

Iranian *Mesobuthus Eupeus* Crude Venom Induces Selective Toxicity in Chronic Lymphocytic Leukemia B-Lymphocytes Through Lysosomal/Mitochondrial Dysfunction and Reactive Oxygen Species Formation

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Abstract

From ancient times to the present-day animal venoms had been used as medicinal and therapeutic agents. Recently it has been reported that the scorpion venom is a potential source of active and therapeutic compounds to design potent drugs against variety of cancerous cells and other diseases. The current study aimed to evaluate the selective toxicity of Iranian *Mesobuthus eupeus* (IMe) crude venom as a potential source of anticancer compounds on cancerous CLL B-lymphocytes and normal lymphocytes. For this purpose, we isolated cancerous CLL B-lymphocytes and normal lymphocytes from chronic lymphocytic leukemia patients and healthy volunteers. Cancerous CLL B-lymphocytes and normal lymphocytes were treated with different concentration (0, 5, 10, 20, 40 and 80 µg/ml) of IMe crude venom for 12 hours and cytotoxicity, reactive oxygen species (ROS) production, collapse of mitochondrial membrane potential (MMP) and lysosomal membrane integrity were determined. The data demonstrated the significant cytotoxic effect of IMe crude venom on cancerous CLL B-lymphocytes, with a concentration value (IC₅₀) that inhibits 50% of the cell viability of 60 µg/ml after 12 h of incubation. MTT assay proved that the IMe crude venom is selectively toxic to cancerous CLL B-lymphocytes, and IMe crude venom induced selective cell death via activation of ROS formation and mitochondrial/lysosomal dysfunction. These finding showed that IMe crude venom has a selective mitochondrial/lysosomal-mediated cell death effect on cancerous CLL B-lymphocytes. Therefore, the IMe crude venom and its fractions may be promising in the future anticancer drug development for treatment of CLL and variety of cancers.

Keywords: Leukemia- Cytotoxicity- Animal Toxins- Anticancer Effect- Scorpion

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Introduction

Since the beginning of civilization, for thousands of years, the natural substances due to their therapeutic properties were used by humans (Dias et al., 2012). Toxins made from poisonous animals are potent and selective to vital physiological processes in hunted animals (Bordon et al., 2020). Venom is defined as a mixture of biologically active proteins, polypeptides and other components include carbohydrates, lipids, amines and etc (Lee et al., 2016). About 3% percent of the used medicines in the market are originated directly or indirectly from animals (Calixto, 2019).

Animal toxins constitute an extremely important

resource to develop new drugs. These natural toxins are used as: I) a direct source of therapeutic agents; II) as taxonomic markers for discovery of new drugs; III) prototypes for design of lead molecules and III) a source of raw material for development of complex, semi-synthetic drugs (Calixto, 2019). As noted above, about 3% of the new drugs approved by the Food and Drug Administration (FDA) are animal sources, but best-selling and many important drugs originated from animals are toxins (Calixto, 2019). There are several successful toxin-derived drugs such as captopril, Enalapril exenatide and ziconotide (Stepensky, 2018). Therefore, it is not surprising that studies focus on animal venoms to evaluate therapeutic potential of crude venoms and identify their

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lead compounds for the development of new therapeutics (Coulter-Parkhill et al., 2021). The use of natural toxins in the process of drug discovery and development has several clear advantages (Peigneur and Tytgat, 2018). First, animal venoms represent chemical novelties and can originate lead drug candidate for complex targets (Peigneur and Tytgat, 2018). Second, despite bi- and tri-dimensional complex structures of biologically active proteins and polypeptides presence in the animal venoms, they can still be easily absorbed and metabolized in the body compared to synthetic compounds (Peigneur and Tytgat, 2018). One of the most valuable sources of these animal toxins are scorpions, which are found in various parts of the world (Utkin, 2015). These animals have a lot of diversity in the world, so studying their therapeutic potential, can be valuable in identifying potential therapeutic sources for different diseases such as cancers.

Scorpions with the 18 families and about 1500 different species are venomous animals which have distributed all over the world except Antarctica (Jalali and Rahim, 2014). Scorpion venom is a mixture of various peptides including some enzymes like, phospholipases, alkaline phosphatases, hyaluronidases, acetylcholinesterase's, sphingomyelinases and proteolytic enzymes, neurotransmitters, amino acids, ions and low molecular weight peptides (less than 10 KDa) (Ortiz et al., 2015). The scorpion venoms are used to cure different diseases like acute and chronic convulsions, cardiovascular diseases, epilepsy, tetanus, subcutaneous nodules, human immunodeficiency virus (HIV), human leukemia cell lines, male impotency, kidney tumor, brain tumor, breast cancer, prostate tumor, skin cancers, rheumatism and pancreatitis (Nabi et al., 2015; Attarde and Pandit, 2016; Tobassum et al., 2020). As mentioned above scorpion venoms are very effective for the treatment of various types of cancer cells (Rapôso, 2017). Scorpion venom-induced antiproliferative and cytotoxic effects are mediated by inducing apoptosis in the cancerous cells (Rapôso, 2017). It has been reported that the venom of *Mesobuthus eupeus* from the family Buthidae (known as a spotted yellow scorpion) has various therapeutic potentials including anti-tumor effects (Hmed et al., 2013). Therefore, the venom of Iranian *Mesobuthus eupeus* can be used against a vast variety of cancers like chronic lymphocytic leukemia (CLL).

It has been reported that approximately 10% of all cancers are hematologic in origin (Hallek, 2019). CLL is the commonest leukemia in western countries with a highly variable clinical course and typically occurs in elderly patients (Hallek, 2019). This disease is initiated by specific genomic mutations that impair apoptosis of clonal B-lymphocytes (Puiggros et al., 2014). Cancerous B-lymphocytes escape apoptosis by different mechanism which can lead to inhibition of apoptosis (Hassan et al., 2014). Previous studies shown that animal toxins obtained to date mainly from snakes, scorpions, snails and sea cucumbers, have a long history of beneficial use for the induction of cytotoxicity in cancerous cells (Rapôso, 2017; Salimi et al., 2017; Bordon et al., 2020; Salimi et al., 2021). Also, it has been reported that about 60-80% of anti-cancer and antibiotics drugs approved by FDA are derived from natural products (Calixto, 2019). Therefore,

the current study aims to evaluate the selective toxicity of *IME* crude venom as a potential source of anticancer compounds on cancerous B-lymphocytes and normal lymphocytes.

Materials and Methods

Chemicals and Solution

2',7'-dichlorofluorescein diacetate (DCFH-DA), MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Bovine Serum Albumin (BSA), Rhodamine123, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), Trypan blue, Acridine Orange, Fetal Bovine Serum (FBS), 2-mercaptoethanol and Antibiotic-Antimycotic Solution were obtained from the Sigma Chemical Co. Ficoll-paque PLUS was purchased from Ge Healthcare Bio-Science Company. RPMI1640 was provided from GIBCO (USA).

Collection of Venom

The scorpions (Iranian *Mesobuthus eupeus*) were collected with UV light at night from around the city of Ardabil (Ardabil, Iran). Crude venom was obtained from Iranian *Mesobuthus eupeus* scorpion by electric stimulation at the end of tail. The protein content of fresh venom was determined by the Bradford assay (Kielkopf et al., 2020). For exposure of the lymphocytes the fresh venom previously was dissolved in distilled water.

Blood Samples Collection

The experiments were performed by using isolated human lymphocytes from CLL patients and healthy subjects. For this purpose, ten diagnosed, untreated CLL patients and age-matched healthy individuals (50-60) were entered to the study. For healthy individuals, the criteria of acceptability to warrant reliability of the study were: receiving any medical therapy, from non-alcoholic and non-smoker subjects, having good health and without serious illness. The CLL patients were diagnosed on the basis of clinical examination, immunological and morphological criteria by Dr. Leila Sadeghi as an oncologist. Blood samples were collected from the patients and healthy individuals after informed consent. The study was approved by the ethics committee of Ardabil University of Medical Sciences with approval code IR.ARUMS.REC.1398.596.

Lymphocytes Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples with Ficoll-Paque Plus. Briefly, blood sample containing anticoagulant (5 ml) was gently mixed with equal volume of normal saline (5 ml) in a sterile tube (15 ml). In the next step 3 ml of Ficoll-paque Plus was added two sterile tubes and 5 ml of diluted blood was gently added to each. The tubes were centrifuged at 800 g at 4 °C for 20 min. After centrifugation, the buffy coat layer between plasma and Ficoll-Paque Plus layers was gently isolated using Pasteur pipette and added to a new sterile tube. The collected layer of buffy coat was centrifuged at 400 g at 4°C for 10 min. The obtained pellet was suspended and incubated in erythrocyte lysis buffer at

37°C for 5 min and then was centrifuged again at 400 g at 4°C for 10 min. The supernatant was discarded, and the pellet were washed with RPMI1640 supplemented with L-glutamine 10% and fetal bovine serum (FBS) and centrifuged at 2000g for 7 min. The isolated lymphocytes were suspended in RPMI1640 medium (containing L-glutamine and 10 % FBS) at 37° C in normal condition with a humidified atmosphere and 5% CO₂. At the beginning of each experiment, the number of the living cells were measured by Trypan Blue and the cell viability percentage of acceptability to ensure reliability of the experiment was more than 95% (Salimi et al., 2016).

Cell Treatment

Human isolated lymphocytes were incubated in RPMI1640 supplemented with L-glutamine 10%, FBS and antibiotics (100 µg/ml penicillin and streptomycin) in a humidified incubator in the presence of 5% CO₂-95% air at 37°C. Human isolated lymphocytes treated with different concentrations of IMe crude venom (0, 5, 20, 40 and 80 µg/ml) for 12 hours. After determining IC₅₀ using MTT assay at 12 hours, the concentrations of 40, 60 and 80 µg/ml were tested for more mechanistic studies. Control cells were treated with vehicle (normal saline) alone.

Cell Viability Measurement

Human isolated lymphocytes (104 cells per well) were incubated in 96-well culture plates in 100 µl of RPMI1640 at 37°C with 5% CO₂ in a humidified atmosphere for 12 hours with different concentrations of IMe crude venom (0, 5, 20, 40 and 80 µg/ml). After treatment with IMe crude venom, 25 µl of MTT (0.5 mg/ml) was added to each well and incubated again for 4 hours at 37°C. in the next step, to dissolve the water-insoluble formazan salt, 100 ml of DMSO was added to each well. The optical density of solved formazan salts was measured at absorbance λ=570 nm with an ELISA microplate reader. The cell viability was represented as the percentage absorbance compared with control group (treated with normal saline). Each experiment was independently repeated three times (Soares et al., 2015). To evaluate the IC₅₀ concentration, we used a series of concentration-response results (different concentrations against cell survival) and the linear (y = mx+n) equation on this graph for y = 50 value x point becomes IC₅₀ value.

ROS Formation Measurement

The generation of ROS was measured by 2',7'-dichlorofluorescein diacetate (DCFH-DA). Inside the cytosol by intracellular esterase, DCFH-DA hydrolyzed to the nonfluorescent DCFH, which in the presence of reactive oxygen species, could be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF). After treatment for 12 hours with the concentrations of IMe crude venom (40, 60 and 80µg/ml), the human isolated lymphocytes were washed with phosphate-buffered saline solution and incubated with DCFH-DA (at final concentration of 5 µM) for an additional 20 min at 37°C in the dark. The isolated human lymphocytes that were previously exposed with DCFH-DA dye, using 1 min centrifugation at 1,000 rpm, were separated from the

medium. The additional DCFH-DA from the medium was removed by the cell washing twice using PBS. The fluorescent intensity of DCF was measured by flow cytometry (Cyflow Space-Partec, Germany) and mean of fluorescence intensity was analyzed by software (FlowJo) (Huang et al., 2017). The data were represented as fluorescent intensity per 104 cells. Each experiment was independently repeated three times.

Mitochondrial Membrane Potential (MMP) Measurement

Rhodamine 123 as a fluorescent probe was used to monitor the membrane potential of mitochondria. Rhodamine 123, as a fluorescent, lipophilic and cationic probe is accumulated in mitochondria. The fluorescence intensity of rhodamine 123 is quenched when this dye is accumulated in mitochondria and its re-release indicates loss of mitochondrial membrane potential and mitochondrial damage. After treatment for 12 hours with the concentrations of IMe crude venom (40, 60 and 80µg/ml), the human isolated lymphocytes were washed with PBS solution and incubated with rhodamine 123 (at final concentration of 5 µM) for an additional 20 min at 37°C in the dark. The isolated human lymphocytes that were previously exposed with rhodamine 123 dye, using 1 min centrifugation at 1,000 rpm, were separated from the medium. The additional rhodamine 123 from the medium was removed by the cell washing twice using PBS. The fluorescent intensity of rhodamine was measured by flow cytometry (Cyflow Space-Partec, Germany) and mean of fluorescence intensity was analyzed by software (FlowJo) (Baracca et al., 2003). The data were represented as fluorescent intensity per 104 cells. Each experiment was independently repeated three times.

Lysosomal Membrane Destabilization Measurement

To measure lysosomal membrane destabilization acridine orange was used. This probe as a lysosomotropic weak base accumulates in intact lysosomes on the basis of proton trapping and emits red fluorescence. Therefore, lysosomal membrane destabilization can be determined by detecting changes in the fluorescence intensity of acridine orange (Boya and Kroemer, 2008). After treatment 12 hours, with the concentrations of IMe crude venom (40, 60 and 80µg/ml), the isolated human lymphocytes were washed with PBS solution and incubated with acridine orange (at final concentration of 5 µM) for an additional 20 min at 37°C in the dark. The isolated human lymphocytes that were previously exposed with acridine orange dye, using 1 min centrifugation at 1,000 rpm, were separated from the medium. The additional acridine orange from the medium was removed by the cell washing twice using PBS. The fluorescent intensity of acridine orange was measured by flow cytometry (Cyflow Space-Partec, Germany) and mean of fluorescence intensity was analyzed by software (Brunk et al., 1995). The data were represented as fluorescent intensity per 10⁴ cells. Each experiment was independently repeated three times.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The GraphPad Prism version 9 were used for all statistical

analyses. Statistical significance was set at $P < 0.05$. Statistical significance was determined using one-way analysis of variance (ANOVA), followed by the post-hoc Tukey test. The flow cytometric results were obtained with Cyflow Space-Partec and analyzed by FlowJo software. All experiments were independently repeated three times.

Results

IME Crude Venom Selectively Decreased Cell Viability in CLL B- Lymphocytes

As shown in Figure 1 A, after 12 hours of treatment the isolated CLL B-lymphocytes with different concentration of IME crude venom (5, 10, 20, 40 and 80 $\mu\text{g/ml}$) decreased cell viability. Treatment with 5, 10, 20, 40 and 80 $\mu\text{g/ml}$ of IME crude venom decreased the cell viability to 88 ± 2.2 , 79 ± 1.5 , 70 ± 2.1 , 58 ± 2.4 and 39 ± 1.7 respectively. Our results suggest that IME crude venom is able to induce cytotoxicity in dose-dependent manner. While after 12 hours of treatment normal human lymphocytes with different concentration of IME crude venom (25, 50 and 100 $\mu\text{g/ml}$) did not decrease cell viability. Treatment with 25, 50, 100 and 200 $\mu\text{g/ml}$ of IME crude venom decreased the cell viability to 98 ± 1.2 , 97 ± 1.7 , 96.3 ± 1.9 and 91 ± 1.5 respectively (Figure 1 B). These results indicate that in the range of toxic concentrations for cancer cells (5-80 $\mu\text{g/ml}$), this crude venom does not have the ability to cause toxicity in normal cells (0-100 $\mu\text{g/ml}$). Therefore, it can be concluded that IME crude venom can selectively induce death in the isolated CLL B-lymphocytes versus normal human lymphocytes.

IME Crude Venom Enhanced ROS Formation in CLL B- lymphocytes

ROS is generated mainly by the mitochondria and excess ROS formation can be the cause of oxidative stress and cell death. ROS formation was detected by

flow cytometry using DCF-DA stain. ROS formation was enhanced after treatment with 60 and 80 $\mu\text{g/ml}$ IME crude venom for 12 hours and flow cytometry showed a considerable shift to the right in the isolated CLL B-lymphocytes. The quantitative results showed that at concentrations of 60 and 80 $\mu\text{g/ml}$ IME crude venom, the fluorescent intensity of DCF in the isolated CLL B-lymphocytes clearly increased, in a dose-dependent manner, to 7.6 ± 0.2 and 10.01 ± 0.5 respectively, compared with that in the control (5.12 ± 0.3). These results suggest that IME crude venom may cause selective toxicity in the isolated CLL B-lymphocytes through ROS formation (Figure 2).

IME Crude Venom induced Mitochondrial Membrane Potential collapse in CLL B- lymphocytes

A rhodamine 123 probe was used to detect alterations in mitochondrial membrane potential after IME crude venom treatment. In the isolated CLL B-lymphocytes treated with 40, 60 and 80 $\mu\text{g/ml}$ IME crude venom for 12 hours, mitochondrial membrane potential was disrupted, and the flow cytometry histogram showed a considerable shift to the right. The quantitative results indicated that at concentrations of 40, 60 and 80 $\mu\text{g/ml}$ IME crude venom, the fluorescent intensity of rhodamine 123 in the isolated CLL B-lymphocytes apparently increased to 78.91 ± 3.90 , 99.17 ± 4.11 and 139.06 ± 4.21 respectively, compared with that in the control (52.39 ± 2.98). These results suggest that IME crude venom may cause selective toxicity in the isolated CLL B-lymphocytes through mitochondrial dysfunction (Figure 3).

IME Crude Venom induced Lysosomal Damage in CLL B- lymphocytes

An acridine orange probe was used to detect alterations in lysosomal membrane destabilization after IME crude venom treatment. Lysosomal membrane destabilization was increased after treatment with 60

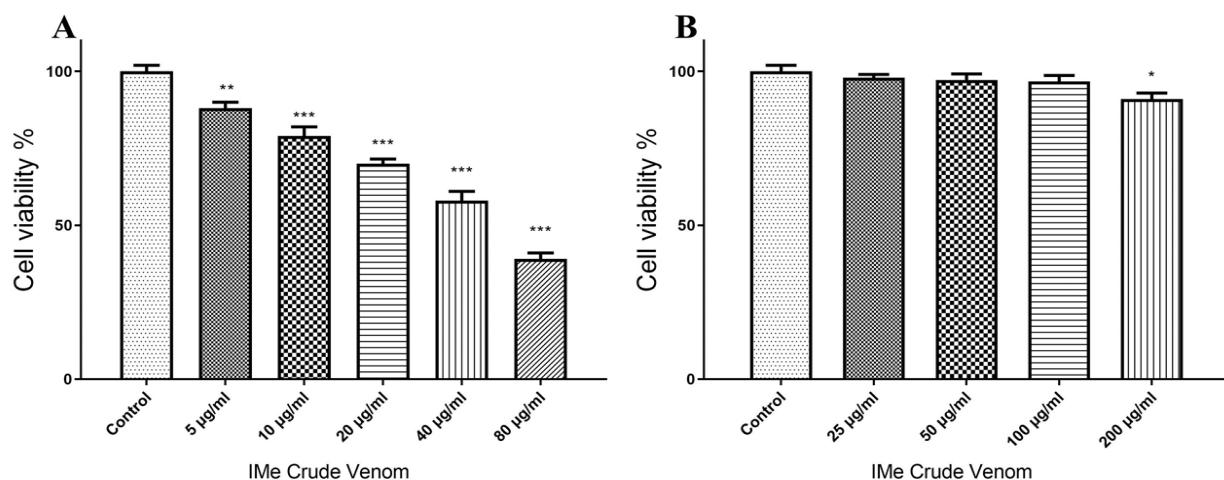


Figure 1. Effect of IME Crude Venom on the Viability of CLL B-lymphocytes and Normal Human Lymphocytes. (A), IME crude venom reduced the viability of CLL B-lymphocytes, which were treated with 5, 10, 20, 40 and 80 $\mu\text{g/ml}$ IME crude venom for 12 hours and assayed using MTT staining to measure cell viability; (B), at the used concentrations (5-80) in CLL B-lymphocytes, IME crude venom did not obviously influence the cell viability of normal human lymphocytes. The data are represented as the mean \pm SD of three independent experiments ($n=3$). Significance was determined using one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. IME, Iranian Mesobuthus eupeus.

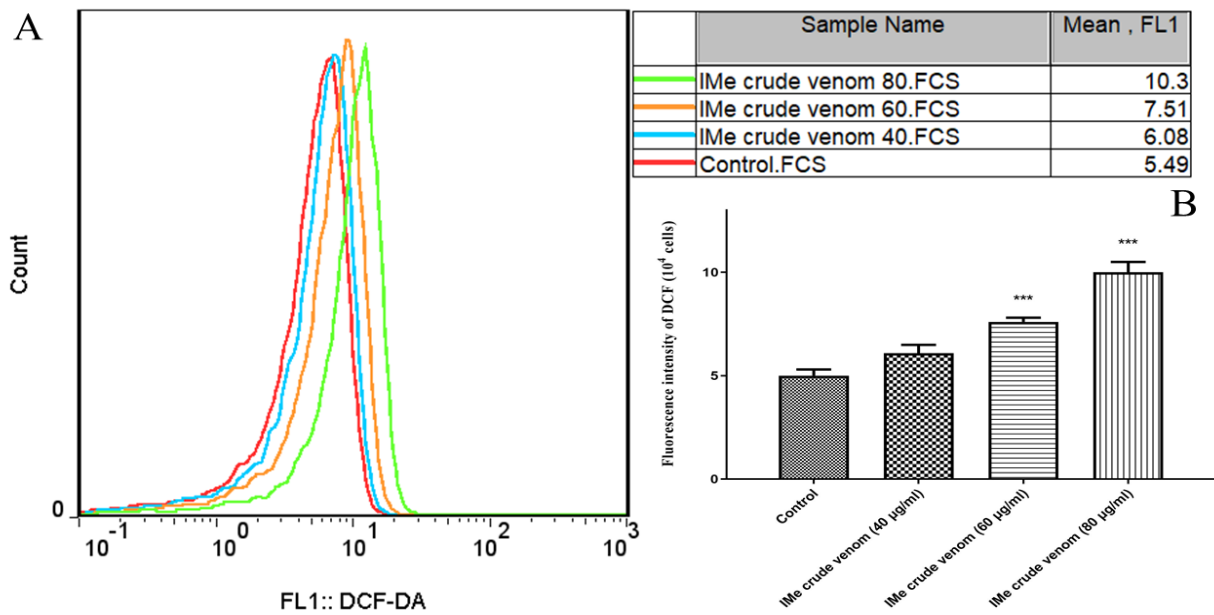


Figure 2. Iranian Mesobuthus Eupeus Crude Venom Induced the Formation of ROS in CLL B-Lymphocytes. (A), The fluorescent intensity of DCF was detected by flow cytometry in the isolated CLL B-lymphocytes treated with different concentrations of IMe crude venom for 12 hours. Histograms showed a considerable shift to the right in the isolated CLL B-lymphocytes; (B), Quantification of ROS formation in the isolated CLL B-lymphocytes was presented in the graph. The values are the mean \pm SD of three independent experiments (n=3). Significance was determined using one-way ANOVA. ***p<0.001. IMe, Iranian Mesobuthus eupeus; ROS, Reactive Oxygen Species; DCF-DA, Dichlorofluorescin Diacetate; FCS, Flow Cytometry Standard.

and 80 μ g/ml IMe crude venom for 12 hours and flow cytometry showed a considerable shift to the right in the isolated CLL B-lymphocytes. The quantitative results showed that at concentrations of 40, 60 and 80 μ g/ml IMe crude venom, the fluorescent intensity of acridine orange

in the isolated CLL B-lymphocytes clearly increased, in a dose-dependent manner, to 27.41 ± 3.21 , 37.39 ± 1.98 and 39.23 ± 2.86 respectively, compared with that in the control (21.87 ± 1.38). These results suggest that IMe crude venom may cause selective toxicity in the isolated CLL

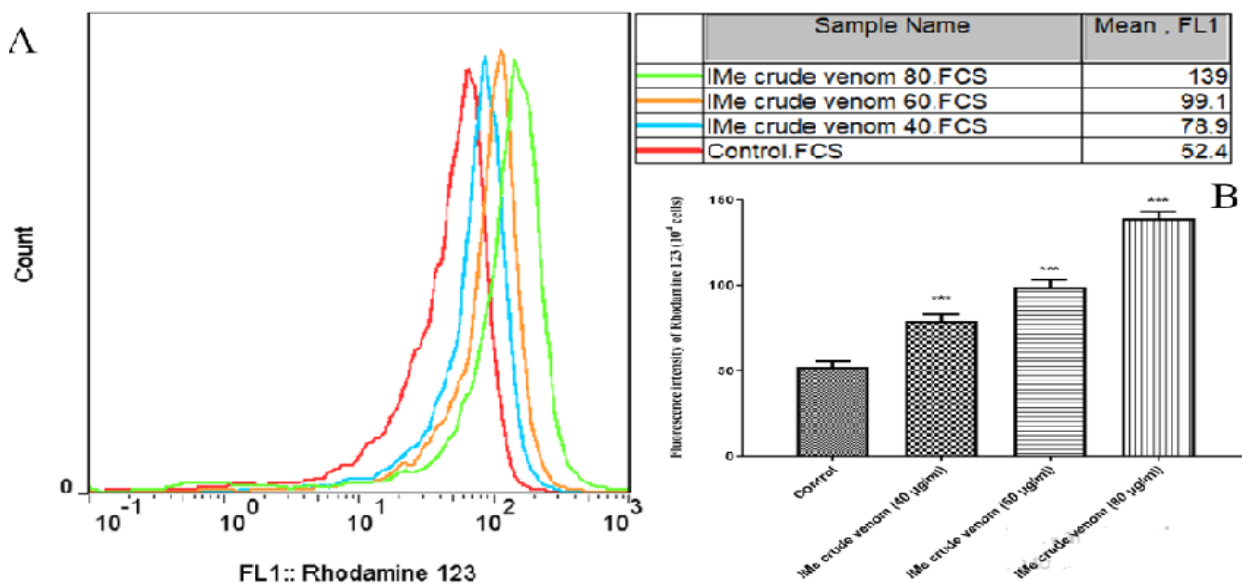


Figure 3. Iranian Mesobuthus Eupeus Crude Venom Induced Mitochondrial Membrane Potential Depolarization in CLL B-lymphocytes. (A), The isolated CLL B-lymphocytes treated with 40, 60 and 80 μ g/ml IMe crude venom for 12 hours. Mitochondrial membrane potential depolarization effects were detected using cell-permeable cationic rhodamine 123 and flow cytometry. Intensity of mitochondrial membrane potential depolarization-disrupted in the isolated CLL B-lymphocytes significantly increased with increasing concentrations of IMe crude venom (40, 60 and 80 μ g/ml); (B), Quantification of mitochondrial membrane potential depolarization in the isolated CLL B-lymphocytes was presented in the graph. The values are the mean \pm SD of three independent experiments (n=3). Significance was determined using one-way ANOVA. ***p<0.001. IMe, Iranian Mesobuthus eupeus; FCS, Flow Cytometry Standard.

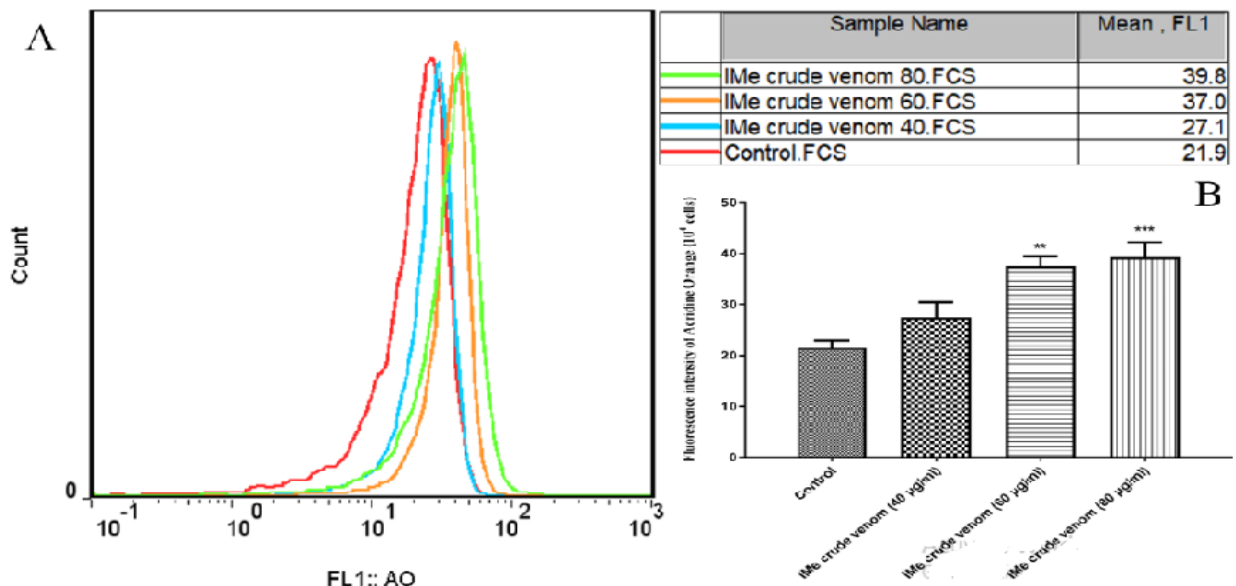


Figure 4. Iranian Mesobuthus Eupeus Crude Venom Induced the Lysosomal Membrane Destabilization in CLL B-lymphocytes. (A), The fluorescent intensity of acridine orange was detected by flow cytometry in the isolated CLL B-lymphocytes treated with different concentrations of IMe crude venom for 12 hours; (B), Quantification of lysosomal membrane destabilization in the isolated CLL B-lymphocytes was presented in the graph. The values are the mean \pm SD of three independent experiments (n=3). Significance was determined using one-way ANOVA. ***p<0.001. IMe, Iranian Mesobuthus eupeus; FCS, Flow Cytometry Standard; AO, Acridine Orange.

B-lymphocytes through lysosomal damage (Figure 4).

Discussion

The literature has been widely reported that the venom of scorpions is the most important source of bioactive polypeptides, proteins and other compounds, for this reason scorpion venoms are serious interest of the pharmaceutical and biotech industries (Ahmadi et al., 2020). Today chlorotoxin as a scorpion-derived therapeutic peptide is the toxin from scorpion venom that has been taken into clinical trials with promising effects (Ojeda et al., 2016). Previous studies have been reported that scorpion venom has cytotoxic, anti-proliferative, immunosuppressive and apoptogenic effects. Therefore, the venom of scorpions can be used against various cancers like, leukemia, human neuroblastoma, brain tumor, glioma, melanoma, breast cancer, human lung adenocarcinomas and prostate cancer (Mishal et al., 2013; Rapôso, 2017). The results of our studies showed that IMe crude venom can induce cytotoxicity in CLL B-lymphocytes as primary cells isolated from CLL patients. There are limited studies to evaluate the potential anti-cancer effects of IMe crude venom in the literature especially primary cells. A recent study showed that IMe crude venom has an antiproliferative effect on the colorectal carcinoma cell line (HT29) by inducing apoptosis through the mitochondria signaling pathway (Valizade et al., 2020). They showed that the IC_{50} of IMe crude venom was 10 μ g/ml within 24 hours in HT-29 cells (Valizade et al., 2020). Our results showed that incubation the isolated CLL B-lymphocytes various concentrations of IMe crude venom (0, 5, 20, 40, and 80 μ g/ml), decreased cell viability to 100%, 88 ± 2.2 , 79 ± 1.5 , 70 ± 2.1 , 58 ± 2.4

and 39 ± 1.7 respectively after 12 hours, respectively. We showed that the IC_{50} of IMe crude venom was 60 μ g/ml within 12 hours in CLL B-lymphocytes. Differences in IC_{50} of IMe crude venom our and mentioned study may be related to exposure time and cell type.

The very important base in the chemotherapy is selective toxicity (Zubrod, 1978). In the cancer treatment selective toxicity refers to cytotoxic agents that are more toxic to cancerous cells than human normal cells (Zubrod, 1978). Selective toxicity is the increasing effectiveness of drugs for the treatment of leukemias such chronic lymphocytes leukemia and other cancerous cell lines (Seydi et al., 2016; Salimi et al., 2017; Salimi et al., 2021). Even previous studies have been reported that such selectivity can be achieved even in the patients (Maeda and Khatami, 2018). Today, the discovery of selective anticancer agents with specific and synergistic effect with existing chemotherapeutics is significantly on the rise (Seydi et al., 2016; Ma et al., 2017). For example, chlorotoxin from the venom of the scorpion *Leiurus quinquestriatus* with selective toxicity potential on cancer cells interacts with chloride channels and has inhibitory effects on migration and invasion of glioma cell (Ojeda et al., 2016). Our results showed that IMe crude venom has significant cytotoxicity and selectivity against CLL B-lymphocytes, while treatment of normal human lymphocytes with different concentration of IMe crude venom did not show significant toxicity in the range of 0-100 μ g/ml. Valizade et al. had previously obtained a similar selective toxicity of IMe crude venom in HT-29 cells (Valizade et al., 2020).

The inhibition of mitochondria-mediated apoptosis or evasion of cell death is a hallmark for chronic lymphocytic leukemia (Sharma et al., 2019). Dysregulation of

mitochondrial functions occur in the tumor cells such CLL B-lymphocytes due to high metabolic demands and rapid cell proliferation (van Bruggen et al., 2019). The difference between cancerous mitochondria and normal mitochondria includes several functional alterations due to mutation of mitochondrial DNA (mtDNA) and differences in the mitochondrial structure, such as higher basicity inside the mitochondrial lumen and higher membrane potential of cancer cell mitochondria (Jeena et al., 2020). It has been reported that the hyperpolarized mitochondrial membrane of healthy mitochondria (-160 mV) compared with that of cancerous mitochondria (-220 mV) facilitates the selective and fast entry of positively charged molecules specifically to the cancer mitochondria (Szabo et al., 2020). These structural and functional alterations in mitochondria of cancer cells are advantageous for the selective targeting and toxicity of cancerous cells (Jeena et al., 2020). Due to the importance of targeting mitochondria to induce selective death in CLL B-lymphocytes (Roy Chowdhury and Banerji, 2018), in the current study we investigated mitochondrial damage in cells exposed to IMe crude venom. Our results showed that IMe crude venom could cause mitochondrial membrane potential depolarization mitochondrial damage possibly the onset of cell death in CLL B-lymphocytes. This study is consistent with the results of previous studies (Zargan et al., 2011; Valizade et al., 2020).

The most prominent sources of ROS within the cells are mitochondria which contribute to oxidative stress and cell death (Perillo et al., 2020). The ROS formation in cancer cells leads to the three major components critical for the opening of the mitochondrial permeability transition pore (PTP), the cyclophilin D, adenine nucleotide translocase (ANT) and voltage-dependent anion-selective channel (VDAC), through the oxidation of specific cysteines in their active sites in the mitochondria (Madesh and Hajnóczky, 2001; Perillo et al., 2020). Although the presence of ROS is beneficial for the normal function and metastasis of tumor cells, but when ROS formation exceeds than the natural need for cancer cells can lead to cell death and apoptosis. Our findings showed that IMe crude venom can increase dose-dependent ROS formation in CLL B-lymphocytes, which can play an effective role in inducing selective toxicity in these cells. These results are consistent with the results of previous studies (Valizade et al., 2020).

Lysosome is a membrane-bound organelle that possesses hydrolase enzymes for the recycling and degradation of essential nutrients to maintain the normal homeostasis within cells (Dielschneider et al., 2017). It has been reported that tumor cells increase lysosomal function to adapt to stressful environments metabolize and proliferate (Dielschneider et al., 2017). This feature in cancerous cells lead to susceptible to lysosomal membrane permeabilization (LMP) (Dielschneider et al., 2017). Dielschneider et al showed that excess sphingosine in CLL cells could contribute to their sensitivity toward LMP. Thus, a novel therapeutic strategy in CLL cells could be targeting the lysosome (Dielschneider et al., 2016). Upon lysosomal disruption, ROS levels increase leading to oxidative stress, mitochondrial dysfunction,

PTPs opening, reactive iron and autophagy (Dielschneider et al., 2017). Cathepsins as proteases are released from the lysosomes causing degradation of cellular structures and macromolecules (Dielschneider et al., 2017). These events finally lead to killing of the cancer cell through different types of cell death (Dielschneider et al., 2017). In the current study we demonstrated that IMe crude venom can lead to in lysosomal membrane destabilization in CLL B-lymphocytes, which can play an effective role in inducing selective toxicity in these cells.

Finally, our results suggest that IMe crude venom can be a promising source for novel anticancer drug candidates which induce selective cell death presumably directly and indirectly in CLL B-lymphocytes through different targets such as mitochondria, lysosome and ROS formation. This study suggests after fractioning of IMe crude venom and identifying its effective components, the exact mechanisms of induction of cell death and its final targets should be studied.

Author Contribution Statement

The authors confirm contribution to the paper as follows: study conception and design: Ahmad Salimi, Hossein Vatanpour and Leila Sadeghi; data collection: Vahed Adhami and Seyyed Hossein Sajjadi Alehashem; analysis and interpretation of results: Ahmad Salimi; draft manuscript preparation: Ahmad Salimi. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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