Journal of Medicinal Plants Research Vol. 5(16), pp. 3725-3732, 18 August, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Allium sativum extract induces apoptosis in *Leishmania major* (MRHO/IR/75/ER) promastigotes

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Accepted 19 May, 2011

Leishmaniasis is a protozoan disease that affects many people in the world, hence researchers are trying to design more effective and safer medicine, because of many limits in present chemical drug. In this regard, botany- derived products are the most attractive materials. The aim of this study was to determine the possible *Allium sativum* - induced cell death in *Leishmania major* (*L. major)* promastigotes. RPMI1640 cultured *L. major* promastigotes were subjected to different concentrations of *A. sativum* and viability of the parasites was assayed by MTT. Annexin V- FLUOS staining was performed to study apoptotic properties of the extract using FACS flow cytometry. Furthermore, metacaspase and PARP gene expression of treated *L. major* was studied. Apoptotic dose-dependent death of *L. major* accompanied by DNA fragmentation, cell contraction and externalization of phosphatidylserine with intact of integrity of plasma membrane was observed. In addition, over-expression of metacaspase and PARP was seen 4 h after treatment. Current study revealed that *A. sativum* extract induced apoptotic phenomenon in standard strain of *L. major*.

Key words: *Leishmania major*, *Allium sativum*, apoptosis.

INTRODUCTION

Leishmaniasis is a protozoan disease that affects many people in the world; 12 million currently infected and around 350 million cases threaded (Croft et al., 2003). At least there are four clinical features of the disease, including cutanouse, disseminated cutaneous, mucocutaneous and visceral leishmaniasis. The current drug for treatment of leishmaniasis is pentavalent antimonials, that possesses many disadvantages such as painful of administration, drug resistance, and long period of treatment (Croft et al., 2003; Desjeux, 2004). Therefore, researchers are continuously looking for the more effective and safer antileishmanial drugs. Botany-derived products are the most attractive materials (Akendengue et al., 1999; Kayser et al., 2003; Kigondu et al., 2009). According to the World Health Organization, approximately 80% of people worldwide rely on conventional medicine for disease administration (WHO, 1999). Hence, integration of the modern and traditional medicines is important in the health care (Dutta et al., 2007). Garlic (*A. sativum*) is one of the plants with antibacterial, antifungal, antiviral and antiparasitic characteristics (Goncagul et al., 2010). A number of studies have been performed to elucidate the mechanism of action of *A. sativum* extract.

Some experimental studies have been performed to explain the mechanism of action of *A. sativum* extract in at least 12 different human and non-human parasites. Sulfur compounds of the plant, such as Allicin, diallyl trisulphide (DAT) and ajoene can reduce developing different protozoan parasites such as *Giardia lamblia*, *Leishmania major*, *Leptomonas colosoma* etc (Anthony et al., 2005). Phytochemical constituents of allium extracts and its water-soluble organosulfur compounds like

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S-allylmercaptocysteine (SAMC) and S-allylcysteine (SAC) can induce apoptosis in several human cell culture models (Iciek et al., 2009). A proteomic investigation provides additional support to this evidence by showing that 50% of organosulfide-sensitive proteins are tightly associated with apoptotic pathways (Zhang et al., 2006). At first, it was believed that, apoptotic event does not happen in unicellular microorganisms but studies confirmed that this trend also occurs in single-cell organisms (Wanderley et al., 2010). Apoptotic cell death occurred in *Leishmana* spp when treated with anticancer drugs such as miltefosine (Shaha et al., 2006). In animal cells, activation of caspases (cysteine aspartate proteases) is an apoptotic execution switch, but no caspase gene has been identified in plants, yeasts, or protozoan parasites so far (González et al., 2009). Probable participation of metacaspase (MCA) in programmed cell death (PCD) has been studied in yeast and plants and showed that MCA plays a crucial role in the induction of PCD (González et al., 2009; Vercammen et al., 2007). The possible role of MCA in a cell death pathway of protozoa, motivate researchers to study this attractive issue. Understanding of the rout resulted in stimulating or preventing apoptosis in *Leishmania* spp. as a potential target that can contribute to development of anti-*Leishmania* drugs. The aim of this study was to determine the probable induction of the cell death process of *A. sativum* extract in *L. major* promastigotes.

MATERIALS AND METHODS

In this experimental study, Annexin-V-FLUOS Staining Kit, Primers and Taq DNA polymerase were purchased from Roche-Applied-Science (Roche Applied Science, Mannheim, Germany). RNX™ isolation reagent was purchased from Cinnagen (Cinnagen Co., Tehran, IRAN) and cDNA synthesis kit obtained from Fermentas (Fermentas Inc., Vilnius, Lithuania). All other chemicals were obtained from Sigma (Sigma, Chemical Co., St. Louis, MO, USA).

Preparation of *A. sativum* extract

Garlic (*A. sativum*) was purchased from Hamadan, Iran. Aqueous *A. sativum* extract was prepared. Briefly, small pieces of garlic were mixed in a ratio of 1 g of garlic to 1 ml of distilled water on the mixer for 48 h; supernatant was passed from filter paper to storage and distribution, and soluble extract was lyophilized with lyophilizers (Labconco 4.5 L).

L. major promastigotes culture

Leishmania m. promastigotes (MRHO/IR/75/ER) (5×10^5 cells/ml) were cultured in the RPMI 1640 media (pH 7.2, containing 25 mM HEPES), 10% heat-inactivated fetal bovine serum and antibiotics at 24°C for 96 h. Then the prepared mixture was subcultured at cell densities of 2×10^7 to 2.5×10^7 cells/ml. Promastigotes was seeded in 96-well culture plates at a density of 2×10^6 cells/ml and treated with *A. sativum* extract in final concentrations ranging from 1-100 mg/ml. The plates were incubated at 25°C for 48 h. All testswere performed in triplicates.

Cell proliferation measurements by colorimetric MTT assay

MTT [3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] is colorimetric assay measured reduction of MTT dye (tetrazolium) into formazan by mitochondrial enzymes in viable cells. Test procedure was done with a brief modification that previously described (Verma et al., 2007). Briefly, $1x10^6$ promastigotes at logarithmic phase were grown for 48 h and treated with various concentrations of *A. sativum* extract. MTT assay was done 48 h later. MTT reagent was added to a final concentration of 400 µg/ml, and the plates incubated for 3 h at 24°C. Cells were centrifuged for 10 min and 100 µl DMSO (dimethyl sulfoxide) was added to pellets and incubated for 15 min. The absorbance read on an Elisa reader at 540 nm. Based on the optical absorbance of the treated and untreated samples and blank, relative numbers of live cells were determined. Results were expressed as the concentration that inhibited parasite growth by 50% (IC_{50}).

Protease activity in *A. sativum* extract-treated cells

In *A. sativum* extract, encountered *L. major* promastigotes the role of caspases and other proteases in induced cell death assessed by MTT assay. Briefly, exponential-phase promastigotes were harvested and re-suspended in medium, and cells seeded ($1x10^5$ in 250 µl per well) in 96-well tissue culture plates. Then the prepared mixture was pre-incubated for 2 h with one of the protease inhibitors, namely aprotinin (3 µg ml^{-1}), leupeptin (100 µM), phenylmethylsulfonylfluoride (PMSF, 1 mM), pepstatin (10 µM), trypsin inhibitor (1 mM), EDTA (10 mM), EGTA (10 mM) or Z-Val-Ala-DL-Asp (methoxy)-fluoromethylketone (Z-VAD-FMK, 100 µM). Afterwards, *Allium* extract (37 µg/ml) was added and co-incubated for a further 72 h. The viability of promastigotes was evaluated using the colorimetric 'modified MTT assay' (Dutta et al., 2005).

Phosphatidyl Serine exposure analysis of treated cells

Apoptotic and necrotic cells events were studied by Annexin-V-FLUOS Staining Kit based on manufacturer's instruction. Practically, the *L. major* promastigotes were washed in cold phosphate-buffered saline (PBS) twice and centrifuged at 1400 g for 10 min. Subsequently, they were incubated for 15 min in the dark and at room temperature in 100 µl of Annexin-V FLUOS in the presence of PI. Then for each sample, FACSCalibur flow cytometer (Becton Dickinson and CellQuest software) was used to determine the percentage of positive cells.

Cell cycle progression of treated promastigotes

Flow cytometric analysis after cell permeabilization and labeling with PI was used to quantify the percentage of pseudohypodiploid cells. Parasites ($1x10^6$ cells) were treated with an IC_{50} dose of 24, 36 and 48 h at 24°C. At each point in time, cells were fixed in chilled 70% ethanol and kept at -20°C until analysis. After washing the cells in PBS, the resultant pellet was re-suspended in 500 ml DNase-free RNase (200 µg) and incubated for 1 h at 37°C. Cells were then stained with PI (40 µg) and incubated in the dark for 20 min at 20–25°C. The data were acquired using a FACSCalibur and analyzed using CELLQUEST PRO software.

Oligonucleosomal-DNA fragmentation assay in the presence of *A. sativum* extract

Agarose gel electrophoresis was used for total gDNA fragmentation qualitative analysis. Briefly, *L. major* promastigotes (5×10^6 cells)

related to different time of growing were incubated and harvested. Then to extract DNA from apoptosis-induced and un-induced cells, an apoptotic DNA ladder kit was used according to the manufacturer's order. Ten microgram of each extracted DNA was electrophoresed in 1.5% agarose gels at 80 V for 2.5 h, visualized using an UV transilluminator and photographed.

Promastigotes' morphology after treatment with *A. sativum* extract

To observe changes in cell morphology, promastigotes treated with or without *A. sativum* extract ($IC5_0$), were examined. Of note cells were centrifuged at low speed (1000 g) and the pellets suspended in PBS. Changes in morphology were observed under ×100 objectives on a light microscope. Alteration of cellular morphology was studied in different time points, and for each sample, at least 10 microscopic fields were observed under ×100 objectives.

Total RNA extraction, cDNA synthesis, and polymerase chain reaction (RT-PCR)

Total RNA was isolated from 1 x 10^6 *L. major* promastigotes in post logarithmic phase using RNX™ isolation reagent according to the manufacturer's instruction. Complementary DNA was prepared from total RNA using reverse transcription. Additionally, 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 h, followed by 10 min at 95°C to inactivate the enzyme. Primers of *L. major* metacaspase and PARP were designed.

The sequences of the primers were as follows: Metacaspase (610 bp): Forward primer 5'-TGCCGGAAGGCGGCTCATTC-3', Reverse primer 5'- CGCAGTGCGTTGCGCATACC-3', PARP (350 bp): Forward primer 5'-TGCCGGAAGGCGGCTCATTC, reverse primer 5' CGCAGTGCGTTGCGCATACC-3'and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (362 bp): Forward primer 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3', reverse primer 5'-GAG GGT AGG CTG GCC TAT AGG CT-3'. 2 µl of the cDNA were amplified with each primer pair, separately. Each reaction contained, in a total of 20 µl, 2 µl cDNA, 2 µl l0X PCR buffer (l00 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 HPRT mRNA amplification was 94°C/30 s, 65°C/45 s and 72°C /30 s for 30 cycles, 32 cycles for amplifying metacaspase, and 30 cycles for the amplification of PARP in a DNA Eppendorf Mastercycler gradient thermal cycler (Eppendorf-Netherland, Hinz, Hamburg, Germany). The cDNAs based on the expression of HPRT products were normalized, depending on whether the gens were to be detected. For quantification of mRNA expression after amplification, the PCR products (8 µl with 2 µl of a tracking dye) were run on a 1.5% agarose gel containing 1 µg/ml ethidium bromide. The products were scanned (Uvidoc, Gel Documentation System, Cambridge, UK) and the quantity of PCR products existing in each lane was measured using a Molecular Analyst software (Bio-Rad, Philadelphia, PA, USA) version 1.4. The intensity of bands was calculated by densitometry and normalized based on the HPRT expression.

Statistical analysis

All tests were done in triplicate and standard errors of at least three experiments were determined. *In vitro* anti-leishmanial activity, expressed as IC_{50} (50% inhibitory concentration), was determined by linear regression analysis.

RESULTS

In vitro anti-leishmanial activity of *A. sativum* extract

Cytotoxic potential of *A. sativum* on *L. major* (MRHO/IR/75/ER) promastigotes was determined using the MTT assay in order to find 50% inhibitory concentration (IC_{50}) of this herbal extract. *A. sativum* showed a dose-dependent cytotoxic effect in *L. major* with almost 100% death at a concentration of 93 µg/ml (Figure 1). Treatment with *A. sativum* extract resulted in a concentration-dependent inhibition of *L. major* promastigotes' viability with an IC_{50} of 37 µg/ml. Observation with light microscopy of the treated cells showed that cell shrinkage starts around 4 h after drug treatment in *L. major promastigotes*. Almost all the treated cells showed cytoplasmic condensation, shrinkage, and reduction in size in comparison to the control samples at the end of 48 h treatment (Figure 2C).

Phosphatidyl serine exposure determination by flow cytometric analysis

Flow cytometric analysis of treated *L. major* promastigotes was performed with 37 µg/ml garlic extract after labeling with Annexin-V- FLUOS. In early stages of metazoan apoptosis, phosphatidylserine (ps) translocates from the inner side of cell membrane to outer side and to assay this occurrence, Fluorescein-conjugated Annexin-V is used as it has a high binding affinity to this phospholipid component. Furthermore, annexin-V-FLUOS can distinguish among apoptotic cells (annexin V positive, PI negative), necrotic cells (annexin positive, PI positive), and surviving cells (annexinV negative, PI negative). After 18 h of incubation of treated *L. major* promastigotes, 2.1% of the treated cells and 2% of the control cells were annexin-VFLUOS positive.

Meanwhile, after 24 h of incubation, annexine-VFLUOS positivity was 14.3% for treated *L. major* promastigotes and 3.52% for the control cells. After 36 and 48 h of treatment, the percent of annexin-positive cells was 65.55 and 86.11% respectively, whereas the control group just showed 3.9% for both time points (Figure 2A and B). Treated *L. major* in several times point did not show necrosis even after a prolonged incubation, as all the cells remained negative on PI.

A. sativum extract induces cell cycle arrest

Cell cycle arrest occurs in response to cellular stress through activation of some signal transduction pathways (checkpoints). These checkpoints activated in the G1/S

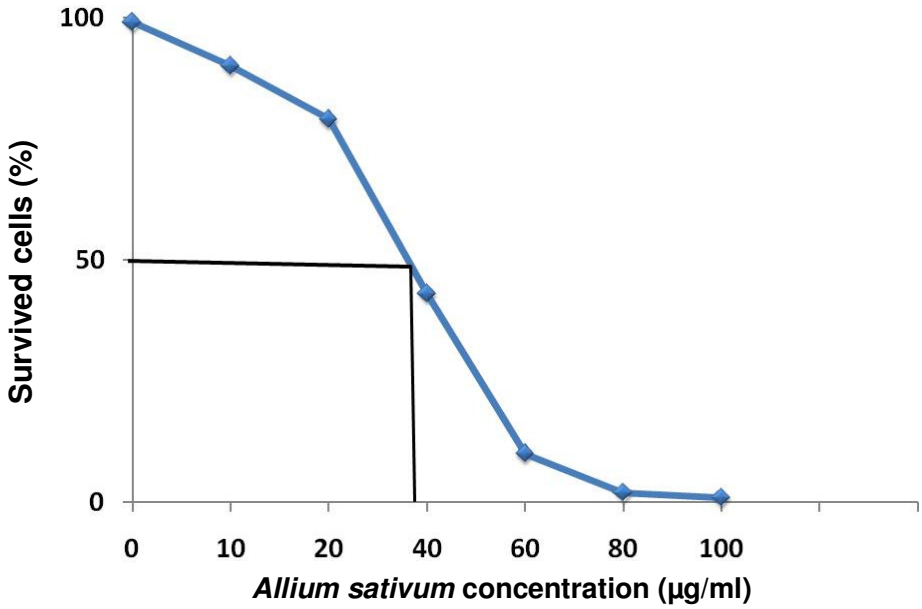

Figure 1. Estimation of IC_{50} of *A. sativum* in *L.major* (MRHO/IR/75/ER) promastigotes. Promastigotes ($1x10^6$/250 µl/well) were incubated with concentrations of *A. sativum* (0-100 µg/ml) for 72 h and viability was measured. Each point corresponds to the mean ± SD of at least three experiments in triplicates.

phase to prevent replication of damaged DNA or in the G2/M phase to prevent segregation of damaged chromosomes during mitosis. In a given cell, the bound dyes correlates with the DNA content and thus DNA fragmentation in apoptotic cells translates into fluorescence intensity lower than that of G0/G1 cells, that is, a sub-G0/G1 peak. In *L. major,* promastigotes incubated with 37 µg/ml *A. sativum* extract for 24 h, the proportion of cells in the sub- G0/G1 phase increased to compare to controls (11.33% vs 2.36%). Following 48 h of *A. sativum* extract treatment, the ratio of promastigotes in the sub-G0/G1 phase increased in comparison to control cells (58.1% vs 3.47%) (Figure 3A). In contrast, increased 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. The increased ratio of cells in the sub-G0/G1 phase, especially after 48 h, showed that *A. sativum* apoptosis in *L. major* and promastigotes resulted in DNA degradation.

DNA fragmentation assay of *A. sativum*-treated cells

DNA fragmentation into nucleosomal units is one of the hallmarks of apoptotic cell death. DNA fragmentation in promastigotes of *L. major* was confirmed by a presence of fragmented DNA in agarose gel electrophoresis. Genomic DNA was isolated from promastigotes exposed to *A. sativum* extract (37 µg/ml) for 0, 12, 24, and 48 h). After *A. sativum* treatment for 12 and 24 h, the degree of fragmented DNA was comparable with control cells. The fragments were in oligonucleosome size (in approximate multiples of 180 - 200 bp) in promastigotes treated with 37 µg/ml *A. sativum* extract for 48 h, whereas untreated cells and treated cells in 12 and 24 h did not show DNA fragmentation (Figure 3B).

Caspas independent *A. sativum* activity against *L. major*

To evaluate the role of caspases and other proteases in garlic-induced apoptosis, promastigotes were preincubated (2 h) with or without protease inhibitors, namely aprotinin, leupeptin, PMSF, pepstatin, trypsin inhibitor, EDTA and EGTA as well Z-VAD-FMK, followed by the addition of *A. sativum* (37 µg/ml, 72 h). The viability was studied by manipulated MTT assay.

The percentage of *A. sativum* extract-induced promastigote killing was 76.58±5.7%. This remained unchanged in the presence of serine protease inhibitors (aprotinin, 81.4±4.41%; PMSF, 70.32±5.33%), a serine and cysteine protease inhibitor (leupeptin, 72.11±6.2%), an aspartic protease inhibitor (pepstatin, 81.76±3.99%), metalloprotease inhibitors (EDTA, 74.2±4.15%; EGTA, 72.71±5.34%) or trypsin inhibitor (81.33±3.32%). Even the broad-spectrum caspase 3 inhibitor Z-VAD-FMK failed to attenuate *A. sativum*-induced promastigote killing (76.22±2.20%), indicating that *A. sativum*-induced apoptosis is caspase-independent.

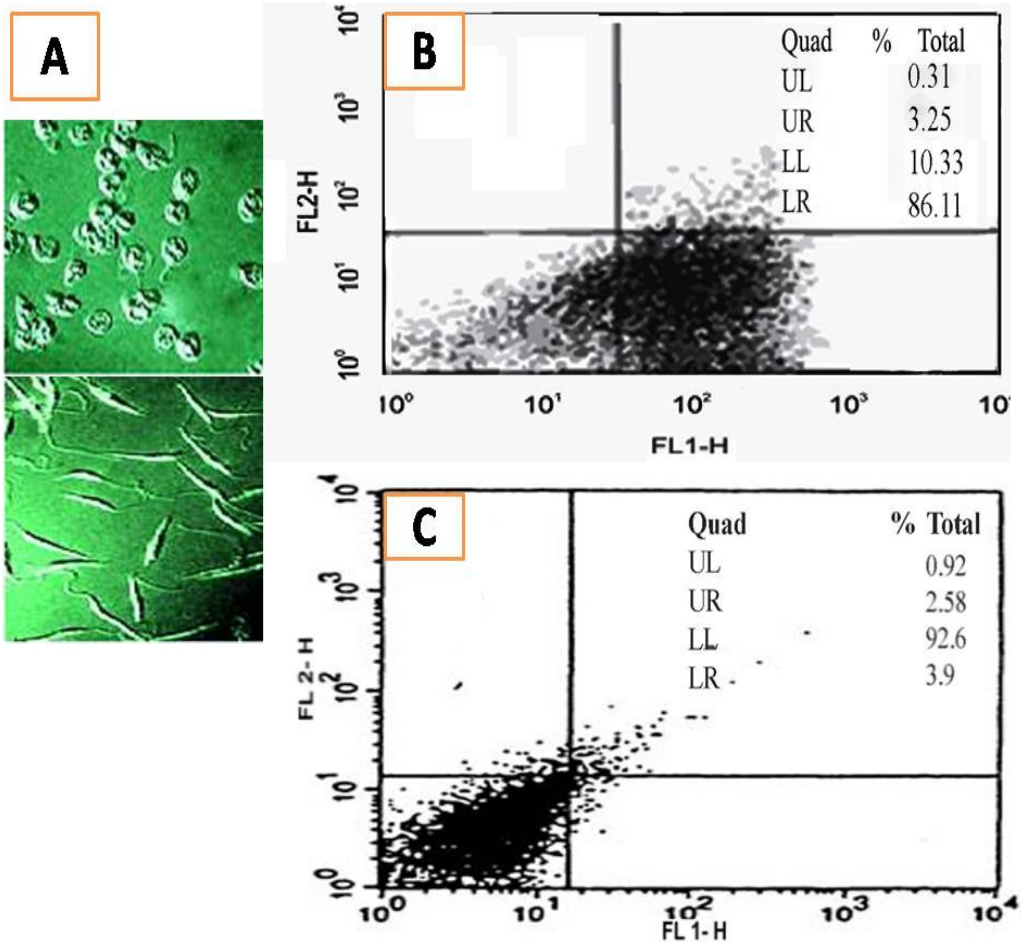

Figure 2. Morphology of *L. major* (MRHO/IR/75/ER) promastigotes after various time period's treatment by *A. sativum* extract (37 µg/ml) in light microscopy (magnification, x 100). A top: Control group 48 h after sub culture and (A down) 48 h after *A. sativum* extract (37 µg/ml) treatment with. (B and C) Flow cytometry analysis of promastigotes after labeling with annexin-V FLUOS and PI in control group (B) and treated cells after 48 h (C). Miltefosine failed to make necrosis in *L. major* (MRHO/IR/75/ER) promastigotes even prolonged administration as all the 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). (B) Control and (C) test sample 48 h after treatment.

L. major metacaspase and PARP gene expression during treatment with *A. sativum*

Metacaspases are caspase-related cysteine-proteases that are present in organisms without caspases such as plants, yeast, and protozoan parasites. Since caspases are important effecter molecules in mammalian apoptosis, the possible role of metacaspases in programmed cell death was evaluated in the *L. major* promastigotes (MRHO/IR/75/ER). After treatment of promastigotes with IC50 (37 µg/ml) of *A. sativum*, metacaspase gene expression was analyzed with RT-PCR 4 h after treatment. Over expression of metacaspase was seen 4 h after treatment. Untreated cells expressed metacaspase gene steady in time point. *Also, L. major* promastigotes were treated with 37 µg/ml garlic extract, the expression of PARP was studied using RT-PCR. PARP expression increased in initial time after exposure of *L. major* to the *A. sativum* extract.

The expression of PARP was detected in promastigotes 4 h post-treatment. However, PARP expression could not be detected in control group (Figure 4).

DISCUSSION

Without any effective vaccine, the only means to treat and control leishmaniasis is an affordable medication. Most of the drugs currently being used for leishmaniasis suffer from one or other limits like exorbitant cost, difficult to manage, high toxicity and development of resistance.

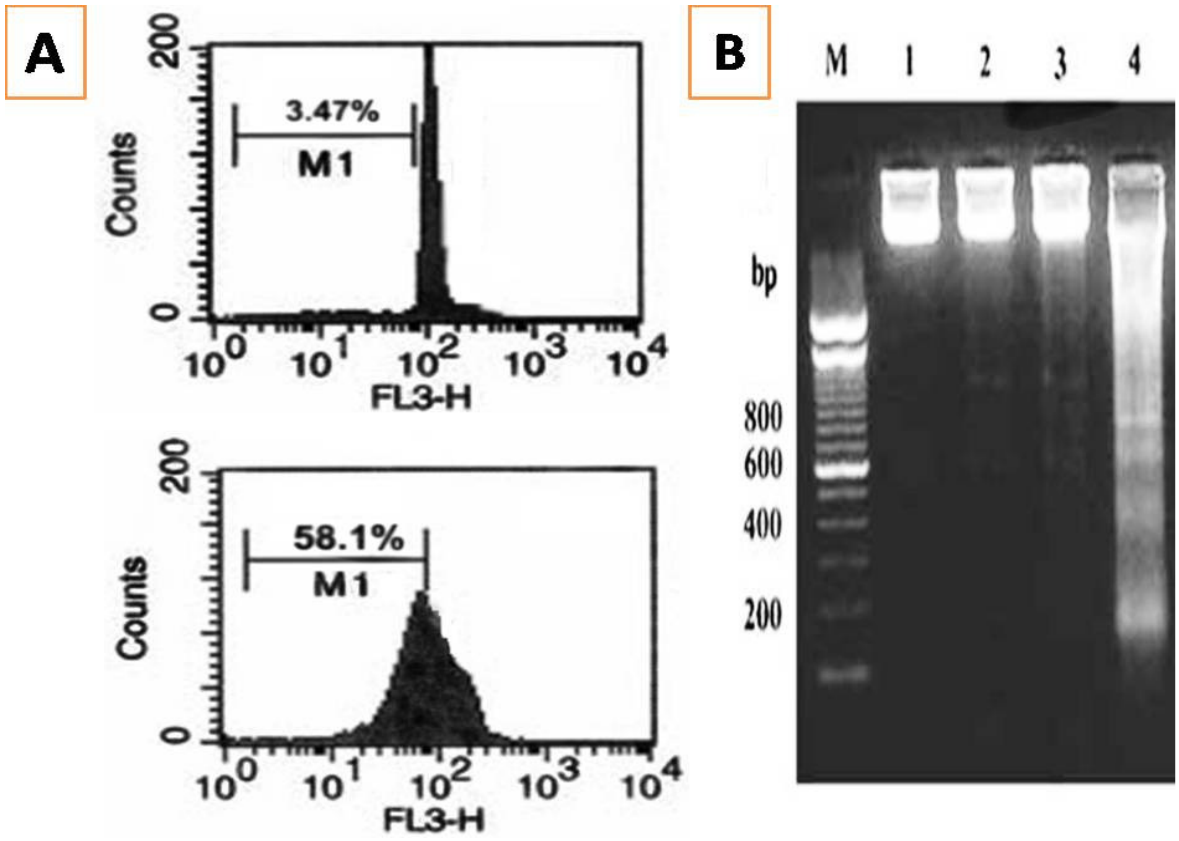

Figure 3. (A) The percentages DNA content of *L. major* (MRHO/IR/75/ER) promastigotes by flow cytometry, found in the sub-G1 peak: untreated (top) or treated with 37 µg/ml *A. sativum* (down). FL3-H, fluorescence intensity. B): DNA fragmentation analysis by agarose gel electrophoresis. The DNA of untreated (Lane 1), 37 µg/ml garlic extract-treated *L. major* (MRHO/IR/75/ER) promastigotes after 6 h and 12 h (Lanes 2 and 3) and *A. sativum* extract-treated *L. major* (MRHO/IR/75/ER) promastigotes after 24 h (Lane 4).

Therefore, there is an urgent need for new, safe, more effective and economically feasible drugs for the treatment of leishmaniasis (Fuertes et al., 2008). The experts in the world are trying to develop a new anti-leishmania medicine and to find new strategies to drug design.

Accordingly, it is interesting to point out that the effectiveness of certain molecules as both anticancer drugs and antiprotozoal agents suggested that this class of compounds and their derivatives might be useful as antileishmanial agents (Fuertes et al., 2008). Garlic contains mainly allicin (diallyl thiosulfinate), diallyl disulfide, vinyl-dithiin and ajoene. Organosulfur compounds originating from garlic stimulate the mitochondrial apoptotic pathway, in various cancer cells in culture and implanted tumors *in vivo* (Iciek et al., 2009). It has been shown that promastigotes of *Leishmania* undergo apoptosis after exposure to some agent - drugs that is, miltefosine (originally anticancer drug) and nutrient deficiencies that cause apoptosis in higher eukaryotes (Khademvatan et al., 2009; Shaha et al., 2006). The anti-leishmanial effect of *A. sativum* component may be due to causing some physiological changes that lead to programmed cell death of the parasite. In the present study, *L. major* promastigotes exposed to garlic extract showed programmed cell death features, including cell shrinkage, DNA fragmentation, and externalization of phosphatidylserine with preservation of integrity of the cell membrane that shares most features associated with metazoan apoptosis (McConkey et al., 1996). Our data showed percentage of killed promastigots does not change in the presence of serin proteas inhibitors (aprotinin, 81. 4±4.41%; PMSF, 70.32±5.33%), a serine and cysteine protease inhibitor (leupeptin, 72.11±6.2%), an aspartic protease inhibitor (pepstatin, 81.76±3.99%), metalloprotease inhibitors (EDTA, 74.2±4.15%; EGTA, 72.71±5.34%) or trypsin inhibitor (81.33±3.32%). The broad-spectrum caspase 3 inhibitor Z-VAD-FMK failed to attenuate *A. sativum*-induced promastigote killing (76.22±2.20%), indicating that garlic-induced apoptosis is caspase-independent. It has been reported that caspase-mediated cell death causes formation of apoptotic bodies and shrinkage of the cell surface, which are absent in caspase-independent

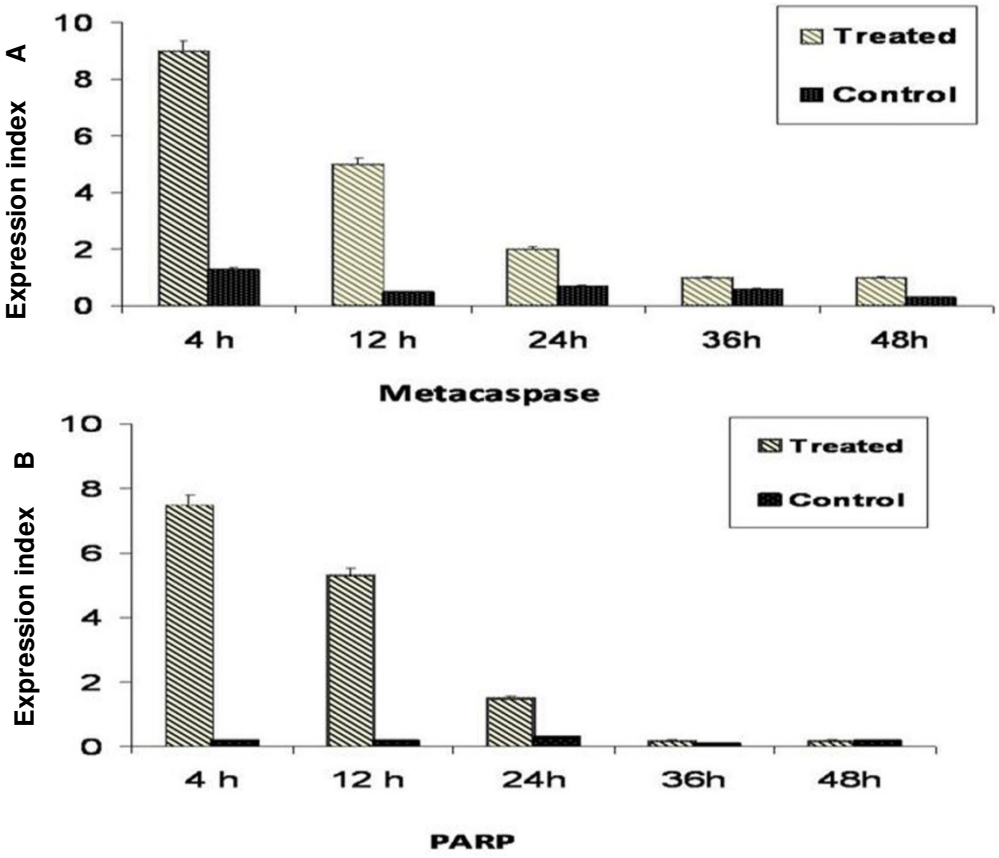

Figure 4. (A, B): Relative gene expression of *L.major* prmastigtes (A) Metacaspase gene expression in treated and control group. (B) PARP gene expression in treated and control group after different time points.The bars show mean value of PARP and metacaspase gene expression in each group. Molecular analyst software was used to analysis of density of each band of PCR products. The results indicate intensity of expression for each gene after different time points. PARP and metacaspase gene expression responses to *A. sativum* extract (37 µg/ml) were also expressed as the expression index defined as the ratio. Results are shown relative to HPRT, defined as 100%. The values (mean± SEM) are derived from triplicate independent tests.

apoptosis (Chipuk et al., 2005). Apoptosis or apoptosis-like changes have been reported for mediating the leishmanicidal action of miltefosine (Verma et al., 2007), amphotericin B (Lee et al., 2002) camptothecin (Lee et al., 2004) and Aloe vera leaf exudates (Dutta et al., 2007).

The part played by metacaspase in a cell death pathway in protozoa is less clear than for plants or yeast (González et al., 2009). Our study showed that *A. sativum* extract induces over-expression of metacaspase in *L. major (*MRHO/IR/75/ER*)* promastigotes. Expression of metacaspase increased 4 h after treatment with IC_{50} of *L. major* but cell death occurs very late in 36 to 48 h after treatment. Its assumed cell death process in *Leishmania* promastigotes depend on metacaspase and in initial time, signaling pathway began. In the previous study, we report DNA fragmentation of treated *L. major* after 48 h that is, end stage of apoptosis (Khademvatan et al., 2009). Ambit et al. (2008) demonstrated that the metacaspase gene of *L. major* (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 (Ambit et al., 2008). González et al. (2009) showed that *L. major* Metacaspase (LmjMCA) has a role in cell death. These researchers showed that LmjMCA was involved in yeast cell death, similar to Saccharomyces cerevisiae metacaspase (YCA1), and that this function depends on its catalytic activity. Breakdown of PARP, a DNA repair enzyme that catalyses the poly (ADP-ribosylation) of various nuclear proteins, is a hallmark of apoptosis. PARP was cleaved by caspase 3 and 7. In present study, PARP gene expression chiefly increased and this progression reached maximum in 18 h but decreased slowly in 24, 36 and 48 h after treatment, which seems to be due to DNA injury. Cell cycle arrest occurs in response to cellular stress through activation of some signal transduction

pathways (Hartwell et al., 1989). Many studies have shown that treatment of various cancer cells with *A. sativum* organosulfur compounds leads to cell cycle arrest (Herman-Antosiewicz and Singh, 2004). In the present study, cells after PI labeling were analyzed with flow cytometry to quantify the percentage of pseudohypodiploid cells. The quantity of fluorescence intensity has a direct correlation with DNA substance of cells and in apoptotic cell's DNA degradation translates into PI the intensity lower than that of G1 cells (sub-G1 peak).

Cells that are exposed to 37 µg/ml *A. sativum* extract after 24 h showed that 37% of the promastigotes were found in the sub-G1 peak region, while in the control group only 3% of promastigotes were found in the sub-G1 peak region. Thus this finding indicates that *A. sativum* extract had induced DNA fragmentation in treated promastigotes. Interestingly, besides the *in vitro* anti-leishmanial activity, garlic ingredients are also reported to have immunomodulatory effect in shifting the cytokine response to a Th-1-type pattern and therefore enhance the protective immune response of the host (Ghazanfari et al., 2006, 2000), which is a paramount step in effective containment of leishmaniasis. This bilateral effect of *A. sativum* component makes it potentially a candidate to treatment of leishmaniasis. Better understanding of mechanisms of *A. sativum* component action may help in finding of new targets for treatment of *Leishmania* parasites. Finally, our findings indicate that garlic may induce apoptosis in leishmania parasite and induced cell death has similarity with metazoan apoptosis. Our data is the first report of apoptosis induction of *A. sativum* in *leishmania* promastigots.

ACKNOWLEDGMENTS

This study was supported by grants No: u-89297 from Ahvaz Jundishapur University of Medical Sciences .The authors would like to thank Miss Bakhshayesh and Parisa Hayat (Iran University of Medical Sciences). The authors declare no conflict of interests.

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