

RESEARCH ARTICLE

Persian Gulf Jellyfish (*Cassiopea andromeda*) Venom Fractions Induce Selective Injury and Cytochrome C Release in Mitochondria Obtained from Breast Adenocarcinoma Patients

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Abstract

Objective: This study was conducted to investigate whether fractions of jellyfish *Cassiopea andromeda* venom, could selectively induce toxicity on mitochondria isolated from cancer tissue of patients with breast adenocarcinomas. **Methods:** Firstly, we extracted two fractions, (f1 and f2) from crude jellyfish venom by gel filtration on Sephadex G-200. Then different dilutions of these extracted fractions were applied to mitochondria isolated from human breast tumoral- and extra-tumoral tissues. Parameters of mitochondrial toxicity including generation of reactive oxygen species (ROS), mitochondrial membrane potential (MMP) collapse, swelling, cytochrome c release, activation of caspase3 and apoptosis were then assayed. **Result:** Our results demonstrate that fraction 2 of *Cassiopea andromeda* crude venom significantly ($P < 0.05$) decreased mitochondrial succinate dehydrogenase activity, increased mitochondrial ROS production, induced mitochondrial swelling, MMP collapse and cytochrome c release, activated caspase3 and induced apoptosis only in tumoral mitochondria, and not in mitochondria obtained from extra-tumoral tissue ($P < 0.05$). **Conclusion:** In conclusion this study suggested that fraction 2 of *Cassiopea andromeda* crude venom selectively induces ROS mediated cytotoxicity by directly targeting mitochondria isolated from human breast tumor tissue.

Keywords: *Cassiopea andromeda* venom- gel filtration- selective toxicity- Isolated mitochondria- Apoptosis

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Introduction

Breast cancer is the most common malignancy in women (23% of invasive cancers) which accounts for 25.2% of cancers diagnosed in women and 1.67 million new cancer cases in 2012 worldwide (Zhu et al., 2014; Ferlay et al., 2015).

Chemotherapy is the common mode of therapy used to treat and control of breast cancer, however severe side effects and drug resistance in patients has been reported. So searching for new cytotoxic natural compounds with low side effects, low drug resistance and high efficacy for prevention and treatment of breast cancer is required (Zhang et al., 2012; Kang et al., 2014).

The prominent cell organelle mitochondria play a vital role in cellular functions such as metabolism, redox signaling, cellular calcium concentration, production of reactive oxygen species (ROS) and cell fate. Mitochondria are also indispensable for ATP production and for the

intrinsic pathway of apoptosis as a regulator (Fulda et al., 2010; Ralph et al., 2010). Mitochondria have been regarded to be a potentially essential target for development of anticancer drugs (Talari et al., 2014). According to Warburg effect phenomenon, there is a remarkable change in cancerous cells metabolism that exerts substantial increase in aerobic glucose metabolism and seems to be independent on mitochondrial pathway for production of ATP and also other molecules needed for cell growth and proliferation (Máximo and Sobrinho-Simões, 2000; Gogvadze, 2011). It should be regarded, that the mitochondrial functional and morphological changes perceived one cancer type or cell line, should not be distributed as usual mitochondrial abnormalities in all tumoral cells. because, it is possible that the particular alterations may be resulted from original tissues or cancerous cell types, disease stages, proliferation and differentiation statement (Gogvadze, 2011).

As a current approach, mitochondria mediated ROS

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formation is a powerful basis for cancer therapy by initiating intrinsic pathway of apoptosis. To identify and introduce new compounds that are highly beneficial against a variety of cancers with selective toxicity, continued research is required. The marine ecosystem has been identified as an unfinished source of bioactive compound with extensive biological and pharmacological activities. Because of chemical complexity and biological diversity, marine-derived compounds are major candidates for the discovery of new therapeutic agents (Leone et al., 2013).

Jellyfish belongs to Cnidarians family increasingly have been introduced as an attractive source of physiologically and pharmacologically active compounds. venom from different jellyfish species were observed to show hemolytic (Kang et al., 2009), anti-microbial (Ovchinnikova et al., 2006), cardiovascular (Bruhn et al., 2001; Ramasamy et al., 2005), insecticidal (Yu et al., 2005a), antioxidant (Yu et al., 2005b) and cytotoxic (Marino et al., 2004) effects. Recently various studies accomplished on jellyfish venom showed that venom (from different jellyfish species) can inhibit the growth of tumoral cells like clavulone, a compound derived from *Clavularia viridis* which is able to inhibit growth of tumor cell in leukemia HL-60. Another research showed the anti cancerous effect of *Chrysaora quinquecirrha* nematocyst venom on animal model of Ehrlich ascites carcinoma (EAC). In this mentioned study, The antitumor influence of a peptide derived of sea nettle nematocyst venom (SNV) was proven by assessing in vitro cytotoxicity, antioxidant parameters and survival time (Honda et al., 1985; Balamurugan et al., 2010). On the other hand some of toxins from Jellyfish species for instance; venoms of *Chiropsalmus quadrigatus*, can even induce apoptosis in glioma and vascular endothelial cell lines (Sun et al., 2002).

Cassiopea andromeda which also called upside-down jellyfish is another member of Cnidarians family that was native the eastern Mediterranean, but due to some anthropogenic effects like eutrophication, global warming, shipping, overfishing, coastal developments and marine transports invasively entered the coastal waters of Persian Gulf in the Nayband Lagoon, from Bushehr- Iran since 2014 (Nabipour et al., 2015).

In the current study in order to explain the selective toxicity of *Cassiopea andromeda* venom fractions we introduced the crude venom fractions on mitochondria isolated from human tumoral breast tissue and monitored the possible upstream events in ROS mediated apoptosis through mitochondrial pathway and compared our findings with those of from mitochondria isolated from autologous extra tumoral breast tissue.

Materials and methods

Patients

The patients that were diagnosed newly with breast adenoma carcinoma ranging in 35-65 years from Surgery Center of Imam Khomeini, Tehran Medical University, Iran, who had not undergone any previous treatment for their tumors, were enrolled into the study. According to patient Selection criteria, the patients were not using,

oral contraceptives and hormones. None of them had concurrent diseases such as liver diseases, rheumatoid Arthritis and diabetes mellitus. Table 1 shows the general characteristics of breast adenoma carcinoma patients. In this study fresh tumor tissue was obtained from the core of the tumor whereas the normal tissue was obtained from neighboring extra tumoral parts at least 5 cm away from the tumor margin in the mastectomy specimen. This study was approved by Ethical Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.PHNM.1394.357). For the present study informed consent was received from all the patients.

Mitochondria isolation from human breast tissue

For mitochondrial isolation, the human breast tissue samples were collected after surgery and were washed with ice-cold buffer consisting sucrose 0.25 M, Tris-HCl 5 mM, pH 7.4, and EDTA 1 mM. After mincing in to tiny section with scissors, they were homogenized gently using homogenizer in chilled buffer. The homogenate was centrifuged at 1,300 ×g for 10 min to discard the pellet containing the nucleus, cell debris and erythrocytes and the supernatant obtained from the first step was centrifuged at 17,000 ×g for 15 min to obtain pellet containing purified mitochondria and then the extracted mitochondria were suspended in cold buffer. All the upon steps were performed at 4 °C (Upadhyay et al., 2002). The protein concentrations of obtained mitochondria were determined by the method of Bradford using bovine serum albumin as standard and all the measured mitochondrial parameters were normalized as (/mg of mitochondrial proteins) (Hosseini et al., 2013).

Crude venom preparation

After collection of *Cassiopea andromeda* specimens from coastal waters in the Nayband Lagoon, from Bushehr, Iran during summer months. Identity of the species was verified by Professor Brenden Holland from the University of Hawaii. For venom extraction, we separated nematocysts and tentacles (Bloom et al., 1998). Briefly, the tentacles were immediately excised manually from living specimen after capture and placed into small glass containers filled with seawater, and directly through the cool container, transferred to the biotechnology laboratory in Bushehr city. After that, they were homogenized using a homogenizer (IKA, Germany), the resultant homogenate was stored in 4°C for 2 days aimed at carrying out the autolysis of the tissues according to available protocol (Burnett et al., 1992) and then centrifuged (Eppendorf, Germany) at 12,000 ×g for 15 min in 4°C to exclude any sediments. The supernatant was freeze-dried (Christ, UK) and kept at -80°C for subsequent experiments.

Gel filtration chromatography

Sephadex G-200 (Pharmacia, USA) gels were poured into a 60 × 2.5 cm diameter. Elution was completed with 0.05 M phosphate buffer (pH 7.2 at 4°C) and flow rate adjusted to 0.5 ml/min. About 200 mg of crude venom of *Cassiopea Andromeda* was dissolved in phosphate buffer. After centrifugation at 12,000 ×g for 15 min at 4°C, the supernatant was loaded on Sephadex G-200,

the toxins eluted with the phosphate buffer and then fractions containing 4 ml were collected with fraction collector apparatus and finally protein absorbance was measured at 280 nm by UV spectrophotometer (Othman and Burnett, 1990).

Determination of succinate dehydrogenase activity

The activity of succinate dehydrogenase as a mitochondrial complex II was assayed by measurement of the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) to its insoluble formazan. Briefly, 100 μ L of mitochondrial suspensions (1 mg protein/ml) was incubated with different concentrations of crude venom and its fractions (0, 50, 100, 200, 500 and 1,000 μ g/ml) at 37 °C for 1 h; then 25 μ L of 0.4% of MTT was added to the medium and incubated at 37 °C for 30 min. The formed crystals were dissolved in 100 μ L DMSO and then the absorbance at 570 nm was measured with an ELISA reader (Tecan, Rainbow Thermo, Austria) (Salimi et al., 2015).

Determination of mitochondrial ROS formation

The mitochondrial ROS determination was performed using the DCFH-DA fluorescent probe. Briefly isolated breast mitochondria were placed in respiration buffer consisting 0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 μ M EGTA, 0.5 mM MgCl₂, 0.1 mM KH₂PO₄ and 5 mM sodium succinate and then DCFH-DA was added at final concentration of 10 μ M. After adding various concentration of *Cassiopea andromeda* venom into mitochondrial samples (1 mg protein/ml) and incubating them at 37 °C for 10 min, the DCF fluorescence intensity was assayed using Shimadzu RF-5,000U fluorescence spectrophotometer at excitation wavelength of 488 nm and emission wavelength of 527 nm (Shaki et al., 2012).

Determination of mitochondrial swelling

Determination of mitochondrial swelling in mitochondrial suspension (1 mg protein/mL) was performed by measurement of decreased absorbance of mitochondrial particles by spectrophotometer at 540 nm (30 °C) which is oppositely is relevant to increased forward light scattering due to mitochondrial enlargement (Rotem et al., 2005). Briefly, isolated breast mitochondria (1 mg protein/ml) were suspended in swelling buffer containing of 70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM tris-phosphates, 5 mM succinate, and 1 μ M rotenone and incubated at 30 °C for one hour with different concentration of venom. The absorbance was measured at 540 nm within one hour, at 15 min time intervals with an ELISA reader (Tecan, Rainbow Thermo, Austria). A decrease in absorbance illustrates an increase in mitochondrial swelling.

Determination of mitochondrial membrane potential (MMP)

Mitochondrial uptake of Rhodamine 123 As a cationic fluorescent dye has been used for the determination of mitochondrial membrane potential. Different concentrations of *Cassiopea andromeda* venom, Rhodamine 123 (final concentration 10 μ M)

was added to the mitochondria suspended (1 mg protein/mL) in MMP assay buffer including 220 mM sucrose, 68 mM MD-mannitol, 10 mM KCL, 5 mM KH₂PO₄, 2 mM MgCl₂, 50 μ M EGTA, 5 mM sodium succinate, 10 mM HEPES, 2 μ M Rotenone. The fluorescence activity was monitored at the excitation and emission wavelength of 490 nm and 535 nm respectively, by using fluorescence spectrophotometer (Schimadzu RF-5,000U) (Baracca et al., 2003).

In our study, in untreated control mitochondria because of highly negative charge of inner membrane, we have maximum uptake of rhodamine 123 and therefore presence of minimum dye in the buffer medium, and for this reason we have the lowest dye fluorescence. But on the other hand in the disturbed mitochondria pending on severity of damage or decrease of inner membrane potential, we have lower mitochondrial uptake of rhodamine 123 and therefore higher presence in buffer medium, the reason why we have higher dye fluorescence compared to untreated control mitochondria.

Determination of cytochrome c releases

The concentration of cytochrome c was measured by using the Quantikine Human Cytochrome c Immunoassay kit provided by R and D Systems, Inc, (Minneapolis, USA). In brief, according to the protocol of the manufacturer, a monoclonal antibody specific for human cytochrome c was pre coated onto the microplate. Seventy-five microliters of conjugate containing of monoclonal antibody specific for cytochrome c conjugated to horseradish peroxidase and 50 μ L of positive control and standard 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 samples were added to two wells of the microplate. After 2 h of incubation, 100 μ L of substrate was added to each well and then incubated for 30 min. After adding 100 μ L of the stop solution to each well, the optical density of each well was monitored by the spectrophotometer set to 450 nm.

Isolation of primary breast cancer cells from tumoral specimens

For cell isolation, the human breast tissue samples were collected after surgery and washed in fresh, ice-cold phosphate-buffered saline (PBS). Following mincing in to tiny section with sterile razor blade, minced tissues were transferred into a 15 ml tube containing 10 ml DMEM (Dulbecco's modified Eagle medium) with 0.1% collagenase and incubated at 37 °C for 30 min. To ensure optimal digestion of the specimens, digested tissue was transferred from the incubator and shaken vigorously by hand every 5 min. After 30 min incubation, the cell suspension was centrifuged at 100 x g for 2 min at 4 °C. Finally the cell suspension were plated in DMEM containing 10% FBS and supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, 10 mM Hepes (Speirs et al., 1998).

Determination of Cytotoxicity by MTT assay

The effect of the fraction 2 of *C. andromeda* venom on cancerous and healthy cells from patients with

breast adenocarcinoma was investigated using MTT assay. Briefly, the cells were plated in 96-well culture plates at a density of 1×10^6 cells per ml. The fraction 2 of *C. andromeda* venom was added to various final concentrations (0- 1,000 $\mu\text{g/ml}$) in triplicates. After 12 h of incubation with

fraction 2 (37°C and 5% CO₂-air), 25 mL of MTT reaction solution (5 mg/ mL) was added to the wells. The optical density was read at 580 nm wavelength in an ELISA plate reader after 4-h incubation of the plates with MTT in an Incubator (Zhang et al., 2012).

Determination of Caspase-3 Activity

The activity of Caspase-3 enzyme in the cell lysates was carried out according to instructions of the Sigma-Aldrich colorimetric assay kit (CASP-3-C; Sigma-Aldrich, Saint Louis, USA). In brief, the base of this colorimetric assay is hydrolysis of substrate peptide, Ac- DEVDpNA by caspase-3 and the absorbance of the p-nitroaniline released from substrate at 405 nm used for evaluation of caspase-3 activity.

Detection of Apoptosis by Propidium Iodide

Analysis of apoptosis by propidium iodide staining is a rapid and simple flow cytometry method for measuring apoptosis in cells. Fundamentally, in this method propidium iodide (PI), a fluorogenic compound used for nuclear staining by binding to nucleic acids and fluorescence emission induced of 488-nm laser beam, is proportional to the DNA content of a cell. Briefly, following the exposure with fraction 2 of *C. andromeda* venom (12 h), we suspended cells at 1×10^6 cells per 1 ml of PBS and centrifuged at 200g for 5 min at room temperature. After that cell pellet was resuspended in 1 ml of fluorochrome hypotonic solution containing 0.1% sodium citrate (wt/v), 0.1% Triton X-100 (v/v), 50 mg/ml PI in deionized/distilled water. Prior to flowcytometric analysis cells were maintained in the dark room at 4°C, for at least 1 hour. Finally cells were analyzed by flow cytometry using 488-nm laser beam for excitation (Riccardi and Nicoletti, 2006).

Statistical analysis

All statistical significance was performed using the Prism software, version 6. Assays were performed in triplicate and the data were showed as mean \pm SD. Comparisons were made using the one-way and two-way analysis of variance (ANOVA) tests. $P < 0.05$ was considered significant.

Results

Gel filtration chromatography

To obtain cytotoxic effect of fractions of jellyfish venom, we first fractionated crude venom using gel filtration chromatography. As shown in Figure 1, the Chromatogram of *Cassiopea andromeda* crude venom on Sephadex G-200 revealed two fractions, with absorbance at 280 nm (f1 and f2).

Mitochondrial succinate dehydrogenase activity

As shown in Figure 2 and Figure 3 (graph A and B),

the inhibiting effect of different concentrations of *C. andromeda* crude venom and its fractions (0, 50, 100, 200, 500 and 1,000 $\mu\text{g/ml}$) on mitochondrial succinate dehydrogenase activity was determined after 60 minutes of exposure with the MTT assay using isolated breast mitochondria from both tumoral and extra tumoral tissues. However, only fraction 2 of venom caused a significant concentration-dependent reduction of mitochondrial succinate dehydrogenase activity ($P < 0.05$) on tumoral but not extra tumoral mitochondria similar to what was seen by the whole crude venom. Crude venom and its fraction 2 only at the highest concentrations (1,000 $\mu\text{g/ml}$) showed inhibitory effect on extra tumoral healthy mitochondria. In contrast to fraction 2, fraction 1 of *C. andromeda* venom didn't decrease Succinate dehydrogenase activity on isolated tumoral mitochondria at any concentration applied ($P < 0.05$) (Figure 4).

To evaluate the mitochondrial damage parameters in isolated mitochondria following the treatment with fraction 2 of *C. andromeda* venom, The IC₅₀ value for this fraction determined at 60 min of treatment with tumoral mitochondria using MTT assay. The IC₅₀ of a compound is defined as the concentration that inhibits 50 percent of mitochondrial succinate dehydrogenase activity following 60 minutes of treatment. IC₅₀ for fraction 2 was approximately 240 $\mu\text{g/ml}$. In our study for determination of mitochondrial parameters following addition of fraction 2 of venom we used 120, 240 and 480 $\mu\text{g/ml}$ concentrations which is corresponding to IC₅₀/2, IC₅₀, and $2 \times$ IC₅₀ of this fraction.

Effect of fraction 2 of C. andromeda venom on mitochondrial ROS production

As shown in Figure 5, different concentrations of fraction 2 of *C. andromeda* venom (120, 240 and 480 $\mu\text{g/ml}$) induced significant increase at ROS generation in isolated breast cancer tumoral mitochondria in a concentration and time dependant manner ($\mu < 0.05$) (Figure 5). On the other hand no (even the highest) concentration of fraction 2 could induce ROS formation in extra tumoral healthy mitochondria following 60 min incubation.

Effects of fraction 2 of C. andromeda venom on Mitochondrial Membrane Potential (MMP)

As shown in Figure 6, different concentrations of fraction 2 of *C. andromeda* venom (120, 240, and 480 $\mu\text{g/ml}$) significantly ($P < 0.05$) decreased the Mitochondrial Membrane Potential (demonstrated as fluorescence intensity units emitted from Rh123) in a concentration and time dependent manner in the tumoral mitochondria. In contrast with tumoral mitochondria, there is no significant collapse in extra tumoral healthy mitochondria following the addition of different concentrations of fraction 2 ($P < 0.05$) within 60 min of incubation.

Effects of fraction 2 of C. andromeda venom on Mitochondrial Swelling

Mitochondrial swelling as a prominent characteristic of damage to mitochondria was monitored by decrease of absorption in mitochondrial suspension at 540 nm.

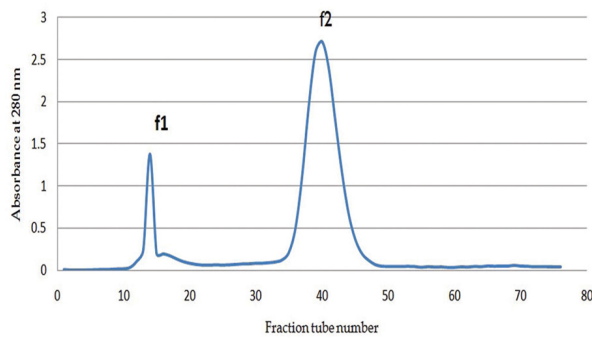


Figure 1. Gel filtration Chromatogram of *Cassiopea Andromeda* Crude Venom on a Sephadex G-200 (60×2.5 cm). 200 mg of Crude Venom Dissolved in 3 ml 0.15 M NaCl (pH 7.2), was Applied and Eluted with the Same Buffer. Flow Rate was 0.5 ml/min and 4 ml/ Tube were Collected at 4 °C

Treatment of mitochondrial obtained from tumoral tissues with different concentrations of venom fraction 2 (120, 240 and 480 $\mu\text{g/ml}$) within 60 min of incubation, led to significant mitochondrial swelling in tumoral but not extra tumoral health mitochondria ($P < 0.05$) (Figure 7).

Effects of fraction 2 of *C. andromeda* venom on Cytochrome c Release

After collapse of Mitochondrial Membrane Potential (MMP) and mitochondrial swelling that could lead to mitochondrial permeability transition (MPT) pore opening, release of cytochrome c from the mitochondria into the cytosolic fraction in response to different concentration of fraction 2 of *C. andromeda* venom, is a subsequent event. As shown in figure 8, fraction 2 of *C. andromeda* venom (240 $\mu\text{g/ml}$) significantly induced release of cytochrom c only in isolated tumoral mitochondria ($P < 0.05$). On the other hand pretreatment of venom fraction 2 (240 $\mu\text{g/ml}$)-treated mitochondria with cyclosporine A (CsA) and butylated hydroxyl toluene

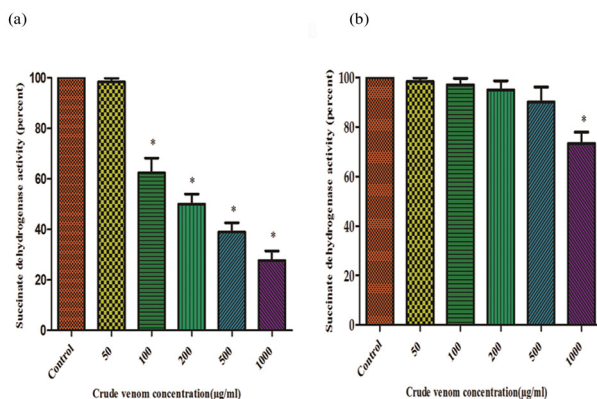


Figure 2. Effect of Crude Venom Concentrations (0, 50, 100, 200, 500 and 1,000 $\mu\text{g/ml}$) on Mitochondrial Succinate Dehydrogenase Activity Measured by MTT Assay Following 60 min of Treatment with the Both Tumoral (A) and Extra Tumoral Healthy (B) Mitochondria. Values are Represented as Mean \pm SD (n = 5). *Significant Difference in Comparison with the Corresponding Control Mitochondria ($P < 0.05$)

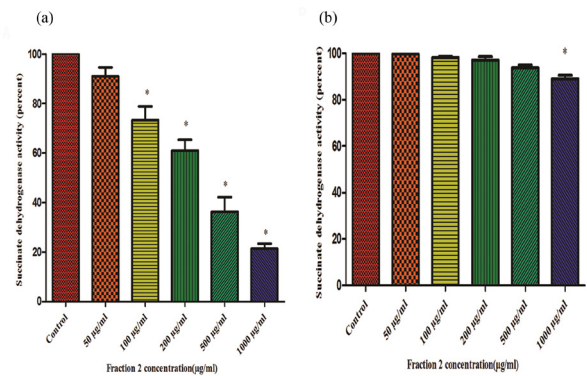


Figure 3. Effect of Fraction 2 Concentrations (0, 50, 100, 200, 500 and 1000 $\mu\text{g/ml}$) on Mitochondrial Succinate Dehydrogenase Activity Measured by MTT Assay Following 60 min of Treatment with the Both Tumoral (A) and Extra Tumoral healthy(B) Mitochondria. Values are Represented as Mean \pm SD (n = 5). *Indicated Significant Difference in Comparison with the Corresponding Control Mitochondria ($P < 0.05$)

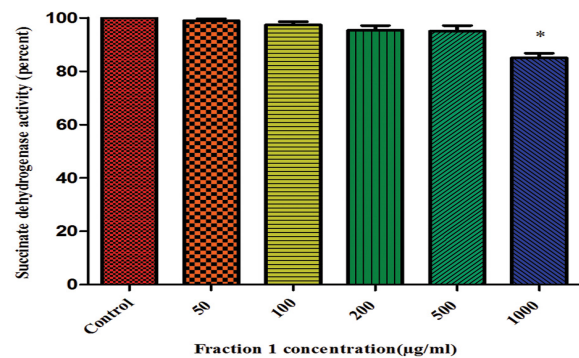


Figure 4. Effect of Fraction 1 Concentrations (0, 50, 100, 200, 500 and 1,000 $\mu\text{g/ml}$) on Mitochondrial Succinate Dehydrogenase Activity Measured by MTT Assay Following 60 min of Treatment with the Tumoral Mitochondria. Values are Represented as Mean \pm SD (n = 5). *Significant Difference in Comparison with the Corresponding Control Mitochondria ($P < 0.05$)

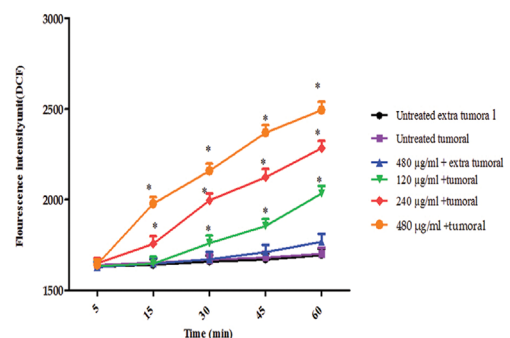


Figure 5. Effect of Various Concentrations of Fraction 2 of *C. andromeda* Venom (120,240 and 480 $\mu\text{g/ml}$) on Mitochondrial ROS Formation at Different Time Intervals within 60 Minutes of Incubation in Tumoral and Extra Tumoral Healthy Mitochondria. Values are Represented as Mean \pm SD (n = 5). *Significant Difference in Comparison with the Corresponding Untreated Control Mitochondria ($P < 0.05$)

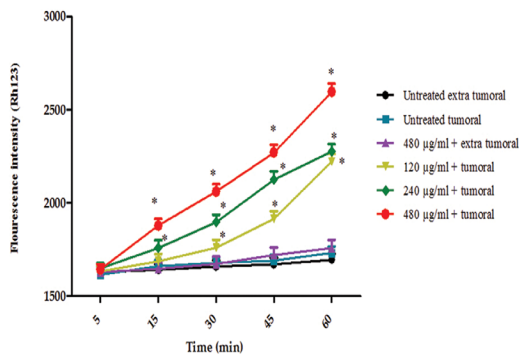


Figure 6. Effect of Various Concentrations of Fraction 2 of *C. andromeda* Venom (120, 240 and 480 µg/ml) on the Collapse of Mitochondrial Membrane Potential (MMP) at Different Time Intervals within 60 min of Incubation in Both Tumoral and Extra Tumoral Healthy Mitochondria. Values are Represented as Mean ± SD (n = 5). *Significant Difference in Comparison with the Corresponding Untreated Control Mitochondria (P < 0.05)

(BHT) as a MPT inhibitor and antioxidant respectively, inhibited release of cytochrome c compared with fraction 2 (240 µg/ml)- treated group (P < 0.05). Indeed these finding indicate that oxidative stress and MPT pore-opening could result in release of cytochrome c following the exposure to fraction 2 of *C. andromeda* venom to the breast cancer tumoral mitochondria.

Effects of fraction 2 of C. andromeda venom on cytotoxicity

As shown in Figure 9 (graph A and B), the inhibiting effect of different concentrations of fraction 2 of *C. andromeda* (0, 50, 100, 200, 500 and 1,000 µg/ml) on cell viability was determined after 12 h exposure with the MTT assay using isolated breast cells from both tumoral and extra tumoral tissues. However, fraction 2 of venom significantly (p < 0.05) reduced cell viability only in tumoral but not extra tumoral cells. Half-maximal inhibitory concentration (IC50) of a 12-hr fraction 2 treatment of cancerous cells was 240µg/ml.

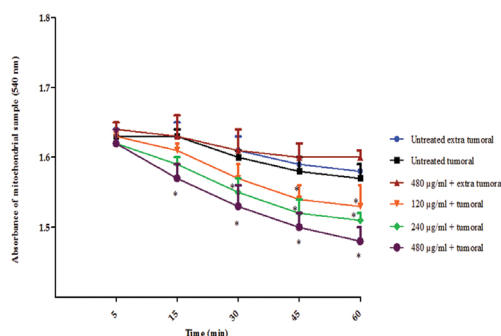


Figure7. Effect of Various Concentrations of Fraction 2 of *C. andromeda* Venom (120, 240 and 480 µg/ml) on Mitochondrial Swelling Measured by Decrease of Mitochondrial Suspensions Absorbance at 540nm at Different Time Intervals within 60 min of Incubation in Both Tumoral and Extra Tumoral Healthy Mitochondria. Values are Represented as Mean ± SD (n = 5). *Significant Difference Compared with the Corresponding Untreated Control Mitochondria (P < 0.05)

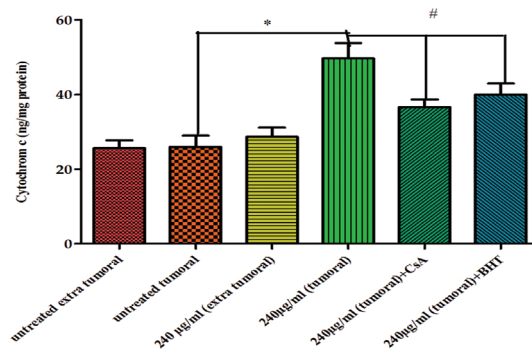


Figure8. Effect of Fraction 2 of *C. andromeda* Concentration (240µg/ml) on the Release of Cytochrome c in Tumoral and Extra Tumoral Healthy Mitochondria. Values are Presented as Mean ± SD.*Significant Difference in Comparison with the untreated tumoral mitochondria (P<0.05). #Significant Differences in Comparison with Fraction 2(240 µg/ml) Treated Tumoral Mitochondria (P < 0.05).

Effect of fraction 2 of C. andromeda venom on Caspase 3 activity.

As shown in Figure 9, fraction 2 of *C. andromeda* venom (240 µg/ml) selectively induced significant increase of caspase 3 activity, as a crucial executioner apoptosis only in cancerous cells (P<0.05). On the other hand pretreatment of venom fraction 2 (240 µg/ml)-treated tumoral cells with Ac-DEVD as a caspase3 inhibitor, prevented activation of caspase3 compared with fraction 2 (240 µg/ml) treated group (P < 0.05).

Effect of fraction 2 of C. andromeda venom on cell apoptosis.

The % apoptosis induced by fraction 2 of *C. andromeda* venom (240 µg/ml) was determined using Flow cytometric analysis of PI-stained cells. As shown in Figure 9 (B) the percentage of apoptotic cells (both early and late apoptosis) and normal cells treated with fraction 2 of *C. andromeda* venom at 12 hr was 59.34% and 3.35%, respectively. On the other hand analysis of PI-stained

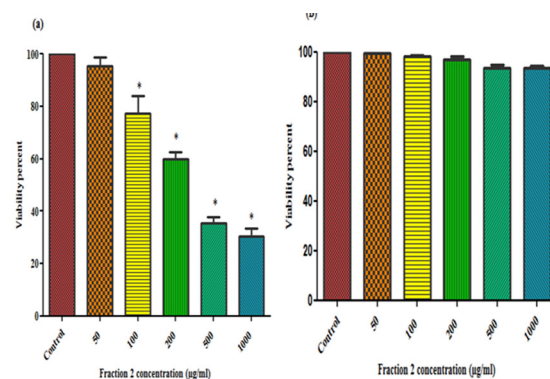


Figure 9. Effect of fraction 2 of *C. andromeda* Concentration (0-1,000 µg/ml) on Cell Viability in Both Tumoral (a) and Extra tumoral Healthy (b) Cells. Cells were Treated with Fraction 2 of *C. andromeda* Venom and Cell Viability was Measured by MTT Assay Following 12 h of Exposure. Values are Presented as Mean ± SD.*Significant Difference in Comparison with the Corresponding Control Cells (P<0.05)

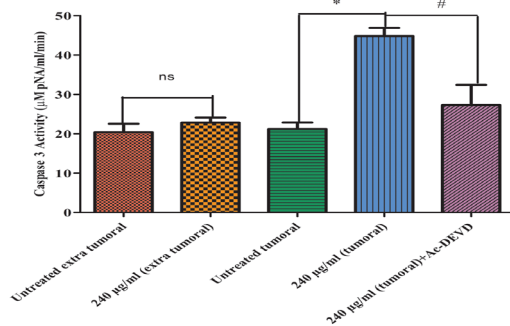


Figure 10. Effect of Fraction 2 of *C. andromeda* Concentration (240µg/ml) on the Activity of Caspase-3 in Tumoral and Extra Tumoral Healthy Cells. Values are Presented as Mean \pm SD.*Significant Difference in Comparison with the Untreated Tumoral cells ($P < 0.05$). #Significant Differences in Comparison with Fraction 2 (240 µg/ml) Treated Tumoral Cells ($P < 0.05$).

apoptotic cells were displayed a broad hypodiploid (sub-G1) peak, which can be easily distinguished from the narrow peak of cells with normal (diploid) DNA content.

Discussion

Mitochondria apply both vital and lethal functions in physiological and pathological events. They are unavoidable for production of energy in eukaryotic cells. On the other hand, mitochondria are prominent regulators of the intrinsic pathway of apoptosis (Fulda et al., 2010). Recently tumoral mitochondria have appeared as novel targets for cancer treatment and also destruction of chemotherapy-refractory cancer cells. A group of compounds with anti-cancer activity that induce apoptosis by means of destabilization of mitochondria, known as mitocans, have been a recent focus of research. Different studies, introduced a universal mitochondria-targeted agents that show the greatest promise for the treatment of human malignancies (Biasutto et al., 2010).

Although cytotoxicity of jellyfish venom has been so far reported in different kinds of tumoral cells (Sun et

Table 1. General Characteristics of Breast Adenocarcinoma Patients

Total number of patients	20
Age range (years)	35-65
Premenopausal	15
Postmenopausal	5
Cancer site	Left/right breast
Clinical stage	Infiltrative/intraductal carcinoma
Morphology	Infiltrative

al., 2002), the cytotoxic mechanism of jellyfish venom is unknown but mitochondrial malfunction and oxidative stress have been suggested as major mechanism for the cell damage caused by jellyfish venom in few published works (Ayed et al., 2011; Morabito et al., 2012). In this study we investigated the potentiality of fraction 2 of *C. andromeda* venom to cause ROS mediated apoptosis through mitochondrial pathway in human breast adenocarcinoma. Mitochondrial apoptosis upstream parameters including mitochondrial ROS, collapse of MMP, mitochondrial swelling and finally cytochrome c expulsion from mitochondria into incubation buffer were assayed. As the first step, we showed that various concentrations of fraction 2 of *C. andromeda* venom (0 - 1,000 µg/ml), could selectively reduce the activity of mitochondrial succinate dehydrogenase in the mitochondria isolated from human breast adenocarcinoma tissue, but not in the mitochondria obtained from the surrounding extra tumoral breast tissue from the same patient. In this report fraction 2 of *C. andromeda* venom significantly ($P < 0.05$) increased ROS production in a time and concentration-related manner only in mitochondria isolated from breast tumoral but not extra tumoral healthy tissue compared to their corresponding untreated mitochondria.

ROS play important role in both normal biological functions and abnormal pathological processes. Intracellular formation of ROS is related to the arrest of cell proliferation. Besides, generation of oxidative damage in response to different external stimuli has been involved in the activation of transcription factors and induction of apoptosis through mitochondrial MPT pore opening and cytochrome c release. Recently several anticancer agents including arsenic trioxide, anthracyclines, cisplatin, bleomycin, have been introduced for cancer treatment can cause increased cellular ROS production (Matés and Sánchez-Jiménez, 2000; Pelicano et al., 2004).

Our results showed that the applied concentrations of fraction 2 of *C. andromeda* venom caused significant MMP collapse in mitochondria isolated from tumoral but not extra tumoral tissue. Alteration of mitochondrial swelling as an indicator of MPT was also monitored in our study. Fraction 2 of *C. andromeda* venom induced significant mitochondrial swelling again only in the mitochondria obtained from tumoral but not extra tumoral tissue.

Regulation of mitochondrial activity highly depends on MMP, and MMP collapse is the starting point for cell death signaling. In brief, damage to mitochondrial membrane leads to MPT pore-opening and the then cytochrome c releases into the cytosol. Subsequently, cytochrome

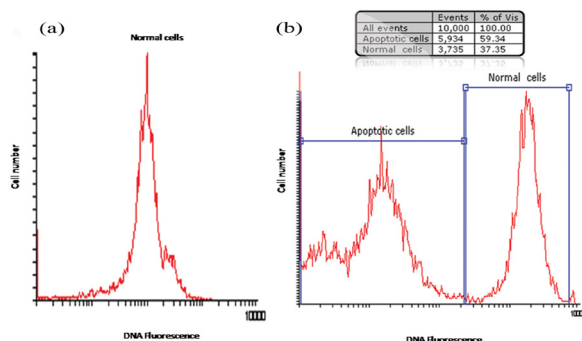


Figure 11. Flow Cytometric Analysis of Apoptotic Cells Using Propidium Iodide. Cells were Incubated with Fraction 2 of *C. andromeda* Venom (240 µg/ml) for 12 h. (A) DNA Histogram of Untreated Tumoral Cells (B) DNA Histogram of Tumoral cells Treated by Fraction 2 of *C. andromeda* Venom (240 µg/ml) for 12 h. The Peak of Hypodiploid Apoptotic Cells Clearly Separated from the Diploid DNA Peak of Normal Cells

c, with apoptotic protease activating factor 1 (Apaf-1) and procaspase-9, forms the apoptosome. Activation of caspase-9 in the presence of ATP, leads to activation of the downstream effector caspase-3, which eventually causes the ultimate steps of apoptosis (Kowaltowski et al., 2001; Circu and Aw, 2010; Qu et al., 2016b).

Our results showed that the applied concentrations of fraction 2 of *C. andromeda* venom induced significant release of cytochrome c from the mitochondria. Moreover, pretreatment with both CsA as an inhibitor of MPT pore and BHT as a ROS scavenger completely blocked the fraction 2 -induced release of cytochrome c from the mitochondria, which proves our assumption that apoptosis induction via fraction 2 of *C. andromeda* venom is due to oxidative stress and relies on the opening of the MPT pore.

Compared to our present results, it was reported that the crude venom from the jellyfish *Pelagia noctiluca* could induce oxidative damage in human colon cancer cells by ROS Production, lipid peroxydation, DNA damage and MMP decrease (Ayed et al., 2011). Other study also indicated that *Pelagia noctiluca* crude venom and its fractions (F1 and F3) were cytotoxic against U87 cells with IC50 values of 125 and 179 µg/ml respectively. (Ayed et al., 2012)

in another study, it was demonstrated that jellyfish *Chiropsalmus Quadrigatus* toxins (CqTX) isolated from box jellyfish *C. quadrigatus* venom induced an apoptotic effect via promotion of p53 expression on the human U251 and rat C6 malignant glioma cells (Sun et al., 2002). In addition, in a recent study crude venom from the jellyfish *Pelagia noctiluca* induced oxidative stress in human neuroblastoma cell line (SH-SY5Y) by inhibition of mitochondrial respiration and reducing oxidative phosphorylation (Morabito et al., 2012).

In other study, the nematocysts venom (NV) from the jellyfish *Chrysaora helvola* Brandt (*C. helvola*) induced an atypical apoptosis-like cell death on the human nasopharyngeal carcinoma cell line confirmed by LDH release assay and Annexin V-FITC/PI staining-based flow cytometry. Interestingly, the NV caused a time-dependent loss of mitochondrial membrane potential ($\Delta\Psi_m$) and increase of intracellular ROS formation in the above mentioned cell line (Qu et al., 2016a).

In another reports, the cytotoxicity of the venom from the nematocysts of jellyfish *Cyanea nozakii* Kishinouye was studied on the three kinds of cell, human hepatoma cells (Bel-7402, SMMC-7721) and human colon cancer cells (H630). The venom had the strongest cytotoxicity on H630 cells with the 50% lethal concentration (IC(50)) of 5.1 µg/ml. However, the IC(50) on Bel-7402 and SMMC-7721 was 17.9 and 24.3 µg/ml, respectively (Cuiping et al., 2012)

Taken together the result obtained in our study by the venom fractions of jellyfish *Cassiopea andromeda* regarding the induction of ROS mediated apoptosis through mitochondrial pathway against breast adenocarcinoma cells and mitochondria were generally in accordance with what was reported in the past with other species of jellyfish on different tumor cell lines.

Mitochondria are necessary for cellular energy, and also participate actively in the regulation of intracellular

Ca²⁺ homeostasis and formation of ROS which might be harmful if produced excessively, so they play important role in the regulation of cell death pathways (Chaabane et al., 2013). Once mitochondrial damage happens, MMP will collapsed, subsequently, ROS and cytochrome c and other pro apoptotic proteins will be released from mitochondrial inter membrane space through MPT pores into cytosol. Excessive ROS production not only attenuate antioxidant enzymes and cell's radical scavenging capacity but also alteration in cell morphology and viability by induction of lipid peroxidation, DNA fragmentation and proteolysis (Zhivotovsky and Orrenius, 2011).

In conclusion, our study demonstrates fraction 2 of jellyfish venom selectively induces apoptosis upstream mitochondrial events including ROS overproduction which subsequently causes MMP decrease, mitochondrial swelling and cytochrome c expulsion in human breast cancerous cells with very ignorable effects on surrounding extra tumoral tissues. Our current results strongly supported the hypothesis that jellyfish venom fraction 2 selectively induces ROS mediated apoptosis in human breast adenocarcinoma cells by directly targeting mitochondria.

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Statement conflict of Interest

The authors declare no conflict of interest.

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