

RESEARCH ARTICLE

Naja Naja Oxiana* Venom Fraction Selectively Induces ROS-Mediated Apoptosis in Human Colorectal Tumor Cells by Directly Targeting Mitochondria*Amir Fakhri¹, Ramesh Omranipour², Sara Fakhri³, Mohammadreza Mirshamsi¹, Fatemeh Zangeneh¹, Hossein Vatanpour^{1*}, Jalal Pourahmad^{1*}****Abstract**

Objective: To investigate the selective effect of *Naja naja oxiana* crude venom and its fractions on human colorectal cancer mitochondria to activate apoptosis signaling. **Methods:** Cells and mitochondria isolated from human cancerous and normal colorectal tissues exposed to *N. oxiana* crude venom and its fractions obtained from size-exclusion chromatography and then mitochondrial parameters related to up-stream cell death signalling such as reactive oxygen species formation, MMP, mitochondrial swelling, cytochrome c release and ATP content as mitochondrial parameters and activation of caspase3 and finally apoptosis/necrosis % were then assayed as cellular parameters. **Result:** Our findings indicated that crude venom (15, 30 and 60 µg/ml) and fraction 3; F3; (10, 20 and 40 µg/ml) of *N. Oxiana* venom induced a significant ($p<0.05$) increase of reactive oxygen species level, swelling of mitochondria, collapse of mitochondrial membrane potential (MMP), release of cytochrome c, activated caspase3 and decrease ATP content only in colon cancer tissue group but not from the healthy colon tissue group. Our results also showed that fraction 3 of venom decreased the percentage of viable cells and induced apoptosis in cancerous colorectal cells. **Conclusion:** F3 fraction of *N. Oxiana* venom is a suitable candidate for further studies as a new drug treatment of colorectal cancer due to its high capacity for induction of apoptosis signaling via mitochondrial pathway.

Keywords: Colorectal cancer- *Naja naja oxiana*- venom- Mitochondria- Apoptosis

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Introduction

Colorectal cancer is the third most common cancer in men and the second most common cancer in women, occurring with a global rate of 10% and 9.5%, respectively. Colorectal cancer has been associated as a disease of developed countries (Ferlay et al., 2010). Observational studies have identified several potentially modifiable risk factors including: diabetes, obesity, tobacco use, excess consumption of alcohol and processed meat, and lack of physical activity (Hagggar and Boushey, 2009). Although chemotherapy has been successful for the treatment of some tumors but its success in the treatment of common epithelial tumors of the colon, lung, and breast has been lacking (Johnstone et al., 2002). Hence, identifying novel cytotoxic natural compounds with low side effects, low drug resistance and high efficacy for prevention and treatment of this cancer is warranted.

Functions of the mitochondria are diverse and interconnected, which includes producing ATP and many biosynthetic intermediates while also contributing to

cellular stress responses such as apoptosis and autophagy (Nunnari and Suomalainen, 2012). Apoptosis, known as programmed cell death, is a carefully controlled, energy-dependent process of cell death. Induction of apoptosis leads to a cascade of key biochemical events resulting in changes in cellular morphology and death. Notable indications of cells undergoing apoptosis include: blebbing, nuclear fragmentation, cell shrinkage, and DNA fragmentation (Cazes, 2010). Common diseases ranging from cancer and neurodegeneration are associated with deregulation of apoptosis (McQueen, 2010). Cancer is a disease arising from genetic and epigenetic alterations in oncogenes and tumor suppressors, many of which are also able to reprogram metabolism (Lu et al., 2015). Mitochondrial dysfunction, including metabolic alterations, has been observed in cancer cells (Roberts and Thomas, 2013). Cancer cells, due to defective mitochondrial oxidative phosphorylation (OXPHOS), switch to glycolysis for ATP synthesis, even in the presence of oxygen. Furthermore, up-regulated glycolysis appears to be for synthesis of biomass and reducing equivalents in

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addition to ATP production (Chen et al., 2015). Because of mitochondrial prominent functions in energy production and in regulation of cell death, mitochondria have been regarded as a potentially essential target for development of anticancer drug.

A key natural source for a variety of bioactive compounds is from animal venoms. A majority of these products are polypeptides that generally target cellular receptors, particular enzymes, or ion channels (Andreotti et al., 2010). Some of these proteins exhibit lethal and debilitating effects as a consequence of neurotoxic, cardiotoxic and tissue necrotizing effects, whereas others induce various pharmacological effects (Kini and Doley, 2010). CTs (cytotoxins) from cobra venom are known to manifest cytotoxicity in various cell types, including erythrocytes, lymphocytes, myocytes, spleen cells and various tumor cells (Chaim-Matyas et al., 1991; Chen et al., 1984; Harvey et al., 1982; Iwaguchi et al., 1985; Stevens-Truss et al., 1996; Tzeng and Chen, 1988). *Naja naja oxiana* (Caspian cobra) belongs to Elapidae family. Its venom is rich in neuro-, cyto- and cardiotoxins (Grishin et al., 1974b; Grishin et al., 1974a) with ability to induce apoptosis in cancerous cells (Ebrahim et al., 2016; Seydi et al., 2017; Strizhkov et al., 1994). The aim of the present study is to further investigate the selective cytotoxicity and mode of cell death caused by the venom of Caspian cobra against human colorectal cancer cells by using their isolated cells and mitochondria and monitoring the possible upstream oxidative stress related apoptosis events.

Materials and Methods

Normal and Colon cancer Tissues

Fresh normal and colon cancer tissue samples were provided by the Surgery Center of Imam Khomeini hospital, Tehran Medical University, Iran. Pathology and histological reports were provided by the pathologist for each tissue sample. All patients examined (n=20, age ranging from 46 to 69 years) had local or locally advanced disease. The patients in the study had not received any prior radiation or chemotherapy. Fresh tumor tissue was obtained from the core of the tumor whereas normal colon tissue samples were taken at sites distant from the tumor by 5 cm and they were controlled for presence of cancer cells (Chekulayev et al., 2015). This study was approved by Ethical Committee of Shahid Beheshti University of Medical Sciences and informed consent was obtained from all the patients.

Mitochondrial Isolation

Tissue samples were collected after surgery and were washed with ice-cold buffer consist of sucrose 0.25 M, Tris HCl 5 mM, pH 7.4, and EDTA 1 mM, then minced and homogenized with glass handheld homogenizer. The nuclei and broken cell debris were sedimented through centrifuging at $1500 \times g$ for 10 min at 4 °C; the pellet was discarded. The supernatant obtained from the first step was centrifuged at $15000 \times g$ for 15 min to obtain pellet containing purified mitochondria and then suspended in cold buffer according to analysis method (Sugiura

et al., 2004). Protein concentrations were determined using the Bradford assay through the Coomassie blue protein-binding method (Chen et al., 2016). The isolation of mitochondria was confirmed by the measurement of succinate dehydrogenase. Mitochondria were prepared fresh for each experiment and used within 4 h of isolation and all steps were strictly operated on ice to guarantee the isolation of high-quality mitochondrial preparation.

Venom preparation

Crude freeze-dried venom of the *Naja naja oxiana* was supplied from Razi Vaccine and Serum Research Institute (Karaj, Iran) and stored at -20 °C, 200 mg of crude venom was dissolved in 10 mL of 0.2 M ammonium acetate. Insoluble material was removed by centrifugation (5,000 g for 10 min) and the supernatant was applied to the column of Sephadex G-50 (2.5×150 cm) equilibrated with 0.2 M ammonium acetate pH 7.4. The elution was carried out with the same solution at a flow rate of 60 mL/h. The effluent was analyzed for protein by absorbance measurement at 280 nm. All fractionation steps were performed at 4 °C (Samel et al., 2008).

Measurement of Mitochondrial succinate dehydrogenase (complex II) activity

Mitochondrial succinate dehydrogenase (complex II) activity was measured by the reduction of MTT to formazan at 570 nm as described in previous studies (Zhao et al., 2010). Briefly, 100 µL of mitochondrial suspensions (1 mg protein/mL) was incubated with different concentrations of crude and fractions venom (0 (control), 5, 10, 25, 50 and 100 µg/mL) at 37 °C for 1h; then, 25 µL 0.4% of MTT was added to the medium and incubated at 37 °C for 30 min. The product formazan crystals was dissolved in 100 µL DMSO and the absorbance at 570nm was measured with an ELISA reader (Tecan, Rainbow hermo, Austria) (Salimi et al., 2015). Based on the calculated IC₅₀ the concentrations of (1/2 IC₅₀, IC₅₀ and 2×IC₅₀) for crude venom and fractions were selected to determine other mitochondrial toxicity parameters (Aghvami et al., 2017).

Measurement of Mitochondrial swelling

Mitochondrial swelling was measured as the absorbance change of the mitochondrial suspensions at 540 nm. Briefly, isolated mitochondria were suspended in swelling buffer (70 mM sucrose, 230 mM mannitol, 5 mM succinate, 3 mM HEPES, 2 mM tris-phosphate, and 1 µM of rotenone) and incubated at 30 °C with various concentration (1/2 IC₅₀, IC₅₀ and 2×IC₅₀) of crude venom and its F3 fraction that obtained from MTT assay. The absorbance was measured in duration 1h with 15 min time intervals at 549 nm with an ELISA reader (Tecan, Rainbow Thermo, Austria). A decrease in absorbance indicates an increase in mitochondrial swelling (Shaki et al., 2012).

Measurement of ROS level

Mitochondrial ROS formation induced by crude venom and its F3 fraction in isolated mitochondria (normalized to 1 mg protein/mL) was measured using dichlorofluorescein diacetate (DCFH-DA) fluorescent probe (final concentration of 10 µM) as a reagent in

modified respiratory buffer, including KCl (130 mM), MgCl₂ (5 mM), NaH₂PO₄ (20 mM), succinate (5 mM) and FeCl₃ (0.1 mM), at pH 7.4. After adding various concentration of crude venom and its F3 fraction and incubating at 37°C for 10 min, the DCF fluorescence intensity was assayed using Shimadzu RF-5000U fluorescence spectrophotometer (Ex: 488 nm and EM: 527 nm) (Pourahmad and O'Brien, 2000).

Measurement of mitochondrial membrane potential (MMP)

The Rhodamine 123 (Rh 123) redistribution technique was used for MMP measurement. Isolated mitochondria (normalized to 100 mg of mitochondrial protein) were suspended in 1 mL of analysis buffer, including 220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH₂PO₄, 2 mM MgCl₂, 50 mM EGTA, 5 mM sodium succinate, 10 mM HEPES and 2 mM rotenone, then Rh 123 (10 µM) was added. Mitochondrial fluorescence and light scattering were analyzed for fluoremetry (Ex: 490 nm and EM: 535 nm) (Baracca et al., 2003).

Measurement of Cytochrome c release

The amount of cytochrome c released to the medium from isolated mitochondria was determined at 450 nm according to the instructions provided by the manufacturer of the Quantikines Rat/Mouse Cytochrome c Immunoassay Kit (Minneapolis, MN). All analysis stages was carried out using an ELISA reader (Infinite M 200, TECAN) at desired concentrations in all groups (Al Maruf et al., 2017).

Measurement of ATP level

The ATP levels were measured using Luciferin/Luciferase Enzyme system (Tafreshi et al., 2007). Bioluminescence intensity was measured using Sirius tube luminometer (Berthold Detection System, Germany).

Isolation of primary colorectal cancer cells from tumoral tissue

The human colorectal tissue samples were collected after surgery and washed in fresh, ice-cold phosphate-buffered saline (PBS). Cut into tiny pieces <0.5 cm², minced tissues were transferred into a 15 ml tube containing 10 ml DMEM (Dulbecco's modified Eagle medium) with 0.25% collagenase and incubated for 1 h at 37 °C. After 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 5% FBS and supplemented with 100 U/ml penicillin, 25 µg/ml gentamycin, 10 mM HEPES, 2 mM glutamine (Bedi et al., 1995).

Determination of Cytotoxicity

The cytotoxicity effect of fraction 3 ; F3; of *N. Oxiana* venom on healthy and cancerous cells (1×10⁶cells/ml) isolated from normal and colorectal cancer tissues, respectively, was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining (Mirshamsi et al., 2017).

Determination of Caspase-3 Activity

The activity of Caspase-3 enzyme in the cell lysates was determined according to instructions of the Sigma's colorimetric caspase-3 assay kit (CASP-3-C; Sigma-Aldrich, Saint Louis, USA). Absorbance of each well's content was read at 405nm and the results were calculated with p-nitroaniline calibration curve, using Tecan-spectra ELISA reader (Sakahira et al., 1998).

Apoptosis versus necrosis assay

The healthy and colorectal cancer cells exposed to F3 and apoptosis versus necrosis % were measured at 12h by flow cytometric double staining analysis (Cyflow Space-Partec) of Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) stained cells. This test was performed using a commercial kit (Immunotech; Beckman Coulter) according to the manufacturer's instructions (Qiu et al., 2012).

Statistical analysis

All statistical significance was performed using the Prism (V.6) software. Data were showed as mean ± SD and assays were performed in triplicate. Comparisons were made using the one-way and two-way analysis of variance (ANOVA) tests. P< 0.05 was considered as significant.

Results

Size-exclusion chromatography (SEC)

N. oxiana crude venom chromatogram on Sephadex G-50 shown four obvious peak (F1-F4) based on their molecular weight with absorbance at 280 nm as shown in Figure 1.

Effect of crude venom and fractions of *N. Oxiana* venom on SDH activity

The effect of crude *N. Oxiana* venom and its fractions (F1-F4) (5, 10, 25, 50 and 100 µg/ml) on SDH was determined by MTT assay. As shown in Figure 2 (B, D, F, H, J) in the colon cancer group, crude and F1-F4 fractions of *N. Oxiana* venom induced significant (P <0.05) reduction in the SDH activity. Our findings show that crude venom and its F3 fraction are more potent than F1, F2 and F4 venom fractions at reducing SDH activity on mitochondria obtained from colon cancer tissue. On the other hand, the mitochondria obtained from the

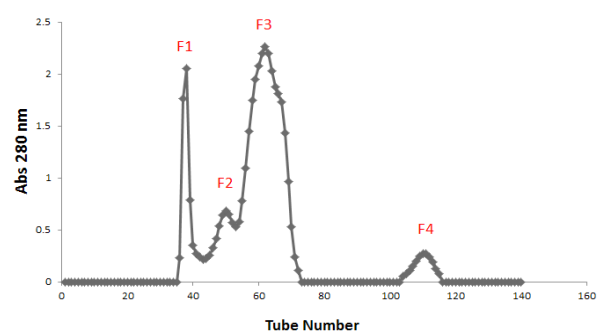


Figure 1. Size-Exclusion Chromatogram of *N. Oxiana* Crude Venom on a Sephadex G-50 Column (2.5*150 Cm). All Fractionation Steps were Performed At 4 °C.

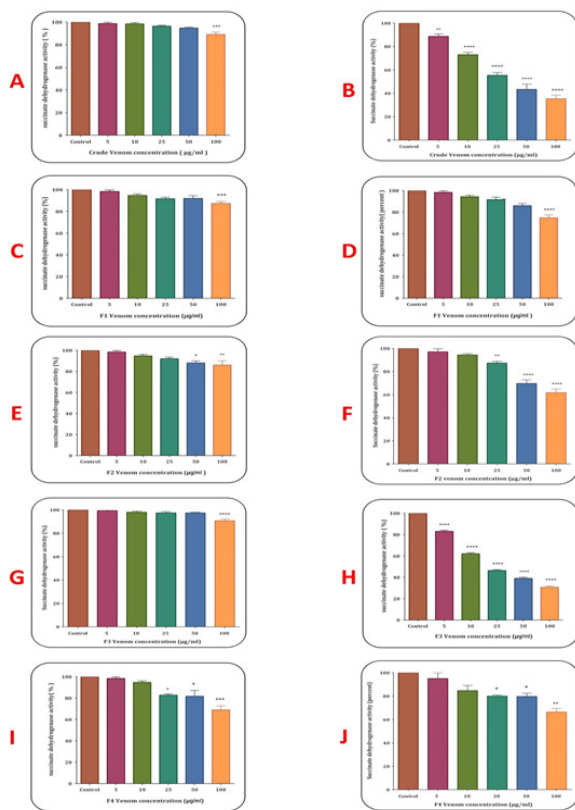


Figure 2. SDH Activity Assay. The Effect of Various Concentrations (0-100 µg/ML) of Crude and Fractions (F1-F4) of *N. Oxiana* Venom on SDH Activity. A, Normal plus crude venom; B, colon cancer plus crude venom; C, Normal plus F1 (fraction 1) venom; D, colon cancer plus F1 venom; E, Normal plus F2 venom; F, colon cancer plus F2 venom; G, Normal plus F3 venom; H, colon cancer plus F3 venom; I, Normal plus F4 venom; J, colon cancer plus F4 venom. Data are showed as mean ± SD (n=3). *, **, *** and **** show a significant difference in comparison with the corresponding control (P<0.05, P<0.01, P<0.001 and P<0.0001, respectively).

extra tumor tissue (normal group), the crude venom and all fractions of *N. Oxiana* venom only at highest dose (50 and 100 µg/ml) induced a significant reduction in SDH activity (Figure 2; A, C, E, G, I). Finally, the IC50 measured for crude venom and F3 fraction was 30µg/ml and 20µg/ml, respectively. Based on the calculated IC50 the concentrations of 15, 30 and 60 µg/ml for crude venom and concentrations of 10, 20 and 40 µg/ml for F3 fraction were selected to determine other mitochondrial toxicity parameters.

Effect of crude and fraction 3 (F3) of N. Oxiana venom on reactive oxygen species

In the next step, we assayed the effect of crude and F3 fraction of *N. Oxiana* venom on the mitochondrial ROS level. Our results showed that crude venom (Figure 3A) and its F3 fraction (Figure 3B) at all applied concentrations induced mitochondrial ROS formation on the mitochondria isolated from the colon cancer but not on the extra tumor group (normal group).

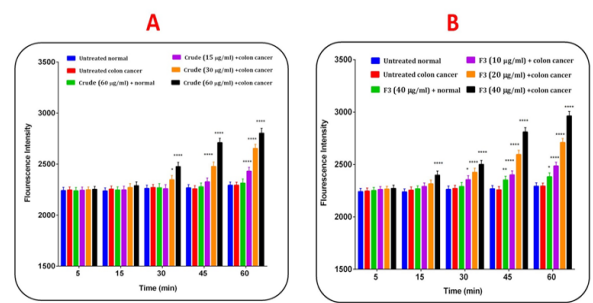


Figure 3. ROS Level Assay. The Effect of Various Concentrations of Crude (15, 30 and 60 µg/ml) and F3 Fraction (10, 20 and 40 µg/ml) of *N. Oxiana* Venom on Mitochondrial ROS Level on the Normal and Colon Cancer Groups. A, Crude venom; B, F3 fraction venom. Data are showed as mean ± SD (n=3). *, ** and **** show a significant difference in comparison with the untreated colon cancer group (P<0.05, P<0.01 and P<0.0001, respectively).

Effect of crude and fraction 3 (F3) of N. Oxiana venom on mitochondria membrane potential (MMP)

As shown in Figure 4A and B, there was a significant difference (P<0.05) in the decline of MMP after exposure to crude (Figure 4A) and its fraction F3 (Figure 4B) of *N. Oxiana* venom at all applied concentrations in the mitochondria from the colon cancer group. However, MMP levels did not significantly decline in normal group, except one time by high dose of venom and F3 fraction (Figure 4A- B).

Effect of crude and fraction 3 (F3) of N. Oxiana venom on mitochondrial swelling

In colon cancer group, our results showed that crude venom at concentration of 30 and 60 µg/ml but not 15 µg/ml induced swelling in the mitochondria (Figure 5A). However, the addition of several concentrations of F3 of *N. Oxiana* venom (10, 20 and 40 µg/ml) to mitochondrial suspensions from the colon cancer group led to significant (P<0.05) swelling in the mitochondria (Figure 5B). In the normal group, all applied concentrations of crude (15, 30 and 60 µg/ml) and F3 (10, 20 and 40 µg/ml) of *N. Oxiana* venom did not significantly raise mitochondrial swelling.

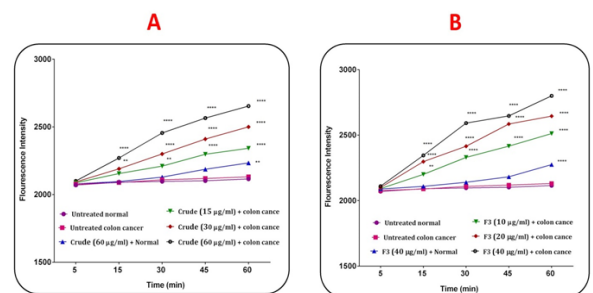


Figure 4. Mitochondria Membrane Potential (MMP) Assay. The Effect of Various Concentrations of Crude (15, 30 And 60 µg/ml) And F3 Fraction (10, 20 And 40 µg/ml) Of *N. Oxiana* Venom on Decline of the MMP on the Mitochondria Isolated from the Normal and Colon Cancer Groups. A, Crude venom; B, F3 venom. Data are shown as mean ± SD (n=3). ** and **** shows a significant difference in comparison with the untreated colon cancer group (P<0.01 and P<0.0001), respectively.

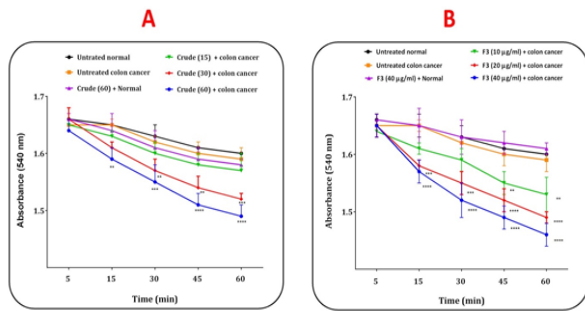


Figure 5. Mitochondrial Swelling Assay. The Effect of Various Concentrations of Crude (15, 30 and 60 $\mu\text{g/ml}$) and F3 Fraction (10, 20 and 40 $\mu\text{g/ml}$) of *N. Oxiana* Venom on the Mitochondrial Swelling in the Normal and Colon Cancer Groups. A, Crude venom; B, F3 venom. Data are shown as mean \pm SD (n=3). **, *** and **** shows a significant difference in comparison with the untreated colon cancer group (P<0.01, P<0.001 and P<0.0001, respectively).

Effect of crude and fraction 3 (F3) of *N. Oxiana* venom on cytochrome c release

Our results show that; only in the mitochondria from colon cancer group; crude and F3 of *N. Oxiana* venom at IC_{50} concentration (30 and 20 $\mu\text{g/ml}$, respectively) induced a significant (P<0.05) release of cytochrome c. Furthermore, as shown in Figure 6A and B, pre-treatment of mitochondria from colon cancer group with cyclosporine A (CsA); as a MPT inhibitors like; and butylated hydroxyl toluene (BHT); as an antioxidant, inhibited the crude and F3 of *N. Oxiana* venom (30 and 20 $\mu\text{g/ml}$, respectively)-induced release of cytochrome c.

Effect of crude and fraction 3 (F3) of *N. Oxiana* venom on ATP content

Mitochondrial electron transfer chain (mETC) is required for mitochondrial ATP production, since crude venom of *N. Oxiana* (15, 30 and 60 $\mu\text{g/ml}$) and its fraction F3 (10, 20 and 40 $\mu\text{g/ml}$) impairs the mETC, we therefore decided to measure the ATP levels in isolated healthy and cancerous mitochondria from colon, following the addition of different concentrations of venom and its fraction F3. As shown in (Table 1), In this study, we showed that crude venom and its fraction are potent to

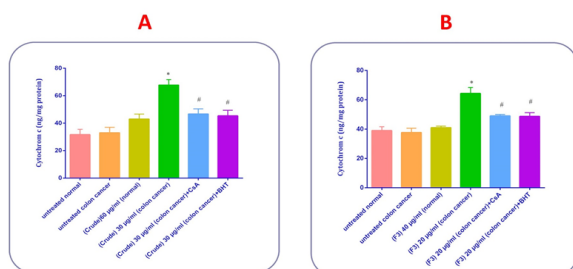


Figure 6. Cytochrome C Release Assay. The Effect of IC_{50} Concentration of Crude (30 $\mu\text{g/ml}$) and F3 Fraction (20 $\mu\text{g/ml}$) of *N. Oxiana* Venom on the Cytochrome C Release. A, Crude venom; B, F3 venom. Data are shown as mean \pm SD (n=3). * shows a significant difference in comparison with the untreated tumoral group (P<0.0001). # Shows significant difference in comparison with crude (30 $\mu\text{g/ml}$) and F3 (20 $\mu\text{g/ml}$) of *N. Oxiana* venom-treated tumoral group (P<0.001)

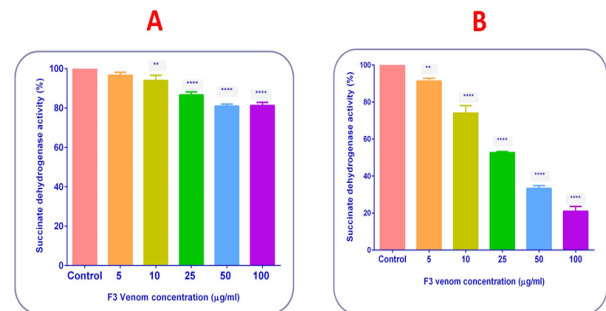


Figure 7. Effect of Fraction 3 of *N. Oxiana* Venom Concentration (0-100 $\mu\text{g/ml}$) on Cell Viability in Both Extra Tumoral (a) and Tumoral (b) Cells. Cells were Treated with Fraction 3 of *N. Oxiana* Venom and Cell Viability was Measured by MTT Assay Following 12 h of Exposure. Values are Presented as Mean \pm SD. ** and **** shows a significant difference in comparison with the untreated colon cancer group (P<0.01 and P<0.0001, respectively).

decrease mitochondrial ATP level in the colon cancer group. In the normal group, all applied concentrations of crude (15, 30 and 60 $\mu\text{g/ml}$) and F3 (10, 20 and 40 $\mu\text{g/ml}$) of *N. Oxiana* venom not significantly decreased ATP level.

Effects of fraction 3 (F3) of *N. Oxiana* venom on cells cytotoxicity

The potential cytotoxicity effect of different concentrations of fraction 3 (F3) venom (0, 5, 10, 25, 50 and 100 $\mu\text{g/ml}$) on cell viability following 12 h of exposure was determined with the MTT assay using isolated colorectal cells (1×10^6 cells/ml) obtained from both tumoral and extra tumoral (healthy) tissues As shown in Figure 7. However, fraction 3 (F3) of venom significantly (p<0.05) reduced cell viability only in tumoral colorectal cells. Half-maximal inhibitory concentration (IC_{50}) of F3 venom for 12h exposure on cancerous cells was 20 $\mu\text{g/ml}$.

Effect of fraction 3 (F3) of *N. Oxiana* venom on Caspase 3 activity

As shown in Figure 8, fraction 3 (F3) venom significantly (P<0.05) increased the activity of caspae-3

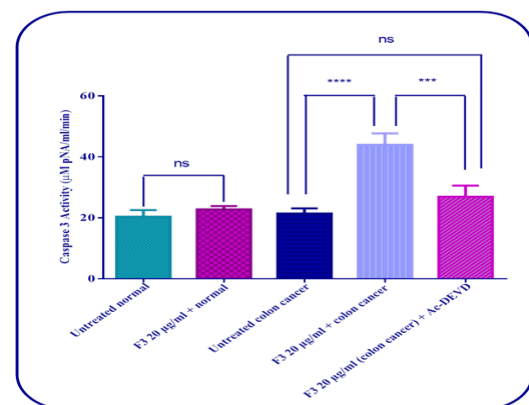


Figure 8. Effect of Fraction 3 of *N. Oxiana* (20 $\mu\text{g/ml}$) on The Activity of Caspase-3 in Tumoral and Extra Tumoral Healthy Cells. Values are Presented as Mean \pm SD. *** and **** Shows a Significant Difference in Comparison with the Treated Tumoral Cells (P<0.001 And P<0.0001, Respectively).

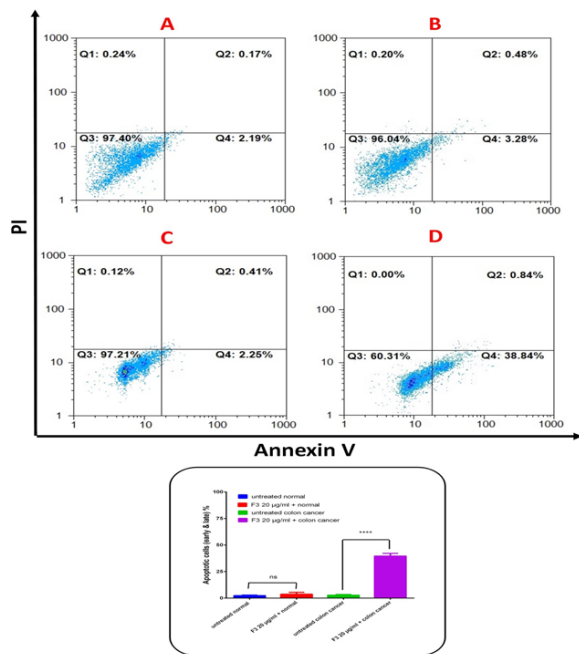


Figure 9. Analysis for Annexin-V and PI Staining of Normal and Cancerous Colorectal Cells Incubated with F3 Venom (20 µg/ml) for 12 h. Only Annexin V-positive (+) and PI-negative (-) cells were defined as apoptotic A: untreated healthy colorectal cells, Annexin V+/PI- = 2.19% B: healthy colorectal cells after 12h exposure of F3 venom, Annexin V+/PI- = 3.28% C: untreated cancerous colorectal cells, Annexin V+/PI- = 2.25% D: cancerous colorectal cells after 12h exposure of F3 venom, Annexin V+/PI- = 38.84%. The summarized apoptotic data was demonstrated at graph. Results are expressed as means±SD, n=3, ****p<0.001 vs. respective control.

as a final mediator of apoptosis only in tumoral colorectal cells. On the other hand our results showed that caspase-3 activation developed by fraction 3 (20 µg/ml) significantly reduced with pre-treatment of Ac-DEVD as a caspase 3 inhibitor in cancerous colorectal cells.

Effect of fraction 3 (F3) of N. Oxiana venom on cell apoptosis

The percentage of cell apoptosis caused by fraction 3 venom (20 µg/ml) was significantly increased according to the result shown in Figure 9.

Discussion

Globally, colorectal cancer is one of the most common cancers in both men and women and remains a prominent cause of cancer death (Thyagarajan et al., 2012). In 2014, approximately 136,830 men and women were diagnosed with colon cancer and 50,310 deaths occurred mostly within the older demographic (Siegel et al., 2014).

Studies have shown that a lack of balance between cell growth and apoptosis plays an important role in the development of colon cancer. Furthermore, defects in the apoptosis process, plays a significant role in the development of colon cancer as well as poor response to treatment, such as chemotherapy. Therapies aimed to stimulate apoptosis signaling in target cells and could play

Table 1. Effect on Mitochondrial ATP Level. colon mitochondria were incubated with various concentrations of crude venom(0, 15, 30 and 60 µg/ml) and its fraction F3 (0, 10, 20 and 40 µg/ml) of *N. oxiana* venom and ATP level were determined using Luciferin/ Luciferase Enzyme System as described in Materials and methods. Values represented as mean±SD (n=3). *p < 0.05; **p < 0.01; ***p < 0.001 compared with Untreated colon cancer mitochondria.

ATP (nmol/mg protein)	
Untreated normal	100±1.1
Untreated colon cancer	73±2
Crude 15 µg/ml+ colon cancer	63±1.2*
Crude 30 µg/ml + colon cancer	54±0.8**
Crude 60 µg/ml + colon cancer	45±1***
ATP (nmol/mg protein)	
Untreated normal	100±1.1
Untreated colon cancer	73±2
F3 10 µg/ml +colon cancer	48.2±1.1**
F3 20 µg/ml + colon cancer	36.21±0.8***
F3 40 µg/ml + colon cancer	21.32±1.4***

a vital role in adjusting evolvment and development of colorectal cancer treatment. Thus, induction of apoptosis is a critical strategy for the treatment of colon cancer (Abraha and Ketema, 2016; De Rosa et al., 2015). The aim of the this study is to further investigate the selective toxicity induced by the crude and fractions of Caspian cobra venom against human colorectal cancer by isolated cells and mitochondria and monitoring the possible mechanism involved.

Within the past years, the designation of choice therapy with high potency and efficacy has led to the raised use of anticancer agent developed from natural resources (Jain and Kumar, 2012). Studies have indicated that many compounds of natural origin are considered as anti-cancer agents (Liu et al., 2014). Snake venoms due to chemical and biological properties have been used for the treatment of numerous types of cancer. In addition, reports indicate that snake toxins are able to induce apoptosis signaling via changing the amount of calcium ion and release of cytochrome c from the mitochondria (Shanbhag, 2015). As a critical organelle, the mitochondria plays a significant role in cell death and Previous studies have shown that many differences (such as, structural and functional) exist between mitochondria in normal cells and cancerous cells. Due to these differences, mitochondria have been used as a therapeutic target and as a means to kill cancer cells(Armstrong, 2006; Seydi et al., 2015, 2016; Talari et al., 2014). Furthermore, mitochondrial DNA (mtDNA) has been shown to play an important role in the development of colon cancer in humans (Thyagarajan et al., 2012). According to our results, crude and its fraction (F3) of *N. Oxiana* venom induced a significant reduction in the activity of SDH activity in the mitochondria from the colon cancer group.

Many studies show that ROS plays an important role in the carcinogenesis process. For example, at low physiological concentrations, plays a role in cell growth

and adjusting pathways. While at high concentrations, plays a role in the oxidative stress process and the induction of apoptosis (Klaunig and Kamendulis, 2004; Yuan et al., 2012). In this study, our results show that crude and F3 of *N. Oxiana* venom increased ROS levels in mitochondria from the colon cancer group and F3 can raise ROS level in lower concentrations in comparison to crude venom. Oxidative stress induced through ROS is supposed to play a vital role in the colorectal carcinogenesis (Thyagarajan et al., 2012). In one study, MCF-7 cancer cell, cytotoxin-II obtained from *N. Oxiana* venom induced a raise in ROS level. Our results are also in line with this study (Ebrahim et al., 2014).

It is well documented that the release of cytochrome c from mitochondria and collapse of MMP plays an important role in the occurrence of apoptosis signaling, which has been presented to be a permanent point towards apoptosis signaling (Ko et al., 2005). Furthermore, we indicate that crude and F3 of *N. Oxiana* venom induced the MMP collapse in the mitochondria from the colon cancer group. Our results are also in agreement with previous studies (Ebrahim et al., 2014, 2016) and F3 can collapse MMP in lower concentrations in comparison to crude venom.

Following these experiment results, we evaluate the mitochondrial swelling as a main pointer of MPT pore opening. In this study, we show that crude and F3 venom are potent at swelling mitochondria in the colon cancer group. Also, release of cytochrome c as a vital indicator of apoptosis induction was assayed. The intrinsic pathway of cell death starts through the collapse of MMP which leads to opening of the MPT pores and the release of cytochrome c (as it triggers the proteolytic activity of caspases cascade) and other apoptosis inducing agents from the mitochondria into the cytosol (Ebrahim et al., 2014). Based on our results, crude and its fraction (F3) of *N. Oxiana* venom induced significant release of cytochrome c only in the mitochondria from human colorectal cancer. Finally, we suggest that F3 of *N. Oxiana* venom and subsequent crude of *N. Oxiana* venom induce the increase of ROS generation, collapse of the MMP, swelling in the mitochondria, release of cytochrome c and decrease ATP content. In fact, ATP performs as a switch between apoptosis and necrosis. We found that crude and F3 of *N. Oxiana* venom induced significant decreased of ATP production on isolated mitochondria from human colorectal cancer and F3 have extremely effect on cancer tissue compare with crude venom (Tafreshi et al., 2007).

Caspase-3 is a downstream effector of the caspase family and implicated as an executioner mediator of apoptosis (Boatright and Salvesen, 2003). In this study, our results showed that F3 induced a significant increase of caspase-3 activity in human colorectal cancer cells and cause of apoptotic enhancement in mode of cell death after treatment with F3 venom. According to our results, F3 of *N. Oxiana* venom is a suitable candidate and warrants consideration as a promising complementary therapeutic agent for the treatment of colorectal cancer.

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Statement conflict of Interest

The authors declare no conflict of interest.

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