RESEARCH ARTICLE

The Effects of Purified Artemia Extract Proteins on Proliferation, Differentiation and Apoptosis of Human Leukemic HL-60 Cells

Abdolkhaleg Deezagi*, Azadeh Chashnidel, Neda Vaseli Hagh, Mahvash Khodabandeh Shahraki

Abstract

There has been an increment in the number of studies focused on marine bioactive materials. Many peptides and other biomaterials with anticancer potential have been extracted from various marine animals. Artemia extracts have found uses in sun-light protection cosmetics and anti-aging products. However, contents of biochemical compounds in Artemia spp. and molecular mechanisms of have not been clearly studied in leukemic cells in vitro. In this work, we isolated and purified proteins of Artemia Urmiana. Six clear fractions (A-F) observed on DEAE-cellulose chromatography were assayed for effects on cell growth, differentiation and apoptosis using the human leukemic HL-60 cell line. Cell proliferation analysis by MTT and BrdU assays indicated that did not affect cells, growth. Cells treated with crude extract and fractions A, B and C, but not E and F (up to 100 μg/mL), exhibited increase of cell growth in a dose dependent manner. Stimulatory effects of fraction D were observed at concentrations of 10 μg/ml and above. In nitro blue tetrazolium (NBT) reduction assays, treatment with 100 μg/mL of fraction E or F for 96 hr increased the fraction of differentiated cells up to 14.8 ± 3.56% and 16.5 ± 2.08% respectively. Combination of those fractions with retinoic acid had significant synergistic effects on the differentiation of cells (56.8 ± 3.7% and 67.4 ± 4.2%, p≤0.01). Annexin-V FITC staining for apoptosis and flow cytometric assays indicated induction of apoptosis by fractions E and F up to 23.8 and 31.8% of cells.

Keywords: Apoptosis- Arteria extract- differentiation- leukemic cells- proliferation

Asian Pac J Cancer Prev, **17 (12)**, 5139-5145

Introduction

Compounds from marine sources have been reported to have bioactive properties with varying degrees of actions such as: anti-tumor, anti-cancer, anti-microtubule, anti-proliferative, anti-hypertensive, cytotoxic as well as antibiotic properties (Aneiros and Garateix, 2004, Wilson-Sanchez et al., 2010; Jimeno et al., 2004). The isolated compounds from marine sources are of varying chemical nature including phenols, alkaloids, terpenoids, polyesters and other secondary metabolites (Chakraborty and Ghosh, 2010). The biodiversity of marine environment far exceeds more than terrestrial environment. Research on the use of marine natural products as pharmaceutical agents has been steadily increasing. There has been an increment in the number of studies focused on marine bioactive materials. Many bioactive peptides and biomaterials with anticancer potential have been extracted from various marine animals like tunicates, sponges, soft corals, sea hares, nudibranchs, bryozoans, sea slugs and other marine organisms (Kim and Wijesekara, 2010; Libes, 2009).

Throughout evolution, marine organisms have developed into very refined physiological and biochemical systems. All these species have developed chemical means to defend against predation, overgrowth by competing species, or conversely, to subdue motile prey species for ingestion. Also, secondary metabolites, which produced by marine invertebrates and bacteria have yielded medicinal products such as novel anti-inflammatory, anti-cancer and antibiotics agents (Haefner, 2003; Guadalupe-Miroslava et al., 2009).

Hyper saline organisms adapt to high salinities by means of various physiologic mechanisms, including osmoregulation and the synthesis and accumulation of various compatible solutes. Artemia (commonly known as brine shrimp) is an aquatic crustacean belonging to the subclass of Branchiopoda. Artemia is the dominant macrozooplankton present in many hypersaline environments. Their ability to survive and even thrive in forbidding environments has long been of interest to biologists (Wurtsbaugh et al., 2001; Clegg and Trotman, 2002). This crustacean, properly called an animal extremophile, has been able to survive in such environments through well-developed osmoregulation involving enhanced Na-K ATPase activity (Holliday et

Department of Molecular Medicine and Biochemistry, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.
**For Correspondence: deezagi@nigeb.ac.ir*

al., 1990; Eads, 2002). Although physiologically able to survive and reproduce in salinities near and below seawater, Artemia is definitely found at salinities below 100 gr/L but the density maybe decreased (Persoone and Sorgeloos, 1980). Artemia found in a wide variety of hyper saline habitats ranging from desert to, tropic to, mountains. Artemia encysted and diapause exhibits a level of stress tolerance such as hypersalinity, very low oxygen tensions and extreme of temperature (Wurtsbaugh, 1992; Tanguay et al., 2004). Few adult animals or their developmental stages tolerate anoxia for an extended time. The response of the well-adapted animals to anoxia is to reduce their metabolic rates to level that are commonly between 1-10% of the aerobic level (Clegg, 2007). Species of the Artemia and genus are found in a variety of very harsh environments and a wide variety of hyper saline habitats ranging from desert to, tropic to, mountains in all continents, except Antarctica (Eads, 2002). Historical overview of Artemia population from Iran was discussed in detail by Abatzopoulos et al. (Abatzopoulos et al., 2006). One of this species (Artemia Urmiana) lives in Lake Urmia in Iran (Urmia or Orumiyeh). Urmia Lake is one of the largest permanent hypersaline lakes in the world and resembles the Great Salt Lake in the western USA in many respects of morphology, chemistry and sediments (Kelts and Shahrabi, 1986; Eimanifar and Mohebbi, 2007). In nature their encysted embryos (Cysts), encounters sever hypersalinity and air desiccation; high dose of ultraviolet radiation; varying degree of hypoxia, inducing anoxia and extremes of temperatures. Information's related to varied uses of several species of the genus Artemia were existed. The nutrient source properties of Nauplii (newly hatched cysts) make it the most widely food item in fish farms. Artemia extract and mud of salt lakes uses in cosmetics as sun light protective and anti-aging agents worldwide too. The cellular and molecular mechanism of these effects are unclear. It has been reported that Artemia proteins confer the thermotolerance in transformed bacteria and mammalian cells such as lens cell, 293H kidney cells and COS-1 cell (Jamil et al., 2005]. Artemia proteins affected on the cells under stress, but the molecular mechanism of these proteins has not been studied clearly in human cells in normal condition.

The sea water, Artemia extract and mud of Urmia Lake have been used as remedies in folk medicine by Iranians for treatment of some inflammatory and skin diseases from many ancients ago. The source and typed of effective materials of them were not clear and did not study to now. Artemia urmiana as the most living organisms in Urmia lake and its extract was studied in this research. uFor these purposes, the total protein of hatched napollies were extracted and purified. Then the human promyelocytic leukemic HL-60 cells were treated by each fraction in a dose dependent manner. Then the cell's proliferation, differentiation and apoptosis were assayed.

Materials and Methods

Cyst hatching and protein extraction

A. urmiana cysts were provided from Urmia lake (West-North of Iran). 10 gr of cysts were hydrated in tap and/or sea water at room temperature for one hour. Then the cysts were decolorized by Sodium Hypochlorite (NaOCl 5% W/V) until the cysts color change from brown to orange. Then the cysts were washed by 500 ml of cold distilled water. The decolorized cysts were hatched in 2 liters of artificial sea water (0.4 M NaCl, 0.009 M KCl, 0.05 M MgCl2, 0.009M CaCl2 and 0.028 M Na2SO4, pH= 8.0) at room temperature and/or 37 ºC for 24 hr under light chamber in a shaking incubator according the procedure reported by Liu and Mclennan (Liu and Mclennan, 1994). Freshly hatched naupliies are phototropism, the napauliies were collected by attraction to light. The collected napuliies were washed with 400 ml of cold distilled water. Embryos were suspended in 50 ml of PBS, pH=7.4 and centrifuged at 3000 rpm for 20 minutes.

1.0 gr of freshly isolated naupliies was dissolved in 5 ml of extraction buffer (Tris- HCl 50 mM, pH 6.8). Total proteins were extracted by liquid homogenization, high frequency sound waves sonication and 3 times freeze/thaw cycles in liquid nitrogen separately. The homogenate was filtered through two layers of Mira cloth into a 50 mL of Falcon tube at room temperature. The filtered homogenate was keep at -20°C until protein purification. In all of experiments total protein concentration was measured by Bradford methods.

Protein purification by anion-exchange chromatography

The proteins content of crude extracts was precipitated with salt extraction by using Ammonium Sulfate 40% (W/V) at 4°C by shaking for 2hr. The precipitate was centrifuged at 5000 rpm for 20 min at 4°C. Then the pellet was dissolved in dialysis buffer (Tris-HCl 50 mM, NaCl 20mM, pH 6.5) and dialyzed against the same buffer for 24 hr at 4 ºC by 2 times change of the buffer.

A 18 × 3 cm column with DE-52 sepharose beads (whatman) was used for chromatography. First, the column was equilibrated d by binding buffer (Tris-HCl 50 mM, NaCl 20 mM pH 6.5). Subsequently, dialyzed crude extract was eluted applying 100 ml of the same buffer. Then salt gradient was subsequently applied by adding 100ml of 0.1, 0.2 , 0.3 and 1.0 M of NaCl in Tris-HCl 50 mM buffer pH 6.5. The samples were collected as 2.0 ml fraction by flow rate about 0.5 ml/min. The samples were monitored at 280 nm continuously. The samples of each fraction were collected and dialyzed. SDS-PAGE electrophoresis was done for analyses of the protein profile of purified fractions on 13% resolving SDS-PAGE gel. The electrophoresis was done by LKB-Pharmacia electrophoresis system (LKB Pharmacia, Uppsala, Sweden). After running; the gels were stained by coomassie blue.

Finally, the fractions were concentrated by freeze-drying and sterilized by 0.2 μm filter papers to use for further analysis in cell culture system.

Clotting Limulus Amoebocyte Lysate (LAL) assay

Determination of endotoxin contamination in all samples was conducted by LAL assay kit (sigma, St. Louis, MO, U.S.A.) according to the manufacture's instruction. The samples were initially pretreated by boiling. The samples with lower than <0.1 ng/ml of endotoxin were used for HL-60 cells treatment.

Cell Culture Conditions and treatment

Human promyelocytic HL-60 cell line was previously provided from American Type Culture Collection (ATCC) (Rockville, M.D., U.S.A). The cells were cultured in RPMI-1640 medium (Gibco, Paisley, U.K.) supplemented with 10 mM HEPES buffer (Sigma, St. Louis, MO, U.S.A.), 2mM glutamine (Merck, Germany) and 10% (v/v) Fetal Calf Serum (FCS) (Gibco, Paisley, U.K.), at 37˚C in a humidified atmosphere of 5% CO2. The cells were subcultured twice a week by being maintained at a density of 1-8 ×105 cells/ml in the logarithmic phase of growth. Cell numbers were counted by using a hemocytometer and cells, viability was assessed by the standard trypan-blue exclusion method. 2 × 105cells/ml were separately treated by increasing concentration (0, 1, 5, 10, 50 and 100 µg/ml) of sterile A. urmiana crude extract and/or partially purified fractions contents in 5 mL of RPMI-1640 medium and 10 % of FCS in 6-wells plates (NUNC, Denmark). The cells were incubated at 37°C for 96 hr.

Cell growth and Proliferation assays

Treated cells cells were collected after 96hr and the total cell number, viability, Bromo deoxyUridin (BrdU) and MTT cell proliferation assays were done as below. Cell number and viability were directly enumerated using a Neobar hemocytometer by trypan blue dye exclusion .

BrdU assay: BrdU cell proliferation assay was done using the BrdU cell proliferation ELISA kit (Roche, Germany) according to the manufacture's instructions. Briefly 100 µl of control and treated cells were transferred to 96 wells microtiter plate and 10 µl of BrdU solution was added to each wells and incubated overnight. Then the microwells were centrifuged and dried by hair drier. Then 200µl of FixDenat was added to each well and plate was incubated for 30 min at 15-25°C. After removing FixDenat, 100µll of anti-BrdU-Peroxidase conjugated antibodies were added to each well and incubated 90 min at room temperature. The solution was completely removed and the wells were washed three times with 200 µl of washing buffer. Finally, 100 µl of Tetra-Methyl Benzidin (TMB) substrate solution was added and incubated at room temperature for 15 min and reaction was stopped by adding 100 µl of stop solution. The absorbance was measured using microtiter plate reader at 450 nm (Titretek multiscan ELISA reader).

MTT assay: After 96 hrs of incubation, 100µl of finely resuspended control and treated cells were transferred to flat bottom 96 microtiter plates. Then 10µl of freshly prepared (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, U.S.A.) solution (5 mg/ml in PBS) was added to each well and were incubated for 4hrs. Finally, 50µl of MTT lysis solution (20% Sodium Dodcyl Sulphate W/V and 50% Dimethy Formamide V/V) was added to each well and incubated overnight. Absorbance was read at 620 nm using an ELISA reader.

Nitro Blue Tetrazolium (NBT) Reduction Assay

Differentiation of HL-60 cells was assayed by NBT reduction test. The cells were treated by A. Urmiana extracts and incubated 96 hr. Then the cells were collected and washed 2 times in culture medium and resuspended in the same medium containing 20% FCS. 100µl of cells suspension (2 × 106 cells/ml) were transferred to 96 micro-wells. Phorbol 13 Myristate Acetate (PMA) (5ng/ml) (Sigma, U.S.A.) was used as positive control stimulator for NBT reduction by cells. The cells were incubated in the presence of freshly prepared NBT solution (1 mg/ml in PBS) for 45 min in a CO2 incubator. Then the cells were washed 3 times with PBS and the percentage of the cells that stained dark blue-black with formazan deposits were determined under a light microscope using a glass slide. Minimum 300 numbers of the cells were scored from each treatment.

Because of low NBT reduction results in using A. urmiana extracts, the cells were treated with different concentrations of Artemia extracts separately and/or in combination by Retinoic Acid (RA)(1µg/ml) too. NBT assayed was done as described above in combination study.

Determination of apoptotic cells by flow cytometry and DNA ladder electrophoresis

For flow cytometry, 1 × 106 HL-60 untreated and Artemia extract treated cells were taken, centrifuged (2500 rpm, 10 min) and washed with washing phosphate buffered saline (PBS). Apoptotic cells were assayed by using Annexin V-FITC apoptotic detection Kit (Roche, Mannenheim, Germany) according the procedure manual of the kit. In brief, washed cells were re-suspended in 100 µl binding buffer, 2 µl Annexin V-FITC and 2 µl Propidium Iodide. Cells were mixed and incubated for 15 min at room temperature in dark. Cell were re-suspended in 500 µl of washing buffer and at least 104 cells and events were analyzed by Flow cytometry (Becton Dickinson, CA, USA). The data was analyzed with Cell Quest version 1.2 software. In manual assay, stained-cells was placed on a slide, covered with a cover slip and observed using a fluorescence microscope equipped with FITC (green) filter by fluorescent microscopy (Zeiss, Germany) too.

For DNA ladder electrophoresis, total DNA was extracted from the treated cells by using an apoptotic DNA ladder kit (Roche, Germany). The purified DNA (1–3 µg) was run out for 60 min at 100V in a 1.5% agarose gel electrophoresis. DNA-laddering was visualized using ethidium bromide staining.

Statistical analysis

Each experiment was performed at least three times for all data, each carried out in duplicated sequences. Data were analyzed using a One-Way Analysis of variance (ANOVA) Values were given as the mean ±1 Standard Deviation (SD) and biological variables were compared by using the students' t-Test. By convention, a α-level of $p<0.05$ was considered to be statistically significant. Finally, the correlation between Artemia extract concentrations and cell proliferation parameters were calculated statistically.

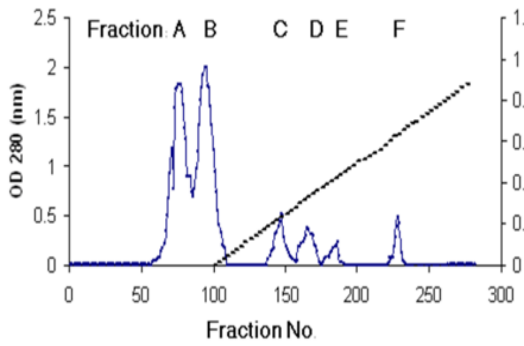

Figure 1. Chromatogram of the A. Urmiana Extracts by DEAE Sepharose Ion Exchange Chromatography. Fractions A and B were eluted by Tris-HCl 50 mM, NaCl 20 mM pH 6.5. Fractions C, D, E and F were eluted by NaCl salt gradient by adding 400ml of 0.1, 0.2, 0.3 and 0.5 M of NaCl in Tris-HCl 50 mM buffer pH 6.5. The samples were collected as 2.0 ml fraction by flow rate about 0.5 ml/min. The samples were monitored at 280 nm continuously.

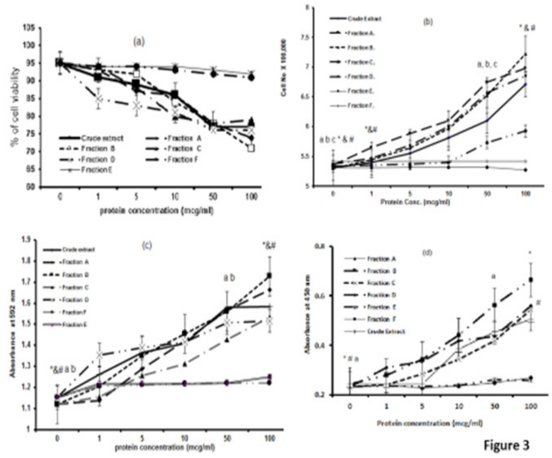

Figure 3. The Effect of Artemia Urmiana Extracts on the Cell Viability and Proliferation of Human Leukemic HL-60 Cells. The cells were treated by Artemia urmiana crude extracts and partially purified fractions of ion exchange chromatography. The cells were incubated for 96 h at 37°C and cell viability and proliferation were assayed as described in methods. a) The cells viability according by trypan blue dye exclusion test b) cell proliferation by cell counting , (c) by MTT assay and (d) by BrdU assay. (n = 4) Values are the means ± 1 SE of data from duplicate cultures. #, &, * indicated to the significance of the concentrations of 100 μg/ml of fraction A, B and C in comparison to untreated control cells (p< 0.01) and in compared to 1.o μg/ml correlate fraction (p,0.05) and a , b and c for cells treated by 5o μg/ml of these fractions in comparison to control cells. (p<0.05).

Results

Hatching, protein extraction and purification

The cysts were decolorizedby Sodium Hypochlorite until the cysts color were changed from brown to orange. This take approximately 2-3 minutes. Freshly hatched nauplii are phototropism; the napauliies were collected by attraction to light. The collected napuliies were washed in cold distilled water. Embryos were suspended and centrifuged as described in methods. The data were summarized in table 1. The hatching efficiency in artificial sea water at pH=8 and 37ºC was about 78%.

In physical used methods the amount of total protein were about, 0.7 mg by liquid homogenization, 1.94 mg by high frequency sound waves sonication and 0.370 mg by 3 times freezing and thawing cycles in liquid nitrogenmg per gram of napuliies wet weight. Therefore, for further studies we used high frequency sound waves sonication method for protein extraction.

Partial purification was done by DEAE sepharose ion exchange chromatography as described in methods. The samples were collected in 2.0 ml fractions by flow rate about 0.5 ml/min. The samples were monitored at 280 nm continuously. Figure 1. indicated to the chromatogram of the purification.

Six obvious fractions (A-F) were observed in the chromatogram. The fractions A and B belong to the cationic proteins which didn't bind to DEAE resin. Fractions C, D, E and F resulted to the salt gradient elution of 0.1, 0.2, 0.3 and 0.5 M of NaCl Respectively.

The purified fractions were concentrated by freeze-drying and analyzed by SDS-PAGE electerophoresis after coomassie blue staining (Figure 2).

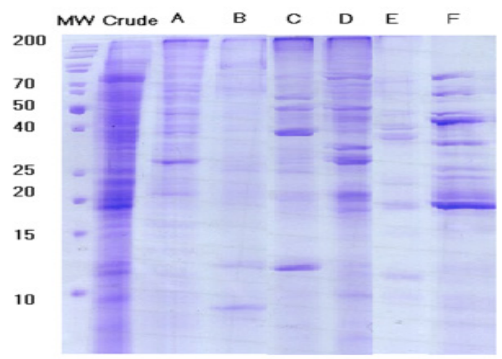

Figure 2. The SDS-PAGE Gel Electrophoresis of Artemia Urmiana Crude Extract and Fractions of Chromatography on 12 % SDS-PAGE Gel and Staining with Coomassie Blue as Described in Methods. MW: Molecular weight marker (kd), Crude extract, A and B samples of cationic proteins, C, D, E and F fractions were eluted by 0.1, 0.2 ,0.3 and 0.5 M of NaCl gradient concentration

HL-60 cells proliferation and growth

To assess the inhibitory/stimulatory effects of crude extract and each fraction on HL-60 leukemic cell growth, initially we determined the cell's viability by trypan blue exclusion method after 96 hr incubation. The results indicated that the treated cells were viable more than 80% in all conditions (Figure 3a).

The cell proliferation and growth rate of treated cells were assessed by cell counting and using two colorimetric (BrdU and MTT) cell proliferation assays for each concentration. The values for each fraction and related concentration were averaged and growth curves were constructed. Figure (3) indicated to the cell proliferation curves of HL-60 in response to different concentration. Counting of the cells by Hemocytometer indicated that fractions E and F did not affect in cell proliferation up to 100 μg/ml, in addition the cells which treated with crude extract and fractions A, B and C exhibit increase of cell's

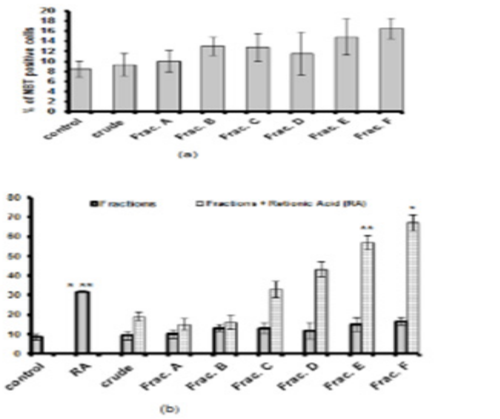

Figure 4. Nitro Blue Tetrazolium (NBT) Reduction Assay for Induction of Differentiation in Human Leukemic HL-60 Cells by A. urmiana extracts. The cells were treated by A. Urmiana crud extract (50 µg/ml) and partially purified fractions of ion exchange chromatography (50 µg/ml) alone (a) or in combination by Retinoic Acid (1µg/ml.) (b). * and ** indicated to fractions F and E which combined by RA in comparison to RA treatment only. (n = 4) Values are the means ± 1 SE of data from duplicate cultures

growth in a dose dependent manner. The stimulatory effect of fraction D was observed in concentration upper than 10 µg/ml (Figure 3b)

For MTT and BrdU assays, the cells were separately treated by extracts (10,000 cell/well in 100 µl in flat bottom 96 wells) and incubated at 37°C for 96 hr. Then MTT dye was added and absorbance was measured. The values for each condition were averaged and growth curves were constructed (Figure 3c). The values for untreated control cells was 1.121 ± 0.114 absorbance units (AU). Results of the treated cells indicated that the cells proliferation was significantly increased with increasing of the protein concentration of crude extract and fractions A, B, C and D. When the cells were treated by fractions E and F up to 100 µg, the cell proliferation potency was not affect too.

In BrdU cell proliferation assay, the absorbance values for each condition were averaged and growth curves were constructed (Figure 3d). Results of the treated cells indicated that the cells proliferation was significantly increased with increasing of the protein concentration of crude extract and fractions A, B, C and D. When the cells were treated by fractions E and F up to 100 µg, the cell proliferation potency was not affect.

Differentiation

In order to determine whether growth inhibition by fractions E and F and partially by fraction D was associated with terminal differentiation of HL-60 cells, we performed a NBT reduction test. Differentiated mature myelocytes of HL-60 leukemic cells can reduce

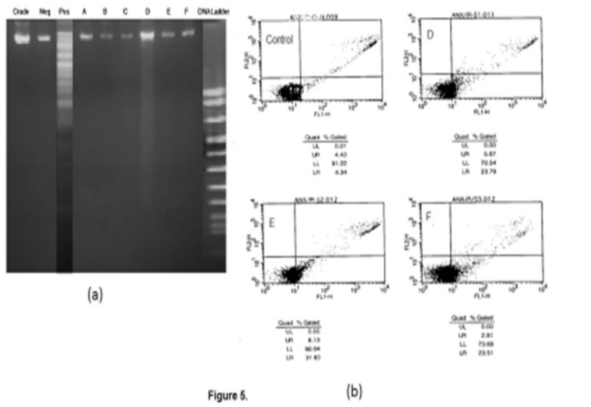

Figure 5. Determination of Apoptotic Cells by Annexin V-FITC Staininig Flow Cytometric Analysis and DNA Ladder Electrophoresis. The cells were treated by A. umania crud extract and partially purified fractions of ion exchange chromatography (50 µg/ml). The cells were incubated at 37°C for 96 hr. a) DNA ladder electrophoresis for apoptosis assay in the HL-60 cells was done as described in materials and methods. The extracted DNA (1–3 µg) was run out for 60 min at 100V in a 1.5% agarose gel electrophoresis. Untreated control cell (Negative control), PMA treated cells (Positive control), cells treated by crude extract and fractions A to E and DNA ladder marker. b) Apoptotic cells were stained by Annexin V-FITC as described in material and methods. 104 cells and events were analyzed by Flow cytometry and analyzed with Cell Quest version 1.2 software. Untreated control cells, D, E and F indicated to cells treated by fractions D, E and F

NBT to dark blue diformazan particles, which can easily be observed under a light microscope. Only 8.4 ± 1.6% (n=6) of untreated HL-60 cells can reduce NBT as shown in figure 4a. Treatment of these cells by each fraction of Artemia Urmiana extract at 100 µg/ml for 96 hr only increased the fraction of NBT positive cells up to 14.8 ± 3.6% by fraction E and 16.5 ± 2.08% by fraction F. This finding indicated that, the obvious inhibition of cell proliferation by fractions E and F was not completely accompanied by differentiation in these conditions and the NBT test was not completely consistent with the cell proliferation findings. Therefore, we used combination of these products by Retinoic Acid (RA, a known differentiation inducing agent in these cells) in the induction of differentiation.

RA (1.0 µM) increased the fraction of positive cells to 31.7 ± 0.5% (n=6; p< 0.001) Figure 4b. The combined treatment of RA by crude extract, fractions A, B and C did not show obvious effects on the differentiation. But combined treatment by fractions D, E and F increased the positive cells up to 43.2 ± 3.7%, 56.8 ± 3.7% and 67.4 ± 4.12% respectively.(n=4; p< 0.001 compared with untreated control cells, (p<0.01 compared with RA

Table 1. The Hatching Efficiency of Artemia urmiana Cysts in Tap and/or Artificial Sea Water at pH=8.0.

Method	Amount of hydrated cysts (gr)	Amount of hatched cysts(gr)	Hatching Efficiency
Tap water at 37 °C	10	4.6	46%
Sea water at 22-23°C	10	6.2	62%
Sea water at 37 °C	10	7.8	78%

treatment).Therefore the combination of these fractions by Retionic Acid had synergistic effects on the differentiation of HL-60 leukemic cells.

Apoptosis

Induction of apoptosis in cells was assayed by DNA ladder electrophoresis and Annexin-V-FITC flow cytometric methods. In gel electrophoresis of DNA, DNA laddering was not observed after exposure to Artemia Urmiana extract (50 µg/ml) except fraction D which indicate slight ladder DNA (Figure 5a). Flow cytometric analysis indicated that the fractions D, E, and F induce apoptotic cells about 23.8, 31.8 and 23.5% of the cells respectively. The apoptotic cells of untreated cells were about 4.4% (Figure 5b). Other fractions did not show significant effect in induction of apoptosis.

Discussion

Finding a potent approach for inhibition the growth of cancerous and leukemic cells is one of the greatest actual challenges for pharmacology and medicine. There is an extensive research effort aimed to obtain efficient compounds from natural origin. Most of the marine peptides subjected to clinical trials are secondary metabolites from animals, but there exists a widely unexplored field in marine protein hydrolysate. Studies on peptides obtained from protein hydrolysate have shown antioxidant, antiproliferative and antimutagenic activities of these molecules. These activities could confer on them anticancer potential; however, more research on the mode of action and molecular mechanism on the arrest of cell cycle and apoptosis of leukemic cell line is open. From this subject the results of this work showed that some of the purified protein fractions of A. Urmiana extracts were effective on inhibition of leukemic cell's proliferation. With consideration the viability of cells, which was more than 75% in higher concentrations, therefore this inhibition could not be because of cytotoxic effect of them. One of the markers of cell proliferation and entrance of cells to cell cycle is induction and/or inhibition of DNA synthesis. This one was assayed by BrdU incorporation assay in new synthesized DNA. Treated cells by some of protein fractions could be effective in cell proliferation. The cells which treated by crude extract and fractions A, B, C and D induce cell proliferatin by BrdU assay slightly. But the fractions E and F inhibited the cell proliferation potency. Therefore the effects of crude and unpurified Artemia extract could be combined stimulatory/inhibitory effects on leukemic cells. Therefore the fractionation and purification of them for probable application is nessesery.

Our finding from induction of differentiation by these protein fractions indicated that, the obvious inhibition of cell proliferation by fractions E and F was not completely accompanied by differentiation in these conditions. The NBT test was not completely consistent with the cell proliferation findings. Therefore, we used combination of these products by Retinoic Acid (RA, a known differentiation inducing agent in these cells) in the induction of differentiation. Result showed that, the combination of these fractions by Retionic Acid had synergistic effects on the differentiation of HL-60 leukemic cells significantly.

As discussed, the fractionation and purification of Artemia extract is nessesery for probably medicinal application. For this reason, isolation and identification of the specific peptides and proteins from A. urmiana source those are responsible for the antileukemic effects carried out in this work. To date, there was not a report about the complete protein profile of A. urmiana. We have partially purified and fractionated them. But we didn't focused on a specific protein in this research. In similar researches by other marines some reported criteria exist. In Solitary tunicate, Jumeri have reported that the low molecular weight peptides have greater molecular mobility and diffusivity than the high molecular weight peptides, which appears to improve interactions with cancer cell components and enhances anticancer activity (Jumeri, 2011). Although a study by Huang on the mechanism of action revealed that modulation of hydrophobicity of peptides plays a crucial role against cancer cells (Huang et al, 2011). Didemnin depsipeptides are cytotoxic to cancer cell lines by inhibiting protein synthesis in vitro (Ahuja et al, 2000). It is suggested that protein synthesis may be inhibited by the binding of Didemnins to ribosome-EF-1α complex, since there is a correlation between inhibiting protein synthesis in cell lysates and in human adenocarcinoma MCF-7 cells (Mayer et al, 2003). Studies in use of Jaspamide in HL-60 human leukemia cell line revealed that nanomolar concentrations of this depsipeptide induced inhibition of cell proliferation and increased polynuclear cells (Nakazawa et al, 2000). It has been reported a small heat shock protein p26 from Artemia Franciscana (Sun and Macrae, 2005). Villeneuve et al. have been reported that, p26 inhibited the apoptosis of embryos of Artemia during Artemia development (Villeneuve et al, 2006).

Artemia extrac uses in cosmetic and health product today. The molecular mechanism and its action was not clearly understood in cellular level. The results of this work showed that, the effects of crude and unpurified Artemia extract could be combined of stimulatory/inhibitory effects on leukemic cells behavior. Therefore we conclude that the fractionation and purification of them and more studying od the protein profiles and focus on the characterization of specific proteins for probable anticancer and antileukemic application in future is nessesery.

Acknowledgment

This work was supported by research funds (No. 442) from National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.

References

Abatzopoulos TJ, Agh N, Van-Stappen G, et al (2006). Artemia sites in Iran. *J Mar Biol Assoc UK*, **86**, 299-307.

Ahuja D, Geiger A, Ramanjulu J, et al (2000). Inhibition of protein synthesis by didemnins: Cell potency and SAR. *J Med Chem*, **43**, 4212-18.

Aneiros A, Garateix A, (2004). Bioactive peptides from marine sources: Pharmacological properties and isolation procedures. *J Chromatogr B Anal Technol Biomed Life Sci*, **803**, 41-53.

Chakraborty S, Ghosh U, (2010). Oceans: a store of house of drugs-A review. *J Pharm Res*, **3**, 1293-96.

Clegg JS, Trotman CAN (2002). Physiological and biochemical aspect of Artemia ecology. In: Artemia; basic and applied Biology edited by: Abatzopoulos THJ et al Kluwer Academic Publishers, pp 129-70.

Clegg JS (2007). Protein stability in Artemia embryos during prolonged anoxia. *Biol Bull*, **212**, 74-81.

Eads BD (2002). Book Review, Artemia: Basic and Applied Biology. *J Exp Biol*, **207**, 2004.

Eimanifar A, Mohebbi F, (2007). Urmia Lake (Northwest Iran): a brief review. *Saline Systems*, **3**, 1-8.

Guadalupe-Miroslava SJ, Armando BH, Josafat-Marina EB, (2012). Bioactive Peptides and Depsipeptides with Anticancer Potential: Sources from Marine Animals. *Mar Drugs*, **10**, 963-86.

Haefner B (2003). Drugs from the deep: Marine natural products as drug candidates. *Drug Discov Today*, **8**, 536-44.

Holliday CW, Roye DB, Roer RD (1990). Salinity induced changes in branchial Na/K – ATPase activity and transepithelial potential difference in the brine shrimp, Artemia salina. *J Exp Biol*, **151**, 279-96.

Huang Y, Wang X, Wang H, et al (2011). Studies on mechanism of action of anticancer peptides by modulation of hydrophobicity within a defined structural framework. *Mol Cancer Ther*, **10**, 416-26.

Jamil K, Macrae TH, Clegg JS, et al (2005). A small stress protein act synergistically with trehalose to confer desiccation tolerance on mammalian cells. *Cryobiology*, **51**, 15-28.

Jimeno J, Faircloth G, Soussa-Faro JF, et al (2004) New marine derived anticancer therapeutics-A journey from the sea to clinical trials. *Mar Drugs*, **2**, 14-29.

Jumeri-Kim SM (2011). Antioxidant and anticancer activities of enzymatic hydrolysates of solitary tunicate (Styela clava). *Food Sci Biotechnol*, 20,1075–85.

Kelts K, Shahrabi M, (1986). Holocene sedimentalogy of hypersaline lake Urmia, northwestern Iran. *Palaeogeogr Palaeoclimatol Palaeoecol*, **54**,105-30.

Kim S, Wijesekara I (2010). Development and biological activities of marine-derived bioactive peptides: A review. *J Funct Foods*, **2**, 1-9.

Libes SM (2009). Organic product from the sea: pharmaceuticals, nutraceuticals, food additives, and cosmoceuticals. In introduction to marine biogeochemistry, 2nd ed.; Libes SM, Ed.; Academic Press: Conway, SC, USA.

Liu J, Mclennan AG (1994). Purification and properties of GTP: GTP guanylyltransferase from encycted embryos of the brine shrimp Artemia. *J of Biol Chem*, **269**, 11787-94.

Mayer AM, Gustafson KR (2003). Marine pharmacology in 2000: Antitumor and cytotoxic compounds. *Int J Cancer*, **105**, 291-99.

Nakazawa H, Kitano K, Cioca D, et al (2000). Induction of polyploidization by jaspamide in HL-60 cells. *Acta Haematol*, **104**, 65-71.

Persoone G, Sorgeloos P (1980). General aspects of the ecology and biogeography of Artemia. In: The brine shrimp Artemia. Ecology, Culturing, Use in aquaculture Volume 3. Edited by: Persoon G, Sorgeloos P, Roels O, Jaspers E. Universal Press, pp 3-24.

Sun Y, Macrae TH (2005). Characterizationof novel sequence motifs within N- and C- terminal extentions of P26, Asmall heat shock protein from Artemia Franciscana. *FEBS J*, **272**, 5230-43.

Tanguay JA, Reyest RC, Clegg JS (2004). Habitat diversity and adaptation to environmental stress in encycted embryos of the crustacean Artemia. *J Bio Sci*, **29**, 489-501.

Villeneuve TS, Ma X, Sun Y, et al (2006). Inhibition of apoptosis by P26: implications for small heat shock protein function during Artemia development. *Cell Stress Chaperones,* **11**, 71-80.

Wilson-Sanchez G, Moreno-Félix C, Velazquez C, et al (2010) Antimutagenicity and antiproliferative studies of lipidic extracts from white shrimp (Litopenaeus vannamei). *Mar Drugs*, **8**, 2795-09.

Wurtsbaugh WA (1992). Food-web modification by an invertebrate predator in the Great Salt Lake, USA. *Oecologia*, **89**,168-75.

Wurtsbaugh WA, Gliwicz ZM (2001). Limnological control of brine shrimp population dynamics and cyst production in the Great Salt Lake, Utah. *Hydrobiologia*, **466**, 119-32.