

Standardized Extract of the Persian Gulf Sponge, *Axinella Sinoxea* Selectively Induces Apoptosis through Mitochondria in Human Chronic Lymphocytic Leukemia Cells

Ahmad Salimi^{1,2}, Mehrnoush Pir Saharkhiz¹, Abbasali Motallebi³, Enayatollah Seydi¹, Ali Reza Mohseni⁴, Melika Nazemi⁵ and Jalal Pourahmad^{1,6,*}

¹Department of Pharmacology and Toxicology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

²Department of Pharmacology and Toxicology, School of Pharmacy, Ardabil University of Medical Science, Ardabil, Iran

³Research and Education and Extension Organization (AREEO) and Iranian Fisheries Research Organization, Ministry of Jihad-e-Agriculture, Tehran, Iran

⁴Department of Laboratory Sciences, Faculty of Paramedicine, Mazandaran University of Medical Sciences, Sari, Iran

⁵Iranian Fisheries Research Institute, Persian Gulf and Oman Sea Ecological Research, Agricultural Research, Education and Extension Organization (AREEO), Bandar Abbas, Iran

⁶Phytochemistry Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract: Sponges are important components of the Persian Gulf animal communities. The marine sponges of the genus *Axinella sinoxea* is a genus of sponges in the family *Axinellidae*. Species of *Axinella sinoxea* occur in the India, Pacific Oceans and also Persian Gulf. Chronic lymphocytic leukemia (CLL) is a disease characterized by the relentless accumulation of CD5⁺ B lymphocytes. CLL is the most common leukemia in adults, about 25–30% of all leukemias. In this study B lymphocytes mitochondria (both cancerous and non-cancerous) were isolated using differential centrifugation from peripheral blood samples and succinate dehydrogenase activity, mitochondrial reactive oxygen species (ROS) production, collapse of mitochondrial membrane potential (MMP), mitochondrial swelling and finally release of cytochrome C were examined following the addition of methanolic extract of *Axinella sinoxea*. Our results showed that only in mitochondria isolated from cancerous BUT NOT normal lymphocytes a significant ($P < 0.05$) increase in mitochondrial ROS formation, MMP collapse, mitochondrial swelling and cytochrome c release. These results showed that *Axinella sinoxea* extract has a selective toxicity on chronic lymphocytic leukemia lymphocytes and their mitochondria and hence may be considered as a promising anti CLL candidate for further studies needed as a supplement for cancer patients in the future.

Keywords: Sponge, *Axinella sinoxea*, Mitochondria, Chronic Lymphocytic Leukemia.

1. INTRODUCTION

Marine organisms have shown to be potential sources of bioactive compounds with pharmacological effect [1]. With bryozoans, tunicates and sponges being the most promising marine organisms as sources of new active compounds for drug development [2]. Because of their prevalence, ease of collection and ability to biosynthesize an array of structurally diverse natural products, marine sponges have been the primary source of biologically active marine natural products [3]. The initial study on the sponge nucleosides spongouridine and spongothymidine in the marine sponge *Cryptotethia crypta* played a major role in promoting the search for marine bioactive molecules

[4]. In the recent years, marine natural products bioprospecting has yielded a considerable number of drug candidates, most still being in preclinical or early clinical development, with only a limited number already in the market [5]. Among these, several anticancer agents derived from marine sources especially sponges have entered preclinical and clinical [6-8]. These compounds (Cytarabine, Halichondrin B, Bryostatin1, Dolastatin 10 and Ecteinascidin 743) have shown cytotoxic activity against various tumor types (leukemia and lymphoma) [9]. Cancer, as one of the most important diseases in humans, has always attracted the scientific and commercial communities with an effort of continuously discovering new anticancer agents from natural products sources [10]. Chronic lymphocytic leukemia (CLL), is a low-grade B-cell malignancy [11] identified by the gradual accumulation of a monoclonal population of CD5⁺CD19⁺ B lymphocytes [12]. Clinically, the CLL is

*Address correspondence to this author at the Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, P.O. Box: 14155-6153, Iran; Tel: +98(21)2255-8786; Fax: +98(21)8820-9620; E-mail: j.pourahmadjaktaji@utoronto.ca

very heterogeneous that some patients may live for many years, while others may rapidly die of progressive and chemotherapy-resistant forms of CLL. At present, there is no effective cure for CLL, and thus novel therapies have been urgently demanded for these patients with poor prognosis [13]. Apoptotic mode of death is mainly characterized by morphological changes such as phosphatidylserine (PS) translocation across the plasma membrane [13], accumulation of reactive oxygen species (ROS) in cells [14] and loss of mitochondrial membrane potential ($\Delta\Psi_m$) [15].

Apoptosis is a tightly regulated, genetically encoded process of cell suicide that leads to the clearance of dead cells without provoking a potentially damaging inflammatory response [12]. Although classically recognized for their role in adenosine triphosphate (ATP) production, mitochondria are currently regarded as central organelles in mediating intrinsic death signals and might provide a novel target for new chemotherapeutics. Mitochondria were not fully appreciated as mediators of apoptosis until it was discovered that cytochrome c a mitochondrial protein, can activate caspases [4, 16, 17]. Once in the cytoplasm, cytochrome c binds Apaf-1 and procaspase 9, leading to the activation of caspase 9 and the initiation of the caspases cascade [18].

In our study we isolated B lymphocytes from CLL patients and normal lymphocytes from healthy donors. Then we investigated cytotoxic effect of methanolic extract of *Axinella sinoxea* on B lymphocytes obtained from CLL patients. Mitochondria as a major pathway that can induce apoptosis in cells were studied. Therefore the known parameters of mitochondrial dysfunction, such as mitochondrial swelling, ROS formation, collapse mitochondrial membrane potential and cytochrome C release as an initiator of induced death on isolated mitochondria from both groups (CLL patients and healthy donors) lymphocytes are researched.

2. MATERIALS AND METHODS

2.1. Sponge Sampling and Identification

Sponge samples of *Axinella Sinoxea* were collected manually from exposed and semi-exposed habitats, at depths of between 0.5 and 14 m, from locations on the coast line of Persian Gulf (southern Iran). Taxonomic designation was based on scanning electron microscope studies and on skeletal slides and dissociated spicule mounts. Specimens of all materials

are deposited in the Department of Fisheries University of Hormozgan, Iran [9].

2.2. Extract Preparation

Methanolic extracts were produced by the following procedure. Sponge materials were ground together with sand and water three times for 30 min. The resulting extract (collected after each 30 min) was subsequently filtered and freeze-dried. The remaining material was sequentially extracted five times with a methanol by maceration over 5 days. The resulting extract solution was then filtered and concentrated in a Rotavapor. For the cytotoxic and mitochondrial assays, methanolic extract was suspended in 0.05% Dimethyl sulfoxide (DMSO) (v/v) [9].

2.3. Selection Healthy Donors or Patients with CLL

10 CLL patients (6 males and 4 females) aged 54-65 years were enrolled in this study. CLL was diagnosed and confirmed according to definition of the World Health Organization (WHO) classification by oncologist. Only those patients showing no previous treatments within the last 6 months were included in the present study. All 10 patients were sampled prior to the commencement of any treatment and at least 2 weeks after the transfusion. Age-matched controls were obtained from 10 healthy donors. This study was approved by the Shahid Beheshti University of Medical Science's ethics committee and all the patients and healthy controls signed an informed consent form [19].

2.4. Isolation Lymphocytes

Blood samples were obtained from the CLL patient and healthy donors during a routine diagnosis at Shohadaye Tajrish Hospital. B lymphocytes were isolated immediately using Ficoll gradient centrifugation. After 1 h of incubation at 37°C in 5% CO₂, adhesive mononuclear cells were removed. Those non-adherent lymphocytes were thoroughly washed with the Hank's solution. T lymphocytes were removed using anti-CD3 dynabeads. The purification of B lymphocytes was assessed by flow cytometry with anti-CD19 antibodies with FACS (Immunotech, Coulter, USA). This cell preparation contained about 95% CD19 (B lymphocyte antigen) positive cells. It was added stromal cell-derived factor-1 to rescue B-lymphocytes from apoptosis [19].

2.5. Cytotoxicity Assay

The effect of the methanolic extract of *Axinella sinoxea* on lymphocytes obtained from healthy

donors or patients with CLL was investigated using MTT assay. Briefly, the cells were seeded in 96-well culture plates at a density of 10,000 cells per well. The methanolic extract of *Axinella sinxensis* was added to various final concentrations (0, 100, 250, 500, 1000 and 2000 µg/ml) in triplicates. After 24 h of incubation with extract, 25 µl of MTT reaction solution (5 mg/ml in RPMI 1640 medium) was added to the wells. The optical density was read at 580 nm wavelength in an ELISA plate reader (Tecan, Rainbow Thermo, Austria) after 4 h incubation of the plates with MTT in an incubator (37 °C and 5% CO₂ air). All determinations were confirmed using replication from at least three identical experiments [20].

2.6. Cell Lysis and Isolation of Mitochondria

Mitochondria were isolated from the lymphocytes by mechanical lysis and differential centrifugation. Briefly, cells were washed with cold PBS at 4°C and centrifuged at 1300 RPM. The pellet was resuspended in cold isolation buffer (75 mmol/L sucrose, 20 mmol/L HEPES, 225 mmol/L mannitol, 0.5 mmol/L EDTA, pH 7.2), and the cells were disrupted by homogenization. Nonlysed cells and nuclei were spun down by centrifugation at 1000 g for 10 minutes. The supernatant was further spun at 20000 g for 25 minutes. The pellet, designated as the mitochondrial fraction, was suspended in related buffer. The isolation of mitochondria was determined by measurement of succinate dehydrogenase [21].

2.7. Determination of Protein Concentration and Normalizing Samples

The Bradford assay is very fast for determination of protein concentration. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. We prepared a standard curve of absorbance versus micrograms protein with albumin (0, 500, 1000, 1500 and 2000 µg/ml) and determined amounts from the curve. Final concentration of isolated mitochondria was adjusted in 1000 µg/ml in corresponding buffer [22].

2.8. Measurement of Succinate Dehydrogenase

Briefly, mitochondria obtained from patients and healthy donors was suspended in assay buffer (140 mmol/L KCl, 10 mmol/L NaCl, 2 mmol/L MgCl₂, 0.5 mmol/L KH₂PO₄, 20 mmol/L HEPES, 0.5 mmol/L EGTA; adjusted to pH 7.2 with KOH). The assay buffer was supplemented with 1 mg/mL rotenone and 10

mmol/L succinate immediately before use. The mitochondria were seeded in 96-well culture plates at a density of 100 µg/ml per well. The extract was added to various final concentrations (0, 50, 100, 200, 500 and 1000 µg/ml) in triplicates. After 1 h of incubation with extract, 25 µl of MTT reaction solution (0.4% MTT in assay buffer including succinate) was added to the wells. The optical density was read at 580 nm wavelength in an ELISA plate reader after 30 minutes incubation of the plates with MTT in an incubator. All determinations were confirmed using replication from at least three identical experiments [23].

2.9. Determination of Mitochondrial ROS in Isolated Mitochondria

The mitochondrial ROS measurement was performed using the fluorescent probe DCFH-DA. Briefly, isolated mitochondria from both groups of lymphocytes and were placed in respiration buffer (0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 µM EGTA, 0.5 mM MgCl₂, 0.1 mM KH₂PO₄ and 5 mM sodium succinate). Following this step, DCFH-DA was added (final concentration, 10 µM) to mitochondria and following addition of various concentration of extract incubated for 10 min. Then, the fluorescence intensity of DCF was measured using Shimadzu RF-5000U fluorescence spectrophotometer at an excitation wavelength of 488 nm and emission wavelength of 527 nm without 1 hour [24].

2.10. Determination of the Collapse of Mitochondrial Membrane Potential (MMP)

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential. The mitochondrial fractions (1000 µg protein/mL) were incubated with 10 µM of rhodamine 123 in MMP assay buffer (220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH₂PO₄, 2 mM MgCl₂, 50 µM EGTA, 5 mM sodium succinate, 10 mM HEPES, 2 µM Rotenone) and following addition of various concentration of extract the fluorescence was monitored using Shimadzu RF-5000U fluorescence spectrophotometer at the excitation and emission wavelength of 490 nm and 535 nm, respectively [25].

2.11. Determination of Mitochondrial Swelling

Purified mitochondria were isolated as described above. Mitochondria suspensions (at 100 µg protein per well) were incubated in 96-well plates at 25° C in assay buffer (140 mmol/L KCl, 10 mmol/L NaCl, 2 mmol/L MgCl₂, 0.5 mmol/L KH₂PO₄, 20 mmol/L

HEPES, 0.5 mmol/L EGTA; adjusted to pH 7.2 with KOH) supplemented with 1 mg/mL rotenone and 10 mmol/L succinate. We added various compounds (different concentrations of extract and negative and positive controls, separately) after 10 minutes of preincubation of mitochondria in swelling buffer. We preincubated our isolated mitochondria just to adapt to our experimental condition. Mitochondrial swelling was measured spectrophotometrically. This method equates mitochondrial membrane permeability transition with high amplitude swelling of the mitochondria. Mitochondrial swelling results in a decrease in absorbance monitored at 540 nm [19].

2.12. Determination of Cytochrome c Release from Isolated Mitochondria

The concentration of cytochrome c was determined through using the Quantikine human Cytochrome c Immunoassay kit (R&D Systems, Inc., Minneapolis, Minn, USA). Briefly, a monoclonal antibody specific for human cytochrome c was pre-coated onto the microplate. 75 μ L of conjugate (containing monoclonal antibody specific for cytochrome c conjugated to horseradish peroxidase) and 50 μ L of control and test group were added to each well of the microplate. One microgram of protein from each supernatant fraction was added to the sample wells. All of the standards, controls and test were added to two wells of the microplate. After 2 h of incubation, the substrate solution (100 μ L) was added to each well and incubated for 30 min. After this step, 100 μ L of the stop

solution was added to each well; the optical density of each well was determined through the aforementioned microplate spectrophotometer set to 450 nm [19].

2.13. Statistical Analysis

Results are presented as mean $\pm$ SD. Assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test when appropriate. Statistical significance was set at $P < 0.05$. And the parameters of mitochondrial dysfunction were analyzed by two way ANOVA and Bonferonie posttest. In all graph were expressed as mean $\pm$ SEM and $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Cell Viability

Determination of the methanolic extract of *Axinella sinxoeais* for potential anti-cancer activity on B lymphocytes cells obtained from CLL patients were carried out by studying the cytotoxicity effects using the MTT assay. All used concentrations decreased cell viability in CLL B lymphocytes and concentration of 500 μ g/mL resulted in the 50% MTT reduction at 24 hours (Figure 1B). Then, the effect of extract on viability of normal (lymphocytes) cells was tested. Toxicity evaluation in normal lymphocytes revealed no significant MTT reduction at the tested concentration (Figure 1A).

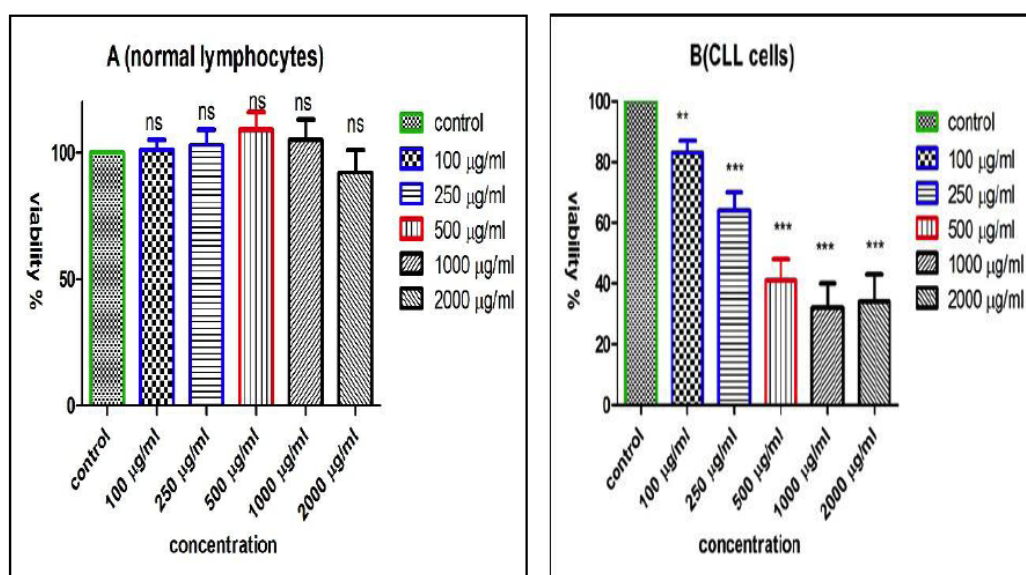


Figure 1: Effect of the methanolic extract of *Axinella sinxoeais* in both normal (A) and cancerous (B) B lymphocytes on cell viability. Cells were treated with the methanolic extract of *Axinella sinxoeais* and cell viability was measured by MTT assay at 24 h. Values were expressed as mean $\pm$ SD of three separate determinations (graph A and B).

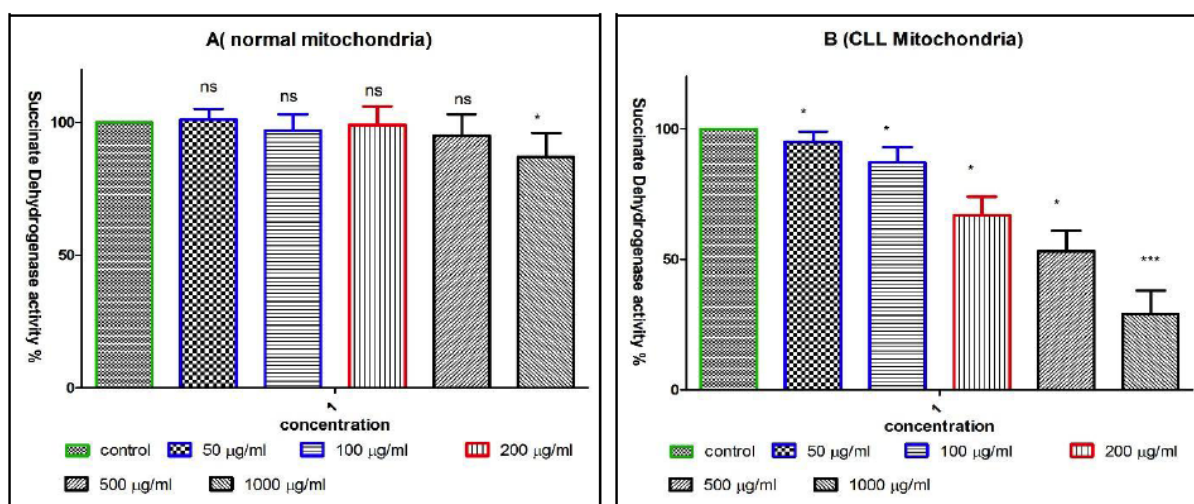


Figure 2: Effect of the methanolic extract of *Axinella sinoxea* in both normal (A) and cancerous (B) mitochondria on succinate dehydrogenase activity. This figure demonstrates the effect of the methanolic extract of *Axinella sinoxea* is on succinate dehydrogenase activity in both normal and cancerous mitochondria. Mitochondrial succinate dehydrogenase activity was measured by MTT assay within 1 h after extract exposure. Values were expressed as mean $\pm$ SD of three separate determinations (graph A and B).

3.2. Succinate Dehydrogenase Activity

Evaluations of the methanolic extract of sponge *Axinella sinoxea* for potential activity on mitochondria obtained from B lymphocytes of CLL patients and normal lymphocytes were carried out by studying its inhibitory effects on mitochondrial succinate dehydrogenase activity using the MTT assay. The methanolic extract of *Axinella sinoxea* strongly inhibited succinate dehydrogenase activity in a dose-dependent manner in the cancerous mitochondria, obtained from CLL patients at a range concentration (50-1000 μ g/ml) while only high concentration of 1000

μ g/ml influenced succinate dehydrogenase activity on normal mitochondria obtained from lymphocytes of healthy donors (Figures 2B, 2A).

3.3. ROS Formation Assay

We examined whether the level of ROS in both cancerous and normal mitochondria were affected by the methanolic extract of *Axinella sinoxea* with DCFH-DA staining. As shown in Figure 3B, treatment with the methanolic extract of *Axinella sinoxea* at 100, 200 and 500 μ g/ml in the interval within 1 hour (15, 30, 45, 60 minutes) significantly increased ROS generation

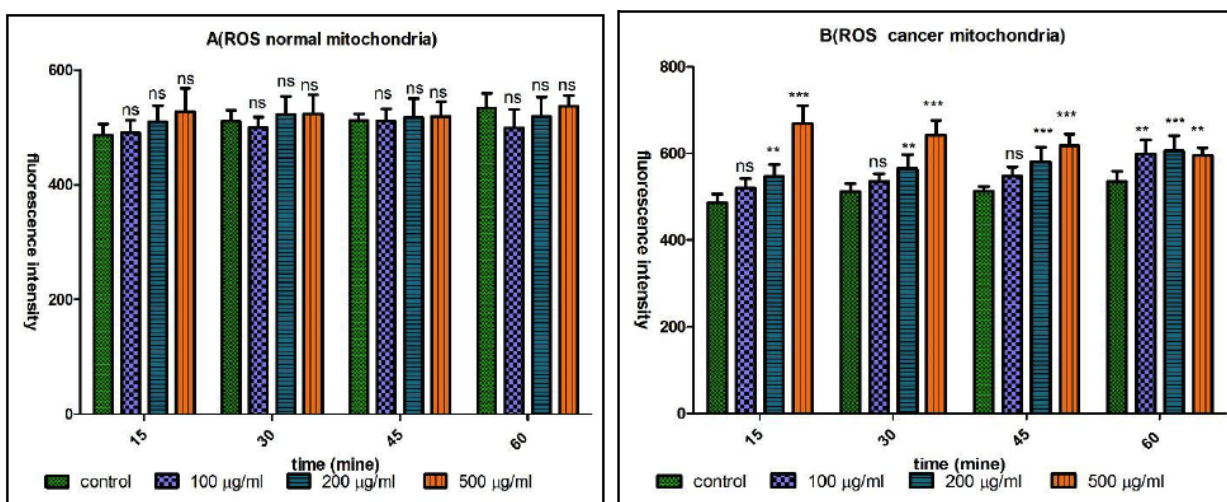


Figure 3: Effect of the methanolic extract of *Axinella sinoxea* in both normal (A) and cancerous (B) mitochondria on ROS formation. Freshly isolated mitochondria were obtained from both healthy donors and CLL patients incubated with the methanolic extract of *Axinella sinoxea* for 1 hour in both groups. ROS was measured by DCFH-DA staining with spectrofluorescence method. The mean fluorescence intensity was enhanced by extract in comparison to control for each group during 1 hour after extract addition. Values were expressed as mean $\pm$ SD of three separate determinations (graph A and B).

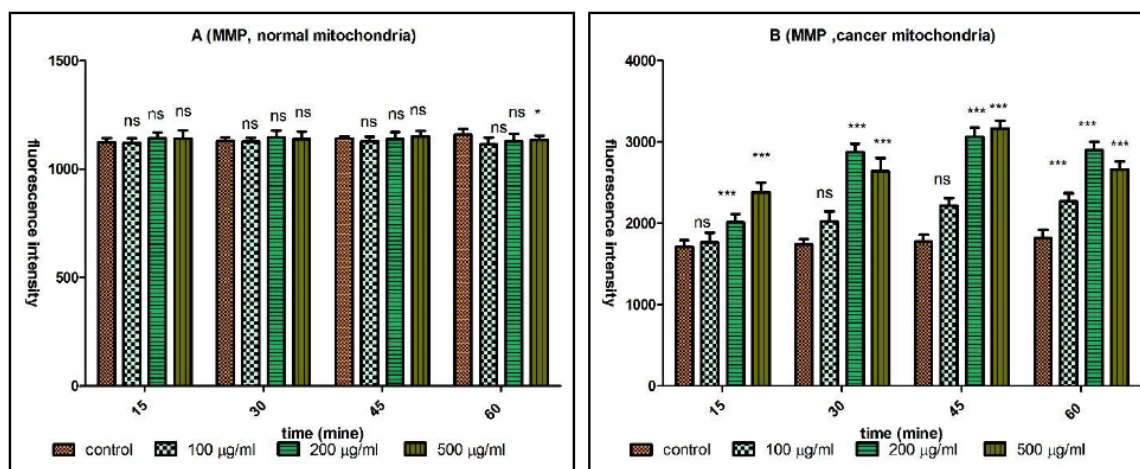


Figure 4: Effect of the methanolic extract of *Axinella sinoxea* in both normal (A) and cancerous (B) mitochondria on $\Delta\Psi_m$. Freshly isolated mitochondria from both healthy and CLL cells were treated as indicated above. $\Delta\Psi_m$ was measured spectrophotometrically by rhodamine 123 staining with. The presented data revealed that the methanolic extract of *Axinella sinoxea* induced a decrease in $\Delta\Psi_m$ only in cancerous mitochondria obtained from CLL patients (graph B) BUT NOT normal lymphocyte mitochondria obtained from healthy donors (graph A). Values were expressed as mean $\pm$ SD of three separate determinations (graph A and B).

($P < 0.05$) only the in cancerous mitochondria. These results suggested that the increasing effect of *Axinella sinoxea* extract on ROS generation might justify its effect on promoting CLL cell apoptosis. However as shown at Figure 3A, treatment with the methanolic extract of *Axinella sinoxea* is at 100, 200 and 500 $\mu\text{g/ml}$ in the interval within 1 hour (15, 30, 45, 60 minutes), did not induce significant ROS elevation in normal mitochondria.

3.4. MMP Assay

To search for the mechanisms involved in apoptosis, we examined the effects of the methanolic

extract of *Axinella sinoxea* is on membrane permeability of mitochondria ($\Delta\Psi_m$) in isolated mitochondria from both groups. The treatment with extract (100, 200 and 500 $\mu\text{g/ml}$ for 1 hour) showed that $\Delta\Psi_m$ decreased only in the mitochondria obtained from CLL B lymphocytes in comparison to their corresponding control (Figure 4B). Our study on normal mitochondria showed that the treatment with extract (100, 200 and 500 $\mu\text{g/ml}$ for 1 hour) did not induce any $\Delta\Psi_m$ collapse in the mitochondria obtained from normal B lymphocytes in comparison to their corresponding control (Figure 4A).

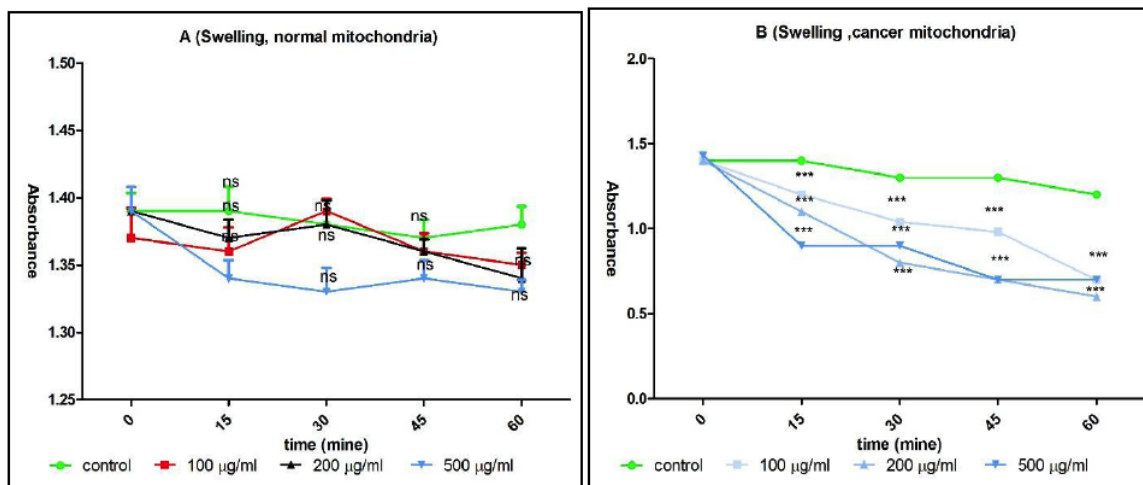


Figure 5: Effect of the methanolic extract of *Axinella sinoxea* in both normal (A) and cancerous (B) mitochondria on mitochondrial swelling. The methanolic extract of *Axinella sinoxea* addition (100, 200 and 500 $\mu\text{g/ml}$) induced mitochondrial swelling in cancerous But NOT normal lymphocyte mitochondria in a concentration depending manner. Mitochondrial swelling suspensions were incubated during for 1 hour. Mitochondrial samples were monitored by following 540 nm absorbance decrease every 15 minutes. Values were expressed as mean $\pm$ SD of three separate determinations (graph A and B).

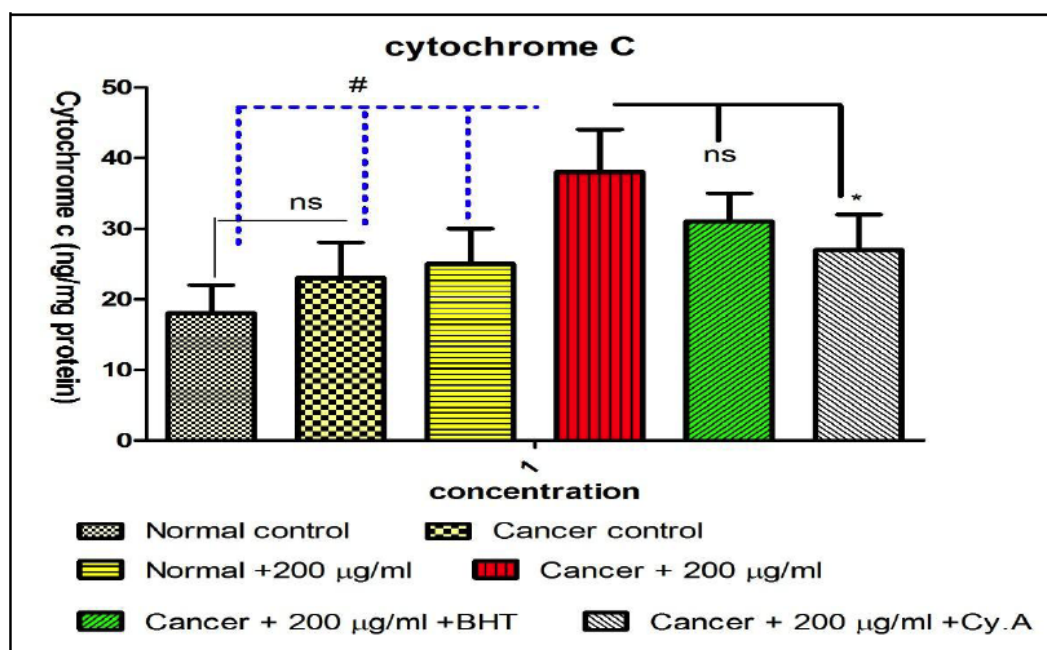


Figure 6: Effect of the methanolic extract of *Axinella sinoxea* in both normal (A) and cancerous (B) mitochondria on the cytochrome c release. As shown in this figure, pretreatment with BHT and CsA significantly inhibited cytochrome c release in the cancerous BUT NOT in normal lymphocyte mitochondria. The amount of expelled cytochrome c from mitochondrial fraction into the suspension buffer was determined using human Cytochrome c ELISA kit as described above. Values were expressed as mean $\pm$ SD of three separate determinations.

3.5. Mitochondrial Swelling

Induction of mitochondrial swelling in mitochondria isolated from lymphocytes was monitored by following 540 nm absorbance. The methanolic extract of *Axinella sinoxea* addition (100, 200 and 500 µg/ml) resulted in an extensive mitochondrial swelling in cancerous mitochondria obtained from B lymphocytes of CLL patients (Figure 5B). Addition of the extract to normal mitochondria (100, 200 and 500 µg/ml) did not result in mitochondrial swelling (Figure 5A).

3.6. Cytochrome c Assay

Our results showed that the methanolic extract of *Axinella sinoxea* is significantly caused mitochondrial swelling and collapse of the mitochondrial membrane potential. These events could result in mitochondrial permeability transition and release of cytochrome c from mitochondria into the cytosolic fraction. As shown in Figures 6, the methanolic extract of *Axinella sinoxea* is (200 µg/ml) induced significant ($P < 0.05$) release of cytochrome c on the B lymphocyte mitochondria isolated from CLL patients but not normal control group. Significantly, the pretreatment of the methanolic extract of *Axinella sinoxea* is-treated mitochondria with the MPT inhibitor of cyclosporine A (Cs.A) and butylated hydroxyl toluene (BHT), an antioxidant, inhibited cytochrome c release as compared with The

methanolic extract of *Axinella sinoxea* is treated group (100 µg/ml) ($p < 0.05$), indicating the role of oxidative stress and MPT pore opening in cytochrome c release.

4. DISCUSSION

At the last estimate, 80% of the world's population still rely on traditional medicines for their health care needs. Without doubt, natural products have been, and still are, the cornerstone of the health care medicines [10]. Moreover, many organisms, both marine and earthly, have developed chemical defense mechanisms, secondary metabolites, which allow a selective advantage and often have distinct biological activities against receptors and enzymes which make them ideal candidates for promising pharmacological investigation [1]. The most interesting phylum with regard to pharmacologically active marine compounds include fungi, bacteria, soft corals and gorgonians, sea hares and nudibranchs, algae, tunicates, bryozoans and sponges [3].

Covering around 70% of the planet surface, the oceans possess a huge potential for the new discovery often on novel molecules [26]. In this report, we investigated the effect of marine sponge, *Axinella sinoxea* (collected from the coast line of Bandarabas, southern Iran), methanolic extract for selective toxic activity on B lymphocytes and their mitochondria

obtained from CLL patients. The results obtained in our study showed that the methanolic extract of *Axinella sinoxea* has selective toxicity on cancerous B lymphocyte and their mitochondria and these effects are depended on concentration and time (data not shown) (Figures 1 and 2).

Our findings on mitochondria isolated from B lymphocytes of CLL patients showed that ROS formation increased following addition of methanolic extract of *Axinella sinoxea* in cancerous mitochondria (Figure 3B) while such a effect has not been observed on normal mitochondria (Figure 3A). Elevated ROS levels in tumor cells compared to normal cells has been exploited experimentally to kill cancer cells specifically by chemically pushing ROS levels over a critical homeostatic threshold that is incompatible with either growth or survival of tumor cells but tolerable by normal cells [27]. Indeed, many current anti-tumor agents used in the clinic, such as cisplatin and certain alkaloids, rely on ROS production for their efficacy [13, 28, 29]. MMP is a universal feature of cell death and is often considered as the “point of no return” in the cascade of events leading to apoptosis [30]. Shortly after the discovery that MMP is frequently impaired in cancer, mitochondria have become an attractive target to induce apoptosis and to overcome resistance to chemotherapy [31-33]. Currently, more than 20 mitochondrion-targeted compounds have been reported to induce apoptosis selectively in malignant cell lines, and some of these already being used in phase II/III clinical trials or validated *in vitro* in pre-clinical settings [31, 34]. These compounds can be classified according to their chemical nature into three main groups: peptide derivatives (Bax 106–134), small molecules (Arsenic trioxide), and cationic lipophilic agents (rhodamine 123). Tested extract at this study had shown that it selectively caused MMP in cancerous but not normal mitochondria (Figure 4). ROS induced promotion of Ca²⁺ dependent mitochondrial permeability transition (MPT), with swelling of the mitochondrial matrix and rupture of the OMM [35, 36] resulted from swelling assay on isolated CLL mitochondria proved again that mitochondrial swelling occurs only in cancerous but not normal mitochondria following the addition of methanolic extract of *Axinella sinoxea* (Figure 5). The mitochondrial respiratory chain on the inner mitochondrial membrane (IMM) is a major intracellular source of reactive oxygen species (ROS). Many studies have connected mitochondrial dysfunction caused by ROS with both apoptotic and necrotic cell death [37]. ROS may promote MPT by causing oxidation of thiol groups on the adenine

nucleotide translocator [31, 38]. Reactive oxygen species (ROS) play a key role in promoting mitochondrial cytochrome c release and induction of apoptosis. ROS induce dissociation of cytochrome c from cardiolipin on the inner mitochondrial membrane (IMM), and cytochrome c may then be released via mitochondrial permeability transition (MPT)-dependent or MPT-independent mechanisms. In our study we showed that cytochrome C release from cancerous mitochondria occurred following their exposure to extract (Figure 6). Stimuli triggering apoptosis through mitochondria pathway, resulting in the release of cytochrome c (Cyt c) from the mitochondrial intermembrane space (IMS) into cytoplasm and activation of downstream effector caspases [39]. Finally we suggest that the methanolic extract of *Axinella sinoxea* contains anti-cancer compounds which could induce apoptosis through mitochondria pathway with increasing ROS production and oxidative stress in cancerous B lymphocytes isolated from CLL patients.

REFERENCES

- [1] Faulkner DJ. Marine pharmacology. Antonie van Leeuwenhoek 2000; 77: 135-145.
<http://dx.doi.org/10.1023/A:1002405815493>
- [2] Faulkner DJ. Marine natural products. Natural Product Reports 2001; 18: 1R-49R.
<http://dx.doi.org/10.1039/b006897g>
- [3] Faulkner DJ. Highlights of marine natural products chemistry (1972-1999). Natural Product Reports 2000; 17: 1-6.
<http://dx.doi.org/10.1039/a909113k>
- [4] Ireland CM, Copp BR, Foster MP, *et al.* Biomedical potential of marine natural products. In: Pharmaceutical and Bioactive Natural Products. Springer 1993; pp. 1-43.
http://dx.doi.org/10.1007/978-1-4899-2391-2_1
- [5] Haefner B. Drugs from the deep: marine natural products as drug candidates. Drug Discovery Today 2003; 8: 536-544.
[http://dx.doi.org/10.1016/S1359-6446\(03\)02713-2](http://dx.doi.org/10.1016/S1359-6446(03)02713-2)
- [6] Mayer AM, Glaser KB, Cuevas C, *et al.* The odyssey of marine pharmaceuticals: a current pipeline perspective. Trends in Pharmacological Sciences 2010; 31: 255-265.
<http://dx.doi.org/10.1016/j.tips.2010.02.005>
- [7] Andavan GSB, Lemmens-Gruber R. Cyclodepsipeptides from marine sponges: Natural agents for drug research. Marine Drugs 2010; 8: 810-834.
<http://dx.doi.org/10.3390/md8030810>
- [8] Miller JH, Singh AJ, Northcote PT. Microtubule-stabilizing drugs from marine sponges: focus on peloruside A and zampanolide. Marine Drugs 2010; 8: 1059-1079.
<http://dx.doi.org/10.3390/md8041059>
- [9] Monks NR, Lerner C, Henriques AT, *et al.* Anticancer, antichemotactic and antimicrobial activities of marine sponges collected off the coast of Santa Catarina, southern Brazil. Journal of Experimental Marine Biology and Ecology 2002; 281: 1-12.
[http://dx.doi.org/10.1016/S0022-0981\(02\)00380-5](http://dx.doi.org/10.1016/S0022-0981(02)00380-5)
- [10] Kinghorn A, Farnsworth N, Soejarto D, *et al.* Novel strategies for the discovery of plant-derived anticancer agents. Pharmaceutical Biology 2003; 41: 53-67.
<http://dx.doi.org/10.1080/1388020039051744>
- [11] Catovsky D, Fooks J, Richards S. Prognostic factors in chronic lymphocytic leukaemia: the importance of age, sex

- and response to treatment in survival. *British Journal of Haematology* 1989; 72: 141-149.
<http://dx.doi.org/10.1111/j.1365-2141.1989.tb07674.x>
- [12] Reed JC. Molecular biology of chronic lymphocytic leukemia: implications for therapy. In: *Seminars in Hematology* 1998. p. 3.
- [13] Rassenti LZ, Jain S, Keating MJ, et al. Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia. *Blood* 2008; 112: 1923-1930.
<http://dx.doi.org/10.1182/blood-2007-05-092882>
- [14] Simon H-U, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 2000; 5: 415-418.
<http://dx.doi.org/10.1023/A:1009616228304>
- [15] Kiss T. Apoptosis and its functional significance in molluscs. *Apoptosis* 2010; 15: 313-321.
<http://dx.doi.org/10.1007/s10495-009-0446-3>
- [16] Newmeyer DD, Farschon DM, Reed JC. Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* 1994; 79: 353-364.
[http://dx.doi.org/10.1016/0092-8674\(94\)90203-8](http://dx.doi.org/10.1016/0092-8674(94)90203-8)
- [17] Liu X, Kim CN, Yang J, et al. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996; 86: 147-157.
[http://dx.doi.org/10.1016/S0092-8674\(00\)80085-9](http://dx.doi.org/10.1016/S0092-8674(00)80085-9)
- [18] Hosseini M-J, Shaki F, Ghazi-Khansari M, Pourahmad J. Toxicity of vanadium on isolated rat liver mitochondria: a new mechanistic approach. *Metallomics* 2013; 5: 152-166.
<http://dx.doi.org/10.1039/c2mt20198d>
- [19] Salimi A, Roudkenar MH, Sadeghi L, et al. Ellagic acid, a polyphenolic compound, selectively induces ROS-mediated apoptosis in cancerous B-lymphocytes of CLL patients by directly targeting mitochondria. *Redox Biology* 2015; 6: 461-471.
<http://dx.doi.org/10.1016/j.redox.2015.08.021>
- [20] Rotem R, Heyfets A, Fingrut O, et al. Jasmonates: novel anticancer agents acting directly and selectively on human cancer cell mitochondria. *Cancer Research* 2005; 65: 1984-1993.
<http://dx.doi.org/10.1158/0008-5472.CAN-04-3091>
- [21] Blattner JR, He L, Lemasters JJ. Screening assays for the mitochondrial permeability transition using a fluorescence multiwell plate reader. *Analytical biochemistry* 2001; 295: 220-226.
<http://dx.doi.org/10.1006/abio.2001.5219>
- [22] Faizi M, Salimi A, Rasoulzadeh M, et al. Schizophrenia induces oxidative stress and cytochrome C release in isolated rat brain mitochondria: a possible pathway for induction of apoptosis and neurodegeneration. *Iranian journal of pharmaceutical research: IJPR* 2014; 13: 93.
- [23] Rezaei M, Salimi A, Taghidust M, et al. A comparison of toxicity mechanisms of dust storm particles collected in the southwest of Iran on lung and skin using isolated mitochondria. *Toxicological & Environmental Chemistry* 2014; 96: 814-830.
<http://dx.doi.org/10.1080/02772248.2014.959317>
- [24] Salimi A, Ayatollahi A, Seydi E, et al. Direct toxicity of amyloid beta peptide on rat brain mitochondria: preventive role of *Mangifera indica* and *Juglans regia*. *Toxicological & Environmental Chemistry* 2015: 1-14.
<http://dx.doi.org/10.1080/02772248.2015.1079016>
- [25] Pirahmadi N, Fazeli M, Zarghi A, et al. 4-(4-(Methylsulfonyl)phenyl)-3-phenoxy-1-phenylazetidin-2-one: a novel COX-2 inhibitor acting selectively and directly on cancerous B-lymphocyte mitochondria. *Toxicological & Environmental Chemistry* 2015; 97: 908-921.
<http://dx.doi.org/10.1080/02772248.2015.1068985>
- [26] Mendola D. *Aquacultural production of bryostatin 1 and ecteinascidin 743*. Karger: Basel 2000.
<http://dx.doi.org/10.1159/000062482>
- [27] Raj L, Ide T, Gurkar AU, et al. Selective killing of cancer cells by a small molecule targeting the stress response to ROS. *Nature* 2011; 475: 231-234.
<http://dx.doi.org/10.1038/nature10167>
- [28] Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. *Molecular Cell* 2012; 48: 158-167.
<http://dx.doi.org/10.1016/j.molcel.2012.09.025>
- [29] Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nature reviews Drug Discovery* 2009; 8: 579-591.
<http://dx.doi.org/10.1038/nrd2803>
- [30] Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004; 305: 626-629.
<http://dx.doi.org/10.1126/science.1099320>
- [31] Belzacq A-S, El Hamel C, Vieira H, et al. Adenine nucleotide translocator mediates the mitochondrial membrane permeabilization induced by Iridomyrmecin, arsenite and CD437. *Oncogene* 2001; 20: 7579-7587.
<http://dx.doi.org/10.1038/sj.onc.1204953>
- [32] Brenner C, Grimm S. The permeability transition pore complex in cancer cell death. *Oncogene* 2006; 25: 4744-4756.
<http://dx.doi.org/10.1038/sj.onc.1209609>
- [33] Brenner C, Le Bras M, Kroemer G. Insights into the mitochondrial signaling pathway: what lessons for chemotherapy? *Journal of Clinical Immunology* 2003; 23: 73-80.
<http://dx.doi.org/10.1023/A:1022541009662>
- [34] Debatin K-M. Apoptosis pathways in cancer and cancer therapy. *Cancer Immunology, Immunotherapy* 2004; 53: 153-159.
<http://dx.doi.org/10.1007/s00262-003-0474-8>
- [35] Byrne AM, Lemasters JJ, Nieminen AL. Contribution of increased mitochondrial free Ca²⁺ to the mitochondrial permeability transition induced by tert-butylhydroperoxide in rat hepatocytes. *Hepatology* 1999; 29: 1523-1531.
<http://dx.doi.org/10.1002/hep.510290521>
- [36] Maciel EN, Vercesi AE, Castilho RF. Oxidative stress in Ca²⁺-induced membrane permeability transition in brain mitochondria. *Journal of Neurochemistry* 2001; 79: 1237-1245.
<http://dx.doi.org/10.1046/j.1471-4159.2001.00670.x>
- [37] Zamzami N, Hirsch T, Dallaporta B, et al. Mitochondrial implication in accidental and programmed cell death: apoptosis and necrosis. *Journal of Bioenergetics and Biomembranes* 1997; 29: 185-193.
<http://dx.doi.org/10.1023/A:1022694131572>
- [38] Zhao K, Zhao G-M, Wu D, et al. Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. *Journal of Biological Chemistry* 2004; 279: 34682-34690.
<http://dx.doi.org/10.1074/jbc.M402999200>
- [39] Desagher S, Martinou J-C. Mitochondria as the central control point of apoptosis. *Trends in Cell Biology* 2000; 10: 369-377.
[http://dx.doi.org/10.1016/S0962-8924\(00\)01803-1](http://dx.doi.org/10.1016/S0962-8924(00)01803-1)