Jundishapur J Nat Pharm Prod. 2021 August; 16(3):e99060. doi: 10.5812/jjnpp.99060.

Published online 2021 July 18. **Research Article**

Apoptosis-Like Cell Death in *Leishmania major* Treated with HESA-A: An Herbal Marine Compound

Jasem Saki [1], Khalil Saki [1] and Reza Arjmand [2, *]

[1] Alimentary Tract Research Center, Imam Khomeini Hospital Clinical Research Development Unit, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2] Parasitology Department, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

[*] *Corresponding author*: Parasitology Department, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Email: arjmand.reza@yahoo.com

Received 2019 October 23; **Revised** 2020 December 26; **Accepted** 2021 January 04.

Abstract

Background: The first drug for the treatment of leishmaniasis is pentavalent antimony compounds which have great side effects.
Objectives: This study aimed to assess apoptosis induction by HESA-A, an herbal marine compound in *Leishmania major* promastigotes.
Methods: *Leishmania major* promastigotes were treated with HESA-A in different increasing concentrations ranged 1.625 - 120 μg/mL, and amphotericin B and the phenomenon of apoptosis in the parasite were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), flow cytometry, and DNA fragmentation tests.
Results: The IC_{50} value of the compound and amphotericin B at 72 h were estimated at 2.81 μg/mL and 40 μg/mL, respectively. After 72 h of the adjacency of *Leishmania major* promastigotes with IC50 dose (2.81 μg/mL), the percentage of promastigotes in early and late apoptosis phases in the treated group was 5.4% and 60.4%, respectively. DNA fragmentation of *Leishmania major* promastigotes treated with 2.81 μg/mL for 72 h was observed.
Conclusions: HESA-A, with significant induction of apoptosis in *Leishmania major* promastigotes, can be plausible in the treatment of cutaneous Leishmaniasis.

Keywords: *Leishmania major*, HESA-A, Apoptosis

1. Background

Apoptosis or programmed cell death (PCD) is a normal and physiological process of cell suicide regulated by several elements. This process has been studied in various cells, such as nematodes, insects, amphibians, and mammals (1-4). This phenomenon involves morphological alterations, such as cell shrinkage, membrane blebbing, cell surface changes, and biochemical changes, including phosphatidylserine (PS) externalization in the plasma membrane and oxidative stress and release of protein from mitochondria (5-7). Apoptosis also results in the condensation of chromatin and fragmentation of chromosomal DNA and nuclear compartments (5). Once, it was believed that PCD emerged with multi-cellularity but did not occur in single-celled microorganisms (2). Recently, several investigations have indicated that apoptosis could also happen in different unicellular eukaryotes (8-15), including various species of *Leishmania* (16-20), especially *Leishmania major* (21). The mechanisms of apoptosis induction in *Leishmania* can be considered as potential targets to produce new anti-leishmaniasis drugs. Leishmaniasis- a complex of protozoan parasitic diseases- has afflicted 12 million people and threatened more than 350 million people worldwide. It has been one of the most important causes of mortality and morbidity amongst different populations. Today, the first drug for the treatment of leishmaniasis is pentavalent antimony compounds in all their forms, including meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostame) (22, 23). These drugs, with an effect on parasitic enzymes, especially interruptions in the phosphokinase enzyme activity, prevent adenosine triphosphate (ATP) production (24). The adverse side effects of these medications and a concerning increase of unresponsiveness to pentavalent antimonial drugs, with more than 50% of individuals infected by different types of *Leishmania* spp. in some areas (25), have led scientists to search for effective medications with minor side effects. HESA-A is a naturally occurring, herbal-marine biological compound invented in Iran. Its mass production has been provided by the Ministry of Health and Medical Education (D5-6638, registration date 2005) (26). This drug is a mixture of *Penaeus* (*Melicertus*), *latisulcatus* (*Pe-*

naeidae), *Carum carvi* L. (*Apiaceae*), and *Apium graveolens* L. (*Apiaceae*) (27). Several investigations have indicated the antitumor activity of certain components in HESA-A (28); these components have also been proven to accelerate the healing process. Numerous in vitro and in vivo studies have been carried out to assess the antitumor properties of HESA-A. These studies have yielded favorable results so far (29, 30). Besides, apoptosis induction of *Leishmania major* promastigotes in some marine organisms, such as marine *Holothuria leucospilota*, has been previously proven (31).

2. Objectives

Hence, the current study aimed to assess apoptosis induced by HESA-A in *Leishmania major* promastigotes.

3. Methods

3.1. Reagents and Chemicals

Promastigotes forms of Iranian standard strain of *Leishmania major* (MRHO/IR/75/ER) were kindly gifted by Dr. Sabery, Isfahan University of Medical Sciences, Isfahan, Iran. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the colorimetric assay kit, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), and L-glutamine were purchased from Sigma Chemical Co. (St. Louis, Missouri, the U.S.A). Besides, the annexin-V FLUOS staining kit and apoptotic DNA ladder kit were purchased from Roche-Applied-Science (Berlin, Germany). Cell culture media of RPMI 1640 (Roswell Park Memorial Institute), penicillin-streptomycin, and fetal bovine serum (FBS) were provided from Gibco (Gibco, New York, the U.S.A). The reference drug, amphotericin B, was purchased by Gilead Sciences (Gilead Sciences, Foster City, the U.S.), kept at 4°C, and diluted with RPMI 1640 medium at the time of incubation.

3.2. HESA-A Preparation

HESA-A is a natural compound, originated from an herbal marine compound. In this study, it was provided by Osveh Drug Co. Iran. Twenty grams of the active HESA-A were dissolved in 200 mL of 1 N HCl to prepare the working solution. The solution was shaken for approximately 24 h. Then, the pH of the solvent was raised to 7.6 by adding NaOH. Finally, the solution was filtered (pore size, 0.45 μm) and stored at 4°C (32).

3.3. Culture of Leishmania major Promastigotes (MRHO/IR/75/ER)

For mass proliferation, *Leishmania major* promastigotes were cultured in an RPMI 1640 medium enriched with 10% FBS, antibiotics (100 μg/mL streptomycin and 100 IU/mL penicillin), and L-glutamine (2 mM). Flask cultures were checked every day to detect bacterial and fungal contamination. Flasks were incubated at 24 $\pm$ 1°C. Finally, when the promastigotes reached a stationary phase, they were used to encounter HESA-A. The trial was repeated three times (33, 34).

3.4. MTT Assay

The anti-*Leishmania*l activity of HESA-A on *Leishmania major* (MRHO/HR/75/ER) promastigotes was quantitatively measured by colorimetric assay using MTT. For MTT reagent preparation, five mg of MTT powder was dissolved in one ml of sterile PBS solution (five mg/mL). A volume of 100 μL of HESA-A at different increasing concentrations ranged between 1.625 - 120 μg/mL, and amphotericin B (40 μg/mL) was added to 2×10^6 cells/mL of promastigotes in a 96-well flat-bottom plate at a final volume of 100 μL. Plates were incubated at 24°C for 72 h. 100 μL of promastigotes (without drug), at a seeding density of 2×10^6 cells/mL, was used as a negative control. All trials were performed in triplicates at three-time points of 24, 48, and 72 h. Subsequently, 20 μL of MTT (5 mg/mL in distilled water) was added to each well, and plates were incubated for 4 h at 24°. Further, to define the cell viability, supernatants were carefully harvested and discarded. Then, 100 μL of DMSO was added to each well to dissolve the colored formazan crystals. In the end, cell viability was estimated by checking the optical density at 540 nm using an ELISA reader device. Growth inhibition percentage was assessed using the below formula:

Viable promastigotes (%) = (AT-AB)/ (AC-AB) $\times$ 100

AT, Absorbance of the exposed promastigotes; AC, absorbance of the unexposed promastigotes; AB, absorbance of the blank.

Finally, the results expressed by IC_{50} (the inhibited half of the promastigotes growth) in the experimental group were compared to positive controls (promastigotes treated with 100 μL of amphotericin B 40 μg/mL) and negative controls (untreated promastigotes) (31, 35).

3.5. Determination of Cellular Morphology in Leishmania major Promastigotes

After 72 h incubation of promastigotes with HESA-A, the organisms' possible changes were investigated under an optical microscope with a magnification level: 100$\times$. In brief, the examination included centrifuging promastigotes at a low speed (1000 g for 3 minutes), suspending in PBS, and observing the morphological changes, including cell shrinkage and motility in experimental and control samples at different times and at least 20 microscopic fields for each sample. Eventually, the treated group was

compared with the untreated group in terms of morphological changes and motility.

3.6. Flow Cytometric Analysis of Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium

3.6.1. Iodide (PI) Binding of Cells

Binding of annexin V-FITC/PI in treated/untreated promastigotes was detected using FITC annexin V Apoptosis Detection kit II (BD Pharmingen™, New Jersey, USA). The whole process was done according to the manufacturer's instructions with minimum modifications. Briefly, 200 μL of promastigotes (1×10^7/mL), previously incubated with different concentrations of HESA-A for 24, 48, and 72 h, were initially collected. Cells were also washed twice using cold PBS, suspended in a 400 μL binding buffer again, and then were transferred to a 5 mL tube. Later, 5 μL of FITC annexin V and 5 μL of propidium iodide (PI) were added, and the cells were incubated for 20 min at room temperature and in the dark. The cells obtained at 24, 48, and 72 h were finally analyzed on a flow cytometer (Becton Dickinson, USA), using CellQuest software.

3.7. DNA Laddering Experiment

For the qualitative measure of fragmentation of DNA as a function of apoptotic cell death, agarose gel electrophoresis was employed, as described before (21). Briefly, *Leishmania major* promastigotes (1×10^7/mL) were, firstly, collected at different growth time points. Then, to extract DNA from induced-apoptosis and non-induced promastigotes, an apoptotic DNA ladder kit was utilized following the manufacturer's protocol. Later, 10 μg from each DNA aliquot was separated on a 1.5% agarose gel containing ethidium bromide (0.5 μg/mL) by electrophoresing for two h at 80 V. Then, DNA bands were observed and photographed by using Gel Doc device (Uvidoc, Gel Documentation System, Cambridge, UK).

3.8. Statistical Analyses

All data were analyzed for significance using SPSS software (version 19). P-values less than 0.05 were assumed as statistically significant.

4. Results

4.1. In Vitro Cytotoxic Effect of HESA-A on Leishmania major Promastigotes by MTT Assay

The inhibitory effect of HESA-A on *Leishmania major* promastigotes was investigated. The IC_{50} value of the compound at three-time points of 24, 48, and 72 h was estimated at 40.35 μg/mL, 13.82 μg/mL, and 2.81 μg/mL, respectively. In addition, HESA-A revealed a dose- and time-dependent activity against parasites in vitro exhibited in Figure 1. In the positive control group, cells were exposed to amphotericin B at several concentrations, and the IC_{50} value at three-time points of 24, 48, and 72 h was obtained 120 μg/mL, 63 μg/mL, and 40 μg/mL by MTT assay.

4.2. Determination of Morphological Alterations in Leishmania major Promastigotes

Investigation of treated (2.81 μg/mL in 72 h) promastigotes, under an optical microscope with magnification level, was as follows: 100× showed intact rounding shapes along with condensed cytoplasm shrinkage of cells and immobility. In addition, the number of cells significantly decreased after treatment. In non-exposed promastigotes, there was neither a change in shape nor a decrease in number (Figure 2).

4.3. HESA-A Induces Externalization of Membrane-Associated PS in Promastigotes of Leishmania major

To differentiate between the necrotic and apoptotic exhibition of cell death in *Leishmania major* promastigotes, double stains annexin V-FITC and PI were simultaneously used in the flow cytometry method. Annexin V is a protein that adheres to PS in apoptotic cells, translocating from inner to outer layers. Unlike annexin V, PI, which does not penetrate live cells, selectively enters permeable necrotic cells and binds to DNA (36).

Moreover, the flow cytometry technique can diagnose progressive cells towards primary apoptosis [annexin V(+), PI (-)], late apoptosis [annexin V (+), PI (+)], necrosis [annexin V (-), PI (+)], and viable cells [annexin V (-), PI (-)]. In the exposed group, after 72 h incubation of treated *Leishmania major* promastigotes with the IC_{50} dose (2.81 μg/mL), the percentage of promastigotes in early and late phases of apoptosis was 5.4% and 60.4%, respectively. In the control group, however, these percentages were 3.7% and 2.3%, respectively. Other data are illustrated in Figure 3.

4.4. DNA Fragmentation in Leishmania major Promastigotes

The laddering pattern of *Leishmania major* promastigotes, treated with 2.81 μg/mL of HESA-A for 72 h, was observed in 1.5% agarose gel electrophoresis (as depicted in Figure 4).

5. Discussion

Several efforts have been made so far for introducing new anti-*Leishmania* agents since there have been increasing drug-resistant *Leishmania* cases. In addition, the side effects of conventional antimonial drugs have remained a concerning issue. Leishmaniasis is a tropical disease

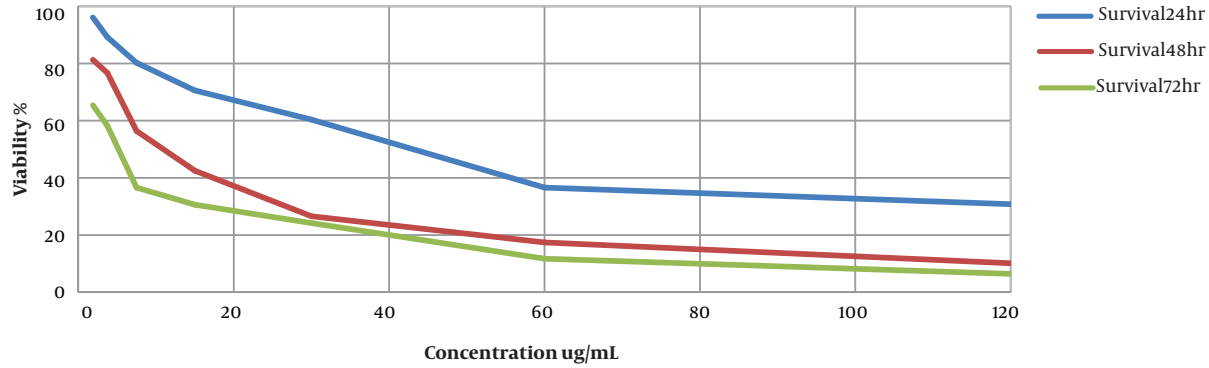

Figure 1. Inhibitory effect of the HESA-A on *Leishmania major* promastigotes at three-time points of 24, 48, and 72 h.

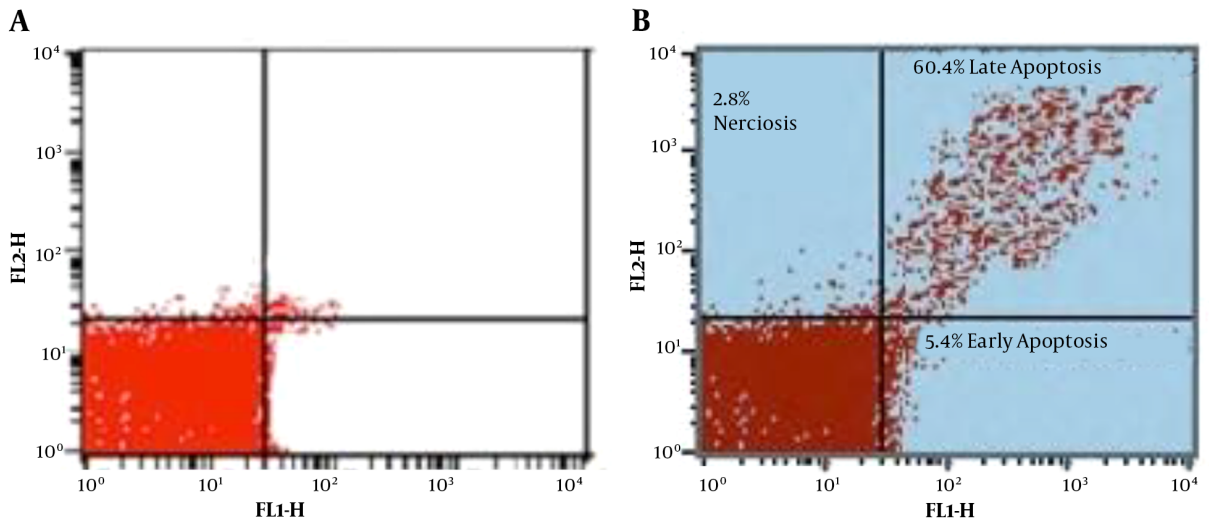

Figure 2. Flow cytometry analysis: Apoptosis occurrence in control (A) and treated (B) *Leishmania major* promastigotes after 72 h treatment with HESA-A (2.81 μg/mL).

treated with non-leishmaniasis medication. Recent research on the treatment of leishmaniasis showed that researchers' focus was more on anti-leishmaniasis medicine in combination with non-leishmaniasis drugs, which can also overcome drug resistance issues. Previous studies reported significant antitumor effects of HESA-A, a new Iranian immunomodulating medication (30, 37). This compound has recently attracted many researchers' attention to investigate the potential antimicrobial possibility of its organic and synthetic elements. Here, we decided to evaluate the induction of apoptosis of HESA-A in *Leishmania major* promastigotes in vitro since several reports on the effects of various elemental components of HESA-A in inducing oxidative stress and cell apoptosis in cancer cells (38). Apoptosis is a strategy for the treatment of cancer cells; anti-cancer drugs are often produced purposefully (39-42). Numerous antitumor medications could eradicate tumor cells by triggering the apoptotic process. This signifies the importance of exploiting the apoptotic mechanism. In this study, HESA-A, as an anti-cancer drug, like other anti-cancer medications, induced apoptosis in the *Leishmania major* promastigotes. Our previous study showed that miltefosine induced apoptosis in *Leishmania major* and *Leishmania ropica* promastigotes (21).

Doroodgar et al. (43) reported that tamoxifen-induced apoptosis of *Leishmania major* in a dose- and time-dependent way. Investigations on the pathways through which apoptosis could be triggered in cell lines have indicated that tamoxifen induces apoptosis by increasing the activation of caspase-3 in a dose- and time-dependent man-

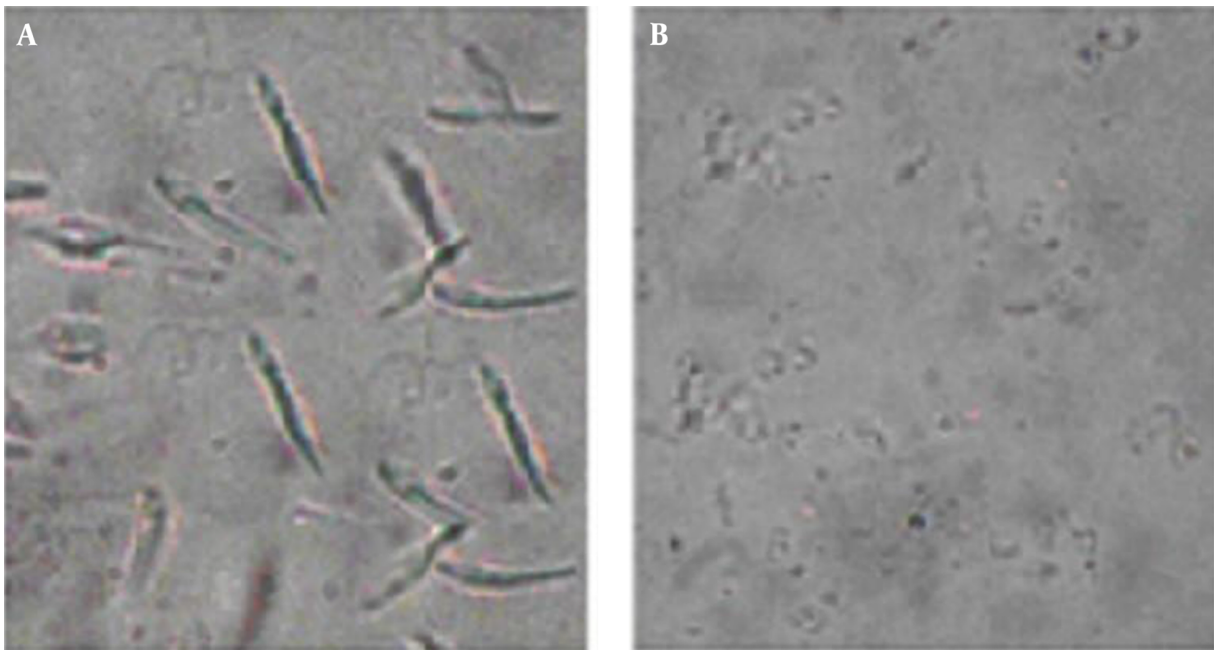

Figure 3. Cellular morphology of HESA-A treated *Leishmania major* promastigotes. A, *Leishmania major* 0 hours after treatment; and B, *Leishmania major* 72 h after treatment.

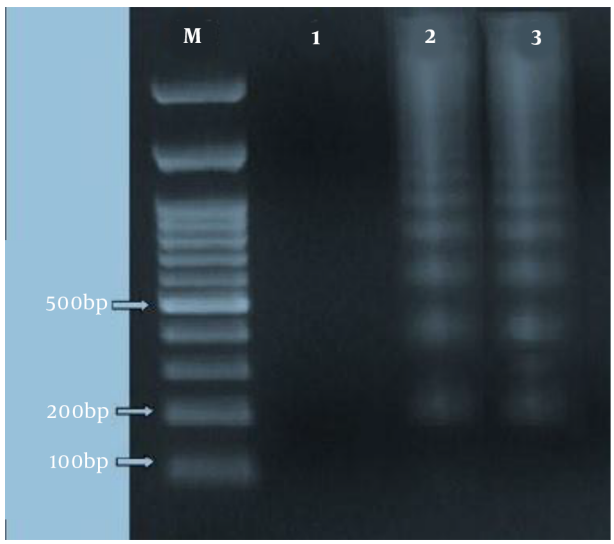

Figure 4. The DNA fragmentation detection of *Leishmania major* on agarose gel electrophoresis. Lanes: Lane M, size marker, lane 1, not treated; lane 2, Positive apoptotic cell (positive control); lane 3, 72 h after treatment with HESA-A.

ner. Flow cytometry analysis also showed that tamoxifen with 50 μg/L concentration after 48 h increased apoptosis from 2% to 59.7% in *Leishmania* promastigotes (43). Our previous study on the possibility of apoptosis-inducing potential of sea cucumbers (*Holothuria leucospilota*), a marine invertebrate animal, in *Leishmania major* promastigotes showed various manifestations of apoptosis after treatment, including cell shrinkage, the formation of apoptotic bodies, blebbing of the cell membrane, and the externalization of PS (not of laddering pattern) (31).

5.1. Conclusions

Our study showed that HESA-A induced apoptotic-like cell death in *Leishmania major* parasites. This significant effect could be due to combining two substances of marine and herbal origin, which probably both simultaneously affect the growth of *Leishmania major* promastigotes. In conclusion, HESA-A, with a significant inhibitory effect on the growth of *Leishmania major* promastigotes and the induction of apoptosis in them, can be a plausible treatment of cutaneous Leishmaniasis. We also recommend further studies on the anti-*Leishmania*l effect of this drug in vivo.

Acknowledgments

This article results from a research project (code RDC-9504), funded by the University's Vice Chancellor for Research and Technology development of Ahvaz Jundishapur University of Medical Sciences. The authors of the manuscript would like to thank the esteemed staff of the university's comprehensive laboratory and the Department of Parasitology, Faculty of Medicine, for their valuable assistance.

Footnotes

Authors' Contribution: Culture: MTT and KS. Data analysis and writing the manuscript: RA. Flow cytometry, DNA fragmentation, and supervision of the project: JS.

Conflict of Interests: The authors declared no conflict of interest.

Ethical Approval: IR.AJUMS.REC.1395.150.

Funding/Support: This manuscript is financially supported by Research Deputy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (code: RDC-9504).

References

1. Ellis RE, Yuan JY, Horvitz HR. Mechanisms and functions of cell death. *Annu Rev Cell Biol*. 1991;**7**:663–98. doi: 10.1146/annurev.cb.07.110191.003311. [PubMed: 1809356].
2. Raff MC. Social controls on cell survival and cell death. *Nature*. 1992;**356**(6368):397–400. doi: 10.1038/356397a0. [PubMed: 1557121].
3. Vaux DL. Toward an understanding of the molecular mechanisms of physiological cell death. *Proc Natl Acad Sci U S A*. 1993;**90**(3):786–9. doi: 10.1073/pnas.90.3.786. [PubMed: 8430086]. [PubMed Central: PMC45754].
4. Steller H. Mechanisms and genes of cellular suicide. *Science*. 1995;**267**(5203):1445–9. doi: 10.1126/science.7878463. [PubMed: 7878463].
5. Heussler VT, Kuenzi P, Rottenberg S. Inhibition of apoptosis by intracellular protozoan parasites. *Int J Parasitol*. 2001;**31**(11):1166–76. doi: 10.1016/s0020-7519(01)00271-5. [PubMed: 11563357].
6. Deponte M. Programmed cell death in protists. *Biochim Biophys Acta*. 2008;**1783**(7):1396–405. doi: 10.1016/j.bbamcr.2008.01.018. [PubMed: 18291111].
7. Shemarova IV. Signaling mechanisms of apoptosis-like programmed cell death in unicellular eukaryotes. *Comp Biochem Physiol B Biochem Mol Biol*. 2010;**155**(4):341–53. doi: 10.1016/j.cbpb.2010.01.010. [PubMed: 20117230].
8. Ameisen JC, Idziorek T, Billaut-Mulot O, Loyens M, Tissier JP, Potentier A, et al. Apoptosis in a unicellular eukaryote (Trypanosoma cruzi): implications for the evolutionary origin and role of programmed cell death in the control of cell proliferation, differentiation and survival. *Cell Death Differ*. 1995;**2**(4):285–300. [PubMed: 17180034].
9. Cornillon S, Foa C, Davoust J, Buonavista N, Gross JD, Golstein P. Programmed cell death in Dictyostelium. *J Cell Sci*. 1994;**107 (Pt 10)**:2691–704. [PubMed: 7876338].
10. Davis MC, Ward JG, Herrick G, Allis CD. Programmed nuclear death: apoptotic-like degradation of specific nuclei in conjugating Tetrahymena. *Dev Biol*. 1992;**154**(2):419–32. doi: 10.1016/0012-1606(92)90080-z. [PubMed: 1426647].
11. Ridgley EL, Xiong ZH, Ruben L. Reactive oxygen species activate a Ca2+-dependent cell death pathway in the unicellular organism Trypanosoma brucei brucei. *Biochem J*. 1999;**340 (Pt 1)**:33–40. [PubMed: 10229656]. [PubMed Central: PMC1220219].
12. Vardi A, Berman-Frank I, Rozenberg T, Hadas O, Kaplan A, Levine A. Programmed cell death of the dinoflagellate Peridinium gatunense is mediated by CO(2) limitation and oxidative stress. *Curr Biol*. 1999;**9**(18):1061–4. doi: 10.1016/s0960-9822(99)80459-x. [PubMed: 10508616].
13. Welburn SC, Lillico S, Murphy NB. Programmed cell death in procyclic form Trypanosoma brucei rhodesiense -identification of differentially expressed genes during con A induced death. *Mem Inst Oswaldo Cruz*. 1999;**94**(2):229–34. doi: 10.1590/s0074-02761999000200020. [PubMed: 10224534].
14. Hamann A, Brust D, Osiewacz HD. Apoptosis pathways in fungal growth, development and ageing. *Trends Microbiol*. 2008;**16**(6):276–83. doi: 10.1016/j.tim.2008.03.003. [PubMed: 18440231].
15. Wanderley JL, Benjamin A, Real F, Bonomo A, Moreira ME, Barcinski MA. Apoptotic mimicry: an altruistic behavior in host/Leishmania interplay. *Braz J Med Biol Res*. 2005;**38**(6):807–12. doi: 10.1590/s0100-879x2005000600001. [PubMed: 15933773].
16. Arnoult D, Akarid K, Grodet A, Petit PX, Estaquier J, Ameisen JC. On the evolution of programmed cell death: apoptosis of the unicellular eukaryote Leishmania major involves cysteine proteinase activation and mitochondrion permeabilization. *Cell Death Differ*. 2002;**9**(1):65–81. doi: 10.1038/sj.cdd.4400951. [PubMed: 11803375].
17. Holzmuller P, Sereno D, Cavaleyra M, Mangot I, Daulouede S, Vincendeau P, et al. Nitric oxide-mediated proteasome-dependent oligonucleosomal DNA fragmentation in Leishmania amazonensis amastigotes. *Infect Immun*. 2002;**70**(7):3727–35. doi: 10.1128/IAI.70.7.3727-3735.2002. [PubMed: 12065515]. [PubMed Central: PMC128075].
18. Moreira ME, Del Portillo HA, Milder RV, Balanco JM, Barcinski MA. Heat shock induction of apoptosis in promastigotes of the unicellular organism Leishmania (Leishmania) amazonensis. *J Cell Physiol*. 1996;**167**(2):305–13. doi: 10.1002/(SICI)1097-4652(199605)167:2<305::AID-JCP15>3.0.CO;2-6. [PubMed: 8613472].
19. Sereno D, Holzmuller P, Mangot I, Cuny G, Ouaissi A, Lemesre JL. Antimonial-mediated DNA fragmentation in Leishmania infantum amastigotes. *Antimicrob Agents Chemother*. 2001;**45**(7):2064–9. doi: 10.1128/AAC.45.7.2064-2069.2001. [PubMed: 11408224]. [PubMed Central: PMC90601].
20. Zangger H, Mottram JC, Fasel N. Cell death in Leishmania induced by stress and differentiation: programmed cell death or necrosis? *Cell Death Differ*. 2002;**9**(10):1126–39. doi: 10.1038/sj.cdd.4401071. [PubMed: 12232801].
21. Khademvatan S, Gharavi MJ, Rahim F, Saki J. Miltefosine-induced apoptotic cell death on Leishmania major and L. tropica strains. *Korean J Parasitol*. 2011;**49**(1):17–23. doi: 10.3347/kjp.2011.49.1.17. [PubMed: 21461264]. [PubMed Central: PMC3063921].
22. Kolodziej H, Kiderlen AF. Antileishmanial activity and immune modulatory effects of tannins and related compounds on Leishmania parasitised RAW 264.7 cells. *Phytochemistry*. 2005;**66**(17):2056–71. doi: 10.1016/j.phytochem.2005.01.011. [PubMed: 16153409].
23. Croft SL, Seifert K, Yardley V. Current scenario of drug development for leishmaniasis. *Indian J Med Res*. 2006;**123**(3):399–410. [PubMed: 16778319].
24. Croft SL, Yardley V. Chemotherapy of leishmaniasis. *Curr Pharm Des*. 2002;**8**(4):319–42. doi: 10.2174/1381612023396258. [PubMed: 11860369].
25. Sundar S. Drug resistance in Indian visceral leishmaniasis. *Trop Med Int Health*. 2001;**6**(11):849–54. doi: 10.1046/j.1365-3156.2001.00778.x. [PubMed: 11703838].
26. Ahmadi A, Mohagheghi M, Karimi M, Seyed Ali G, Naseri M. Anticancer effects of HESA-A in patients with metastatic colon cancer. *Integr Cancer Ther*. 2009;**8**(1):71–4. doi: 10.1177/1534735408327995. [PubMed: 19147644].
27. Sadeghi-Aliabadi H, Ahmadi A. Cytotoxicity and Antitumor Properties of a Marine Compound, HESA-A, on Cancer Cells. *DARU J Pharm Sci*. 2003;**11**(3):82–7.
28. Sadeghi Aliabadi H, Ahmadi A. Cytotoxicity and antitumor properties of a marine compound on cancer cells (HESA-A). *Med J Islam Acad Sci*. 2001;**13**:55–61.
29. Ahmadi A, Mohagheghi MA, Fazeli MS, Nahavandian B, Bashardoost N, Jarahi AM, et al. HESA-A: new treatment for breast cancer and choroidal metastasis. *Med Sci Monitor*. 2005;**11**(6):CR300–3.
30. Ahmadi A, Mohgheghi MA, Sharif-Tabrizi A. Introducing the therapeutic effects of HESA-A on osteosarcoma included in rabbits. *Proceedings of the First National Congress on Cancer Research, Orumiah, Iran*. 2001. p. 5–8.

31. Foroutan-Rad M, Khademvatan S, Saki J, Hashemitabar M. Holothuria leucospilota Extract Induces Apoptosis in Leishmania major Promastigotes. *Iran J Parasitol*. 2016;**11**(3):339–49. [PubMed: 28127339]. [PubMed Central: PMC5256050].
32. Vahabpour R, Sadat S, Zabihollahi R, Ahmadi A, Keivani H, Amini S, et al. In vitro inhibitory effects of the herbal-marine compound HESA-A against replication of human immunodeficiency virus-1. *Jundishapur J Microbiol*. 2012;**5**(1):315–9.
33. Sadeghi-Nejad B, Saki J, Khademvatan S, Nanaei S. In vitro antileishmanial activity of the medicinal plant-Satureja khuzestanica Jamzad. *J Med Plants Res*. 2011;**5**(24):5912–5.
34. Yousefi E, Eskandari A, Gharavi MJ, Khademvatan S. In vitro activity and cytotoxicity of Crocus sativus extract against leihmania major (MRHO/IR/75/ER). *Infect Disord Drug Targets*. 2014;**14**(1):56–60. doi: 10.2174/1871526514666140827101901. [PubMed: 25159304].
35. Feily A, Saki J, Maraghi S, Moosavi Z, Khademvatan S, Siahpoosh A. In vitro activity of green tea extract against Leishmania major promastigotes. *Int J Clin Pharmacol Ther*. 2012;**50**(3):233–6. doi: 10.5414/cp201571. [PubMed: 22373836].
36. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods*. 1995;**184**(1):39–51. doi: 10.1016/0022-1759(95)00072-i. [PubMed: 7622868].
37. Ahmadi A. Method of preparation of an antitumor herbal drug (HESA-A). *Islam Republ Iran Patent*. 2002;**27741**.
38. Qiao B, He B, Cai J, Lam AK, He W. Induction of oxidative stress and cell apoptosis by selenium: the cure against oral carcinoma. *Oncotarget*. 2017;**8**(69):113614–21. doi: 10.18632/oncotarget.22752. [PubMed: 29371933]. [PubMed Central: PMC5768350].
39. Zedan W, Mourad MI, El-Aziz SMA, Elsabaa HM, Abou Elkhier MT. Evaluation of Caspase 3 as a Target for Apoptosis induced via Chemotherapy in Rats. *Int J Adv Res*. 2015;**3**:1591–601.
40. Chen DL, Engle JT, Griffin EA, Miller JP, Chu W, Zhou D, et al. Imaging caspase-3 activation as a marker of apoptosis-targeted treatment response in cancer. *Mol Imaging Biol*. 2015;**17**(3):384–93. doi: 10.1007/s11307-014-0802-8. [PubMed: 25344147]. [PubMed Central: PMC4874215].
41. Bosserman L, Rogers K, Willis C, Davidson D, Whitworth P, Karimi M, et al. Application of a drug-induced apoptosis assay to identify treatment strategies in recurrent or metastatic breast cancer. *PLoS One*. 2015;**10**(5). e0122609. doi: 10.1371/journal.pone.0122609. [PubMed: 26024531]. [PubMed Central: PMC4449169].
42. De Saint-Hubert M, Bauwens M, Verbruggen A, Mottaghy FM. Apoptosis imaging to monitor cancer therapy: the road to fast treatment evaluation? *Curr Pharm Biotechnol*. 2012;**13**(4):571–83. doi: 10.2174/138920112799436320. [PubMed: 22214506].
43. Doroodgar M, Delavari M, Doroodgar M, Abbasi A, Taherian AA, Doroodgar A. Tamoxifen Induces Apoptosis of Leishmania major Promastigotes in Vitro. *Korean J Parasitol*. 2016;**54**(1):9–14. doi: 10.3347/kjp.2016.54.1.9. [PubMed: 26951973]. [PubMed Central: PMC4792327].