] The role played by metacaspase in the cell death pathway in protozoa is less clear than for plants or yeast. Our study showed that miltefosine induces overexpression of metacaspase in *L. infantum* promastigotes. Expression of metacaspase increased 6 hr after treatment with 7 µM miltefosine, but cell death only occur 36 to 48 hr after treatment. It is assumed that cell death process of *Leishmania* promastigotes depends on metacaspase beginning the initial signaling pathway.

González et al.[11] showed that *L. major* metacaspase has a role in cell death. These researchers showed that metacaspase gene of *Leishmania major* (LmjMCA) was involved in yeast cell death, similar to *Saccharomyces cerevisiae* metacaspase (YCA1), and that this function depends on its catalytic activity. These results suggest that in spite of probable differences in their catalytic activity, metacaspases are members of a family of peptidases that have a role in cell death.[25] As metacaspases have a role in cell death they may become potential targets for anti-*Leishmania* medications. In mammalians, caspases do not only function in PCD, but they also have functions in cell proliferation and differentiation.[11]

Ambit et al.[26] found that LmjMCA is expressed in actively replicating amastigotes and procyclic promastigotes, but at a lower level in metacyclic promastigotes. Over expression of LmjMCA in promastigotes leads to severe growth retardation. Also they implicated *L. major* active metacaspase has effective role in the separation of the nucleus and the kinetoplast, function that could be independent of PCD.[26]

In this study, PARP and metacaspase gene expression increased simultaneously. This evolution could repair cell DNA damage. In the present study, increase of PARP gene expression reached its maximum 18 hr after exposure to miltefosine, but the slow decrease observed in the following several time points seemed to be due to DNA injury.

Breakdown of PARP, a DNA repair enzyme that catalyses the poly (ADP-ribosyl)ation of various nuclear proteins is a hallmark of metazoan apoptosis.[27] Western blot analyses reported by Das et al.[19] showed cleavage of a PARP-like protein during apoptosis in *Leishmania* treated by H_2O_2. This process blocked caspase inhibitors. Molecular size of

Table 1. Percentages of* L. infantum *promastigotes DNA content after treatment with 22 µM miltefosine for 24, 36 and 48 hours, respectively

Group	Sub-G0/G1 (M1)	G0/G1 (M2)	G0/M (M3)
24 hours			
Control	3.22*	59.13	40.12
Miltefosine	16.5	61.68	23.33
36 hours			
Control	3.4	61.32	38.5
Miltefosine	18.3	45.3	11.5
48 hours			
Control	4.1	66.85	35.02
Miltefosine	49.9	30.11	8.85

*Values are percentages.

the PARP-like protein that was detected in *Leishmania* by antibody is smaller than the sizes of proteins in mammalian systems (78-kDa intact protein and 63-kDa cleaved fragments in *Leishmania* and 113-kDa intact protein and 85-kDa cleaved fragments in mammalian). *L. major* cysteine proteinase has been reported to process human nuclear PARP into a 40-kDa fragment.[11]

Verma et al.[22] found that in the presence of miltefosine there is no cleavage of *L. donovani* PARP protein.[22] Likewise, there is no cleavage of PARP in the presence of novobiocin.[28]

Flow cytometry analysis after labeling with PI showed that the amount of bound dye correlates with the DNA content and thus DNA fragmentation in apoptotic cells translates into fluorescence intensity lower than that of G0/G1 cells, i.e. a sub-G0/G1 peak. In promastigotes treated with miltefosine (7 μM in 24, 36 and 48 hr), the proportion of cells in the sub-G0/G1 phase increased compared to controls continuously (Table 1). Increase in sub-G0/G1 phase was accompanied by a decrease in the number of cells in the G2/M phase compared with untreated cells in several time points. Increased cells in sub-G0/G1 phase confirmed DNA degradation and apoptosis in *L. infantum* exposed to miltefosine. In another study Khademvatan et al.[29] showed DNA degradation of treated *L. infantum* with DNA ladder assay after 48 hr, that is at the end stage of apoptosis.[29]

In the present study, we have determined an IC_{50} of 7 μM for *L. infantum* promastigotes (MCAN/IR/96/LONDON49), which is lower than what had been reported for other *Leishmania* species. The reported IC_{50} for two strains of *L. donovani* are different from our study. Verma et al.[22] found IC_{50} of 13 μM for *L. donovani* (MHOM/80/IN/Dd8), whereas Paris et al.[24] found to be 25 μM IC_{50} for promastigote of *L. donovani* (MHOM/ET/67/HU3/L82).[24] In contrast, this study showed that the Iranian strain of *L. infantum* promastigote was very sensitive to miltefosine. In another study, we showed that *L. infantum* was more sensitive compared to standard strain of *L. major*i.[29]

In conclusion our findings indicate that miltefisine can induce genes expression related to PCD.

The induction or inhibition route of apoptosis in *Leishmania* spp. is an interesting subject to study as it can identify potential targets for improving anti-*Leishmania* medications.

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