

RESEARCH ARTICLE

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Induction of Apoptosis by an Extract of Persian Gulf Marine Mollusc, *Turbo Coronatus* through the Production of Reactive Oxygen Species in Mouse Melanoma Cells

Fatemeh Zangeneh¹, Amir Vazirizadeh², Mohammad Reza Mirshamsi¹, Amir Fakhri¹, Mehrdad Faizi^{1*}, Jalal Pourahmad^{1*}

Abstract

Objective: A variety of approaches such as surgery, chemotherapy, radiotherapy, hormonal therapy and immunotherapy are used to treat melanomas, but unfortunately in most case, the response is very weak and often side effects are serious. This study concerns selective toxicity of an extract of *Turbo coronatus* on cells and mitochondria from a syngeneic mouse model of melanoma. **Methods:** Cells and mitochondria isolated from extra tumoral and melanoma tissues were exposed to *T. coronatus* crude extract and fractions obtained by gel-filtration chromatography and assayed for mitochondrial and cellular parameters. **Result:** Crude extract (375, 750 and 1,500 µg/ml) and fraction 1; F1; (275, 550 and 1100 µg/ml) of *T. coronatus* extract induced a significant ($p<0.05$) increase of the reactive oxygen species (ROS) level, swelling of mitochondria, collapse of mitochondrial membrane potential (MMP), release of cytochrome c and caspase-3 activation only in the mitochondria and cells obtained from melanoma but not extra tumoral tissues. In addition, the F1 fraction decreased the percentage of viable cells and induced apoptosis in melanoma cells. **Conclusion:** For the first time we could demonstrate that the F1 fraction of a *T. coronatus* extract, selectively induces ROS mediated cytotoxicity by directly targeting mitochondria in melanoma tissues and it may be a suitable candidate for novel drug treatment of malignant melanomas.

Keywords: Melanoma- turbo coronatus- mitochondria- apoptosis- gel filtration- Persian Gulf

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Introduction

Cancer can be defined as an uncontrollable proliferation of an unusual cell group with disregard to cell division of normal rules. One of the most dangerous types of skin cancer, originating from melanocytic cells, is melanoma (Dana et al., 2015). Epidemiological studies have shown that the incidence of melanoma is increasing at a faster rate than any other cancers worldwide (Hoel et al., 2016). It is predicted 91,270 new cases of melanoma will be diagnosed and 9,320 deaths occurred from melanoma in 2018 only in the U.S.A (Siegel et al., 2018). Moreover, although melanoma can be treatable at the early stage of diagnosis, invasive melanoma has a poor prognosis due to various resistance mechanisms to treatment options such as chemotherapy (Looi et al., 2013). Most chemotherapy drugs eventually affect through induction of apoptosis (Grossman and Altieri, 2001). Studies have shown that the inability to induce apoptosis in melanoma cells due to impairment of the pathway and the level of pro/anti

apoptotic factors such as defective in cytochrome c release and high levels of the apoptosis inhibitor FLIP (Grossman and Altieri, 2001; Tian et al., 2012). Therefore all the efforts should be focused on new cytotoxic agents with the ability to induce apoptosis in melanoma cells. Marine environment covers more than 70% of the Earth's surface (Ruiz-Torres et al., 2017). It is a unique resource of potent bioactive compounds with special chemical and structural properties that are not found in terrestrial natural products. Recently, the enormous number of novel potential compounds with pharmacological features isolated from various marine species such as microorganisms as well as invertebrates (sponges, bryozoans, tunicates, molluscs, etc.) (Chen et al., 2015; Malve, 2016; Ruiz-Torres et al., 2017). Many of these bioactive compounds are currently in clinical phases as antineoplastic candidates (Malve, 2016; Ruiz-Torres et al., 2017).

Turbo coronatus is a kind of turban snail, belongs to the turbinidae family, which is widely distributed in the Persian Gulf rocky shores. Recent research indicates

¹Department of Pharmacology and Toxicology, Faculty of Pharmacy and Pharmaceutical Sciences, Research Center Shahid Beheshti University of Medical Sciences, Tehran, ²The Marine Biology and Fishery Science Department, Persian Gulf Institute, Persian Gulf University, Bushehr, Iran. *For Correspondence: j.Pourahmadjaktaji@utoronto.ca, m.faizi@sbm.ac.ir

that IV injection of the crude extract *T. coronatus* to rats produced rhabdomyolysis and hepatocytes injury (Vazirizadeh et al., 2017). Turbostatins 1-4 (type of cerebrosideare) novel antineoplastic agents are isolated from *T. stenogyrus*. All four agents exhibited significant cancer cell growth inhibition against the murine P388 lymphocytic leukemia and a panel of human cancer cell lines (Pettit et al., 2005). Another study on the Genus *marmorata* led to the identification and isolation of turbotoxin A and B, Diiodotyramine derivative which revealed acute toxicity against ddY mice and turbotoxin A inhibits acetylcholinesterase (Kigoshi et al., 2000). The aim of the present study is was to investigate the selective cytotoxicity of *T. coronatus* crude extract and its fractions against the cancerous cells and mitochondria isolated from a mouse model of melanoma and discovering the possible upstream events leading to ROS mediated apoptosis and compared our findings with those of from autologous extra cancerous skin cells and mitochondria isolated from the same mouse model of melanoma.

Materials and Methods

Sample Collection and crude extract Preparation

A total of 2.2 kg of the mollusk *T. coronatus* was collected from tidal and rocky shores of the Persian Gulf beaches from Bandar Bushehr (28.9234° N latitude and 50.8203°E longitude). The samples were immediately transported to the laboratory and the viscera were removed by breaking the shells. The whole body muscles of the sample (450 g) were ground to a powder under liquid nitrogen and homogenized with 30 mL of 0.15M saline solution at 4°C. The homogenate in obtained was centrifuged (12,000 × g 30'). The supernatant was immediately freeze-dried (Christ, UK) and kept on -20°C until further use (Vazirizadeh et al., 2017). The protein concentration of the *T. coronatus* extract was determined by the Bradford method (Bradford, 1976).

Gel Filtration Chromatography (GFC)

Sephadex G-50 (Pharmacia, USA) gels were poured into a 2.5×150 cm diameter. The elution was completed with 0.2 M ammonium acetate (pH 7.1 at 4°C) and flow rate adjusted to 1 ml/min. About 600 mg of crude extract of *T. coronatus* was dissolved in 10 ml of 0.2 M ammonium acetate. After centrifugation at 5,000 × g for 10 min at 4°C, the supernatant was loaded on the column of Sephadex G-50. Fractions were collected and protein absorbance was measured at 280 nm by UV spectrophotometer. Eventually the lyophilized fractions and were stored until use at -20°C (Samel et al., 2008).

Animals

Female C57BL/6 mice (8–10 weeks old, weighting 16–18 g) were purchased from the Pasture Institute of Iran. The animals were housed in a regulated condition (22±2 °C, 50±5% humidity) with a 12h light/dark cycle (8 AM–8 PM). All the experiments were conducted according to instructions of ethical standard and Institutional Animal Care and Use Committee (IACUC) of Shahid Beheshti University of Medical Sciences,

Tehran, Iran.

Induction of syngeneic melanoma model

Using published protocols mice received subcutaneous injections cells (1×10^5 in 0.2 M phosphate buffered saline) B16-F10 in the right flank. When the syngeneic implantation was established (usually 14 to 21 days) based on the visibility of tumor the mice were sacrificed. After removing the tumors, they are cut into 2 mm pieces and were implanted in the right flank C57BL/6 mouse by using a 15-gauge trocar 12 mm long (Overwijk and Restifo, 2001).

Mitochondrial Isolation

After induction of melanoma, on day 7–10 after subcutaneous implantation of tumor the animals were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). For mitochondria isolation from melanoma and extra tumoral tissues, samples were washed with cold isolation buffer (sucrose 0.25 M, TrisHCl 5 mM, pH 7.4, and EDTA 1 mM), then chopped and homogenized with glass hand-held homogenizer. The homogenate was centrifuged at 2,000 × g for 10 min at 4 °C for discard the pellet containing the nuclei, unbroken cells, tissue debris. The supernatant obtained from the first step was centrifuged at 10,000 × g for 10 min to obtain pellet containing purified mitochondria and then suspended in cold Tris buffer (Tris-HCl, 0.25 M of sucrose, 20 mM of KCl, 2.0 mM of MgCl₂, and 1.0 mM of Na₂HPO₄; (pH=7.4) at 4°C) (LampI et al., 2015). Protein concentrations were determined using the Bradford assay through the coomassie blue protein-binding method using BSA as the standard.

Succinate Dehydrogenase (SDH) activity assay

The activity of SDH (complex II) was measured by the reduction of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) to formazan at 570 nm. Briefly, to carry out this assay, 100 µl of mitochondrial suspensions (1 mg protein/mL) was incubated with different concentrations of crude extract and its fractions (0, 250, 500, 1,000, 1,500 and 2,000 µg/ml) at 37 °C for 1 h; then, 25 µl 0.4% of MTT was added to the medium and incubated at 37 °C for 30 min. Finally the formed crystals of formazan, were dissolved in 100 ml DMSO and then the absorbance at 570 nm was measured with an ELISA reader (Tecan, Rainbow Thermo, Austria) (Salimi et al., 2015). In the present study for determination of the other mitochondrial toxicity parameters, we applied concentrations of 1/2 IC₅₀, IC₅₀ and 2 IC₅₀ for crude extract and selected fractions (Fakhri et al., 2017).

ROS (reactive oxygen species) formation assay

Briefly, isolated mitochondria from cancerous and extra cancerous tissues were suspended in dichlorofluoresceindiacetate (DCFH-DA) fluorescent probe (final concentration of 10 µM) as a reagent and respiration assay buffer (0.32 mM of sucrose, 10 mM of Tris, 20 mM of Mops, 50 µM of EGTA, 0.5 mM of MgCl₂, 0.1 mM of KH₂PO₄, and 5 mM of sodium succinate) and incubate with various concentrations of

crude extract and its F1 fraction at 37 °C for 10 min. Then, the DCF fluorescence intensity was assayed using Shimadzu RF-5,000U fluorescence spectrophotometer at an excitation wavelength of 488 nm and emission wavelength of 527 nm (Seydi et al., 2016a).

Mitochondrial swelling assay

Mitochondrial swelling was evaluated from decrease in absorbance measured by spectrophotometer at 540 nm which indicates an increase in mitochondrial swelling. The obtained mitochondria from cancerous and extra cancerous tissues were suspended in swelling buffer (70 mM of sucrose, 230 mM of mannitol, 3 mM of HEPES, 2 mM of Tris-phosphate, 5 mM of succinate, and 1 μ M of rotenone) and incubated at 30°C with different concentrations of crude extract and its F1 fraction at 30°C for one hour. The absorbance was measured at 5, 15, 30, 45 and 60 min time intervals using an ELISA reader (Tecan, Rainbow Thermo, Austria) (Talari et al., 2014).

Mitochondria membrane potential (MMP) assay

In this study, the effect of crude extract and fraction 1; F1 on the mitochondria membrane potential (MMP) was assayed using re-distribution technique of Rhodamine 123 (Rh 123). In the first step, the isolated mitochondria from cancerous and extra cancerous tissues were placed in 1 ml of MMP assay buffer (220 mM of sucrose, 68 mM of D-mannitol, 10 mM of KCl, 5 mM of KH_2PO_4 , 2 mM of MgCl_2 , 50 μ M of EGTA, 5 mM of sodium succinate, 10 mM of HEPES, and 2 μ M of rotenone) for 1 hour. Then various concentrations of crude extract, its F1 fraction and Rh 123 (10 μ M) were added to it. The fluorescence activity of the cytosolic Rh 123 was determined at the excitation wavelength of 490 nm and emission wavelength of 535 nm, by using a fluorescence spectrophotometer (Schimadzu RF-5,000U) (Eskandari et al., 2012).

Cytochrome c release

The concentration of cytochrome c released from the mitochondria into the cytosolic fraction in response to various concentrations of crude extract and its F1 fraction of *T. coronatus*, was measured at 450 nm according to the instructions provided by the manufacturer of the Quantikine Human Cytochrome c Immunoassay kit (R and D Systems, Inc., Minneapolis, USA). All analysis stages were carried out using an ELISA reader (Infinite M 200, TECAN) (Naserzadeh et al., 2017).

Determination of Cytotoxicity by MTT assay

Healthy and cancerous cells (1×10^6 cells/ml) isolated from extra tumoral and melanoma tissues, respectively, were plated in 96-well plates and treated with 0-2,000 μ g/ml concentrations of) The F1 fraction of *T. coronatus* extract for 12 hours (Cells were maintained in RPMI 1640, supplemented with 10% FBS and antibiotics (50 U/ml of penicillin and 50 μ g/ml streptomycin). After treatment, 25 mL of MTT (5 mg/ml in RPMI 1640) reagent was added to each well. After 4 h, the reaction was stopped by the addition of 100 μ l of DMSO. The optical density was read at 580 nm wavelength in an ELISA plate reader.

Caspase-3 activity assay

The effect of crude extract and F1 fraction on the activation of caspase-3 on the mitochondria from melanoma and extra tumoral cells were evaluated according to the instructions of the Sigma's caspase-3 colorimetric assay kit (CASP-3-C; Sigma-Aldrich, Saint Louis, USA). Eventually, the concentration of the p-nitroaniline (pNA) released from the substrate at 405 nm was calculated for assessment caspase-3 activity or from a standard curve prepared with defined p-nitroaniline solutions (Seydi et al., 2016b).

Apoptosis versus necrosis assay

Cells obtained from the extra tumoral and melanoma tissues (1×10^6 cells/ml) were exposed to 500 mg/ml (IC₅₀) concentration of F1 fraction in duration 12 hours. Briefly double staining with Annexin V-fluorescein isothiocyanate (FITC) / propidium iodide (PI) was performed using a commercial kit (Immunotech; Beckman Coulter) and the fluorescence signals of Annexin V and PI were measured by flow cytometry (Cyflow Space-Partec) (Qiu et al., 2012).

Statistical analysis

Assays were performed using the GraphPad Prism software (version 6). Data were showed as mean \pm SD and assays were performed in triplicate. Comparisons were made using the one-way (followed by the post-hoc Tukey test) and two-way (Bonferroni post-hoc test) ANOVA tests. $P < 0.05$ was considered as level of significance.

Results

Gel filtration chromatography (GFC)

To investigate the cytotoxic effect of fractions, we first fractionated *T. coronatus* crude extract using gel filtration chromatography. As shown in Figure 1, the Chromatogram of *T. coronatus* crude extract on Sephadex G-50 revealed four obvious peak (F1-F4) with absorbance at 280 nm.

Effect of crude extract and fractions of T. coronatus on SDH activity

The effect of crude extract *T. coronatus* and its fractions (F1-F4) (0, 250, 500, 1000, 1500 and 2000 μ g/ml) on SDH after 60 minutes was determined by MTT assay. As shown in Figure 2 (B, D, F, H, J) in the mitochondria obtained from melanoma tissue, crude extract and F1-F4 fractions of *T. coronatus* induced significant ($P < 0.05$) reduction in the SDH activity. However, only F1 fraction of extract caused a significant ($P < 0.05$) concentration-dependent reduction of mitochondria succinate dehydrogenase activity on cancerous, but no, not cancerous mitochondria, which was similar to what was seen by the whole extract (Figure 2; C, D). On the other hand, the mitochondria obtained from the extra tumoral tissue, the crude extract and all fractions of *T. coronatus* only at the highest dose (1,000, 1,500 and 2,000 μ g/ml) induced a significant reduction in SDH activity (Figure 2; A, C, E, G, I). Finally, the IC₅₀ measured for crude extract and F1 fraction was 750 μ g/ml and 550 μ g/ml, respectively. Based on the calculated IC₅₀ the concentrations of 375, 750 and 1,500

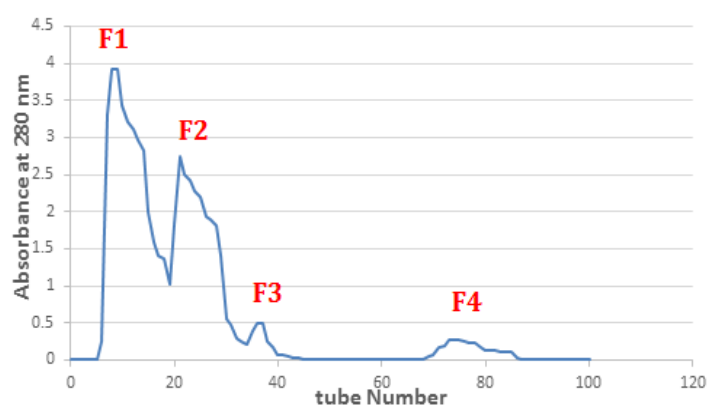


Figure 1. Gel Filtration Chromatogram of *T. coronatus* Crude Extract on Asephadex G-50 Column (2.5×150 cm). All Fractionation Steps were Performed at 4 °C.

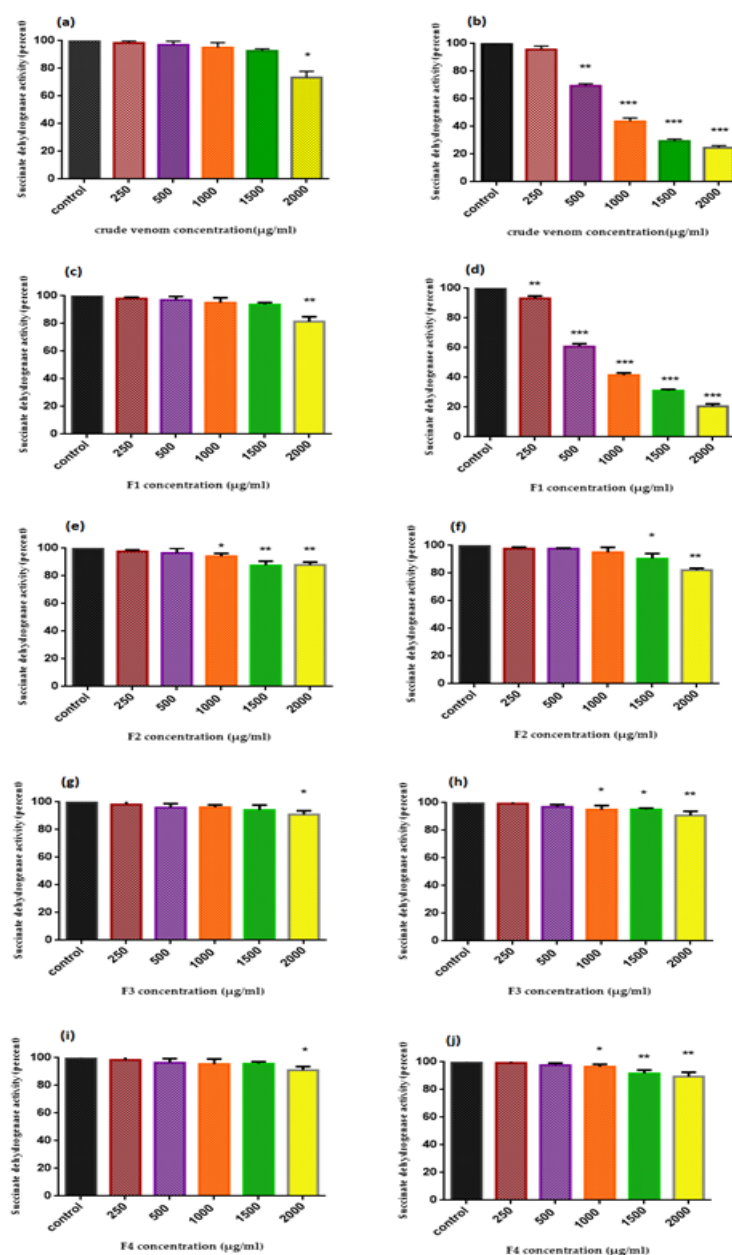


Figure 2. SDH Activity Assay. The effect of *T. coronatus* crude extract and its fractions (F1-F4) on mitochondrial succinate dehydrogenase activity measured by MTT assay. A (non-cancerous plus crude extract); B (EOC plus crude extract); C (non-cancerous plus F1 fraction); D (EOC plus F1 fraction); E (non-cancerous plus F2 fraction); F (EOC plus F2 fraction); G (non-cancerous plus F3 fraction); H (EOC plus F3 fraction); I (non-cancerous plus F4 fraction); J (EOC plus F4 fraction). Data are shown as mean \pm SD (n=5). *, **, *** 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).

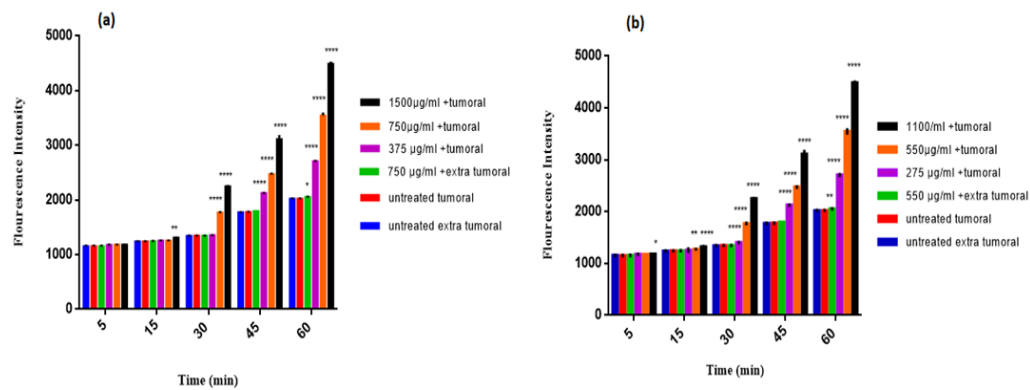


Figure 3. ROS Level Assay. The effect of various concentrations of crude extract (375, 750 and 1500 µg/ml) and F1 fraction (275, 550 and 1100 µg/ml) of *T. coronatus* extract on mitochondrial ROS level obtained from the extra tumoral and melanoma tissues. A (Crude extract); B (F1 fraction). Data are shown as mean \pm SD (n=5). *, ** and *** show a significant difference in comparison with the untreated extra tumoral mitochondria (P<0.05, P<0.01 and P<0.0001, respectively).

µg/ml for crude extract and concentrations of 275, 550 and 1,100 µg/ml for F1 fraction were selected to determine other mitochondrial toxicity parameters.

Effect of crude extract and fraction 1 (F1) of *T. coronatus* on ROS formation

As shown in Figure 3 (A-B), Our results indicated that the ROS generation in the mitochondria isolated from the extra tumoral tissue after 1 hour of incubation with different concentrations of crude extract (375, 750 and 1500 µg/mL) and F1 fraction (275, 550 and 1,100 µg/mL) was significantly (P<0.05) raised, but not on the extra tumoral group, except for the extra tumoral groups treated with 1500 µg/mL of crude extract and 1100 µg/mL of F1 In the 60 minutes.

Effect of crude and fraction 1 (F1) of *T. coronatus* on mitochondria membrane potential (MMP)

Results in Figure 4(A-B) showed that, there was a significant collapse (P<0.05) in the MMP demonstrated by the re-distribution of the cationic fluorescent probe, Rhodamine 123 from the mitochondrial matrix into the suspension buffer after exposure to different concentrations of crude extract (Figure 4A) and F1 fraction

(Figure 4B) only in the mitochondria isolated from the extra tumoral tissues. Re-distribution of Rhodamine 123 from the mitochondria into the suspension buffer ends the quenching effect and increases the fluorescence intensity recorded by the fluorescence spectrophotometer. However, MMP levels did not significantly decline in extra tumoral mitochondria, except one time by high dose of crude extract and F1 fraction.

Effect of crude and fraction 1 (F1) of *T. coronatus* on mitochondrial swelling

Addition of crude extract (375, 750 and 1,500 µg/ml) (Figure 5A) and F1 fraction (275, 550 and 1,100 µg/ml) (Figure 5B) to mitochondria obtained from the melanoma tissues led to the significant (P<0.05) mitochondrial swelling within 60 minutes of incubation. On the other hand, the addition of the same concentrations of crude extract and F1 fraction to the mitochondria obtained from the extra tumoral mitochondria did not induce any mitochondrial swelling.

Effect of crude extract and fraction 1 (F1) of *T. coronatus* on cytochrome c release

As shown in Figure 7(A-B), crude extract (750 µg/ml)

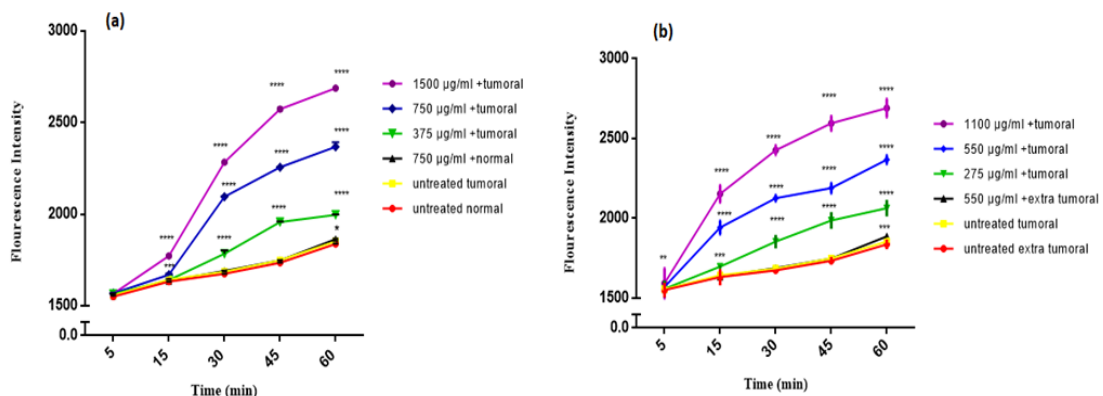


Figure 4. Mitochondria Membrane Potential (MMP) Assay. The effect of various concentrations of crude extract (375, 750 And 1500 µg/ml) and F1 fraction (275, 550 And 1100 µg/ml) of *T. coronatus* on the decline of the MMP on the mitochondria isolated from the extra tumoral and melanoma tissues. A (Crude extract); B (F1 fraction). Data are shown as mean \pm SD (n=5). *, ** And **** shows a significant difference in comparison with the untreated extra tumoral mitochondria (P<0.05, P<0.01 and P<0.0001, respectively).

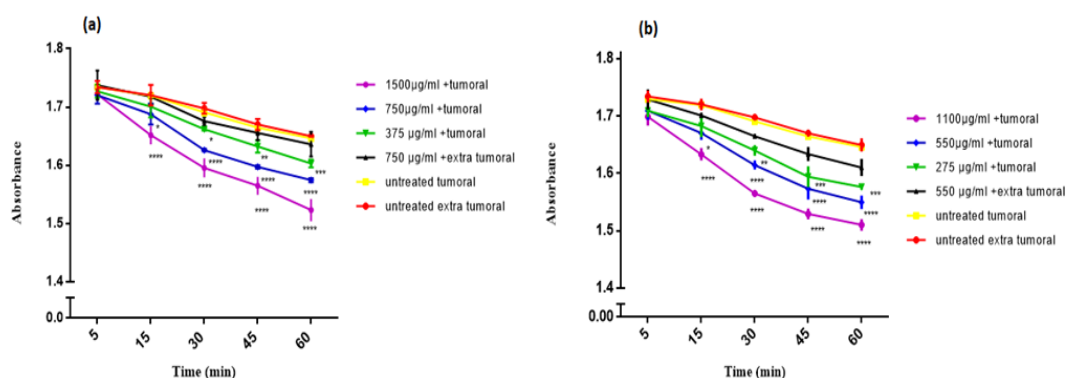


Figure 5. Mitochondrial Swelling Assay. The effect of various concentrations of crude extract (375, 750 and 1500 µg/ml) and F1 fraction (275, 550 and 1100 µg/ml) of *T. coronatus* extract on the mitochondrial swelling in the extra tumoral and melanoma tissues. A (Crude extract); B (F1 fraction). Data are shown as mean \pm SD (n=5). *, **, *** and **** shows a significant difference in comparison with the untreated extra tumoral mitochondria ($P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively).

and F1 fraction (550 µg/ml) induced a significant ($P < 0.05$) release of cytochrome c only in the mitochondria obtained from the melanoma tissue. Alternatively, pretreatment of crude extract and F1 fraction treated melanoma mitochondria by MPT inhibitors and antioxidants, such as cyclosporine A (Cs.A) and butylated hydroxyl toluene (BHT) significantly inhibited cytochrome c release compared to melanoma mitochondria treated with crude extract and F1 fraction alone.

Effects of fraction 1 (F1) of *T. coronatus* on cell cytotoxicity

As shown in Figure 6 (A and B), the inhibiting effect of different concentrations of fraction F1 of *T. coronatus* (0, 250, 500, 1,000, 1,500 and 2,000 µg/ml) on cell viability was determined after 12 hours exposure with the MTT assay using isolated melanoma cells (1×10^6 cells/ml) from both melanoma and extra tumoral tissues. However, F1 fraction of the extract significantly ($p < 0.05$) reduced cell viability only in melanoma but not extra tumoral cells. IC_{50} of F1 fraction for 12 hours exposure on melanoma

cells was 500 µg/ml.

Effect of fraction 1 (F1) of *T. coronatus* extract on Caspase-3 activity

Our results showed that F1 fraction at a concentration of 500 µg/ml induced a significant ($P < 0.05$) increase in activity of caspase-3 only in the mitochondria obtained from the melanoma cells, but not extra tumoral mitochondria (Figure 8). On the other hand, our results showed that caspase-3 activation developed by fraction F1 (500 µg/ml) significantly reduced with pre-treatment of Ac-DEVD-CHO as a caspase-3 inhibitor in cancerous melanoma cells.

Effect of fraction 1 (F1) of *T. coronatus* extract on cell apoptosis

The apoptosis was further quantified by the externalization of PS, assessed by annexin V-PI double staining at 12 hours following the exposure with F1 fraction of *T. coronatus* (500 µg/ml). The percentage of cell apoptosis in cancerous tissue caused by F1 fraction

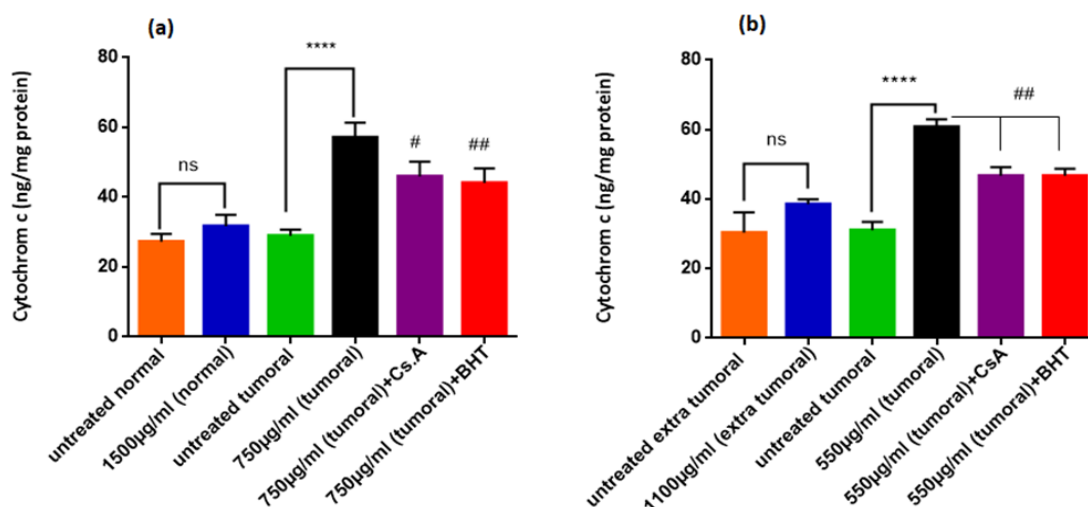


Figure 6. Cytochrome C Release Assay. The Effect of IC_{50} Concentration of Crude (750 µg/mL) and F1 Fraction (550 µg/ml) of *T. coronatus* on the Cytochrome C Release. A (crude extract); B (F1 fraction). Data are shown as mean \pm SD (n=5). **** shows a significant difference in comparison with the untreated tumoral ($P < 0.0001$). # and ## show significant difference in comparison with crude extract (750 µg/ml) and F1 (550 µg/ml) of *T. coronatus* extract-treated tumoral mitochondria ($P < 0.05$ and $P < 0.001$).

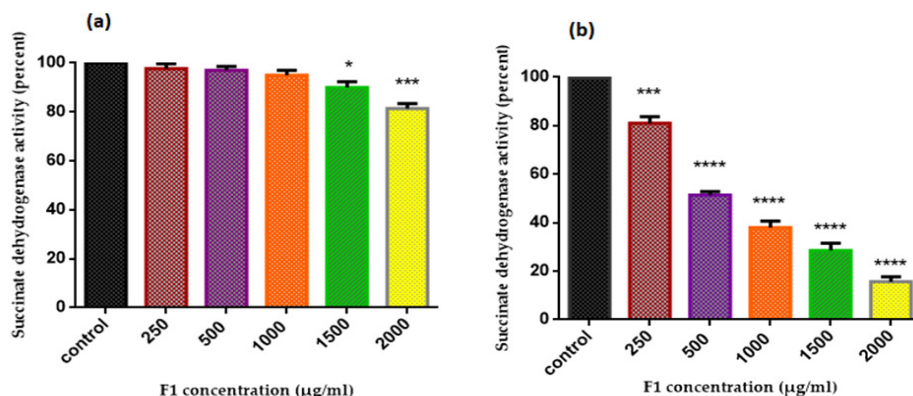


Figure 7. Effect of F1 Fraction of *T. coronatus* Extract Concentration (0-2000 µg /ml) on Cell Viability in Both Extra Tumoral (A) and Tumoral (B) Cells. Cells were treated with F1 fraction of *T. coronatus* and cell viability was measured by MTT Assay Following 12 h of exposure. Values are presented as mean \pm SD (n=5). *, **, *** and **** shows a significant difference in comparison with the untreated tumoral and extra tumoral cells (P<0.05, P<0.01, P<0.001 and P<0.0001, respectively).

was significantly (P<0.05) increased, according to the result shown in Figure 9.

Discussion

Malignant melanoma of the skin is one the most aggressive type of skin cancer that incidence and mortality rate rising in worldwide particular in light-skin populations (Vaid et al., 2016; Ye et al., 2016). That remains a great challenge due to its significant resistance to conventional chemotherapy, which is mostly caused by an intrinsic resistance of neoplastic melanocytes to apoptosis (Barbi de Moura et al., 2012; Kalal et al., 2017). Therefore correcting the dysfunctional apoptotic program in melanoma cells may be a key requisite to overcome drug resistance and improve clinical outcome (Wong, 2011). The aim of this study is to investigate the selective toxicity and apoptotic effect of crude extract

and fractions of the *T. coronatus* extract on cells and mitochondria isolated from the syngeneic animal model of melanoma tissue. Natural sources or synthetic analogues of these products remain a rich source of promising chemotherapeutic or chemo-preventive agents (Cragg and Pezzuto, 2016). Over the past decades, several new experimental anticancer agents derived from marine sources have entered preclinical and clinical trials. At present, the various bioactive compounds from different invertebrates (cone snails, soft coral, mollusk, sponges and other marine organisms) have been recognized as important sources of antitumor agents (Ruiz-Torres et al., 2017). In addition, reports indicate that approximately 75% of the 20,000 marine natural products are derived from marine invertebrates (Hu et al., 2011; Chen et al., 2015; Ruiz-Torres et al., 2017). Studies have reported that invertebrates are able to induce apoptosis signaling via decreasing Bcl-2 protein expression and also increasing

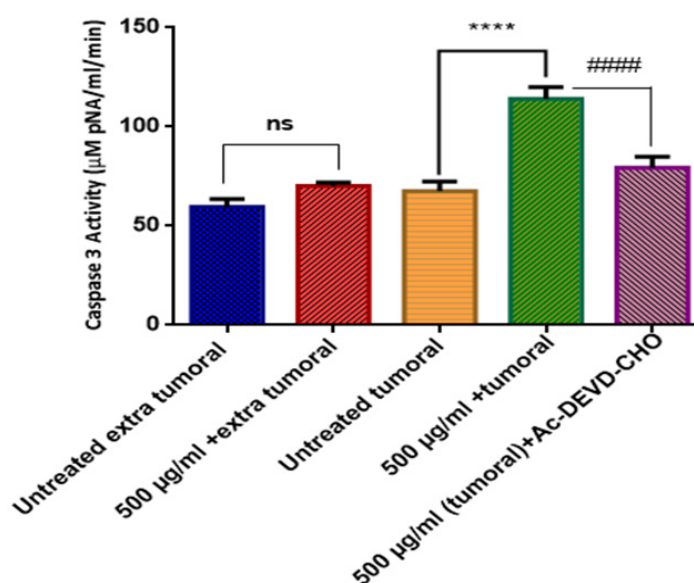


Figure 8. Effect of F1 fraction of *T. coronatus* (500 µg/ml) on the activity of caspase-3 in melanoma and extra tumoral cells. Values are presented as mean \pm SD (n=5). **** show significant difference in comparison with the untreated melanoma cells (P<0.0001). #### show significant differences in comparison with F1 fraction (500 µg/ml) treated melanoma cells (P<0.0001).

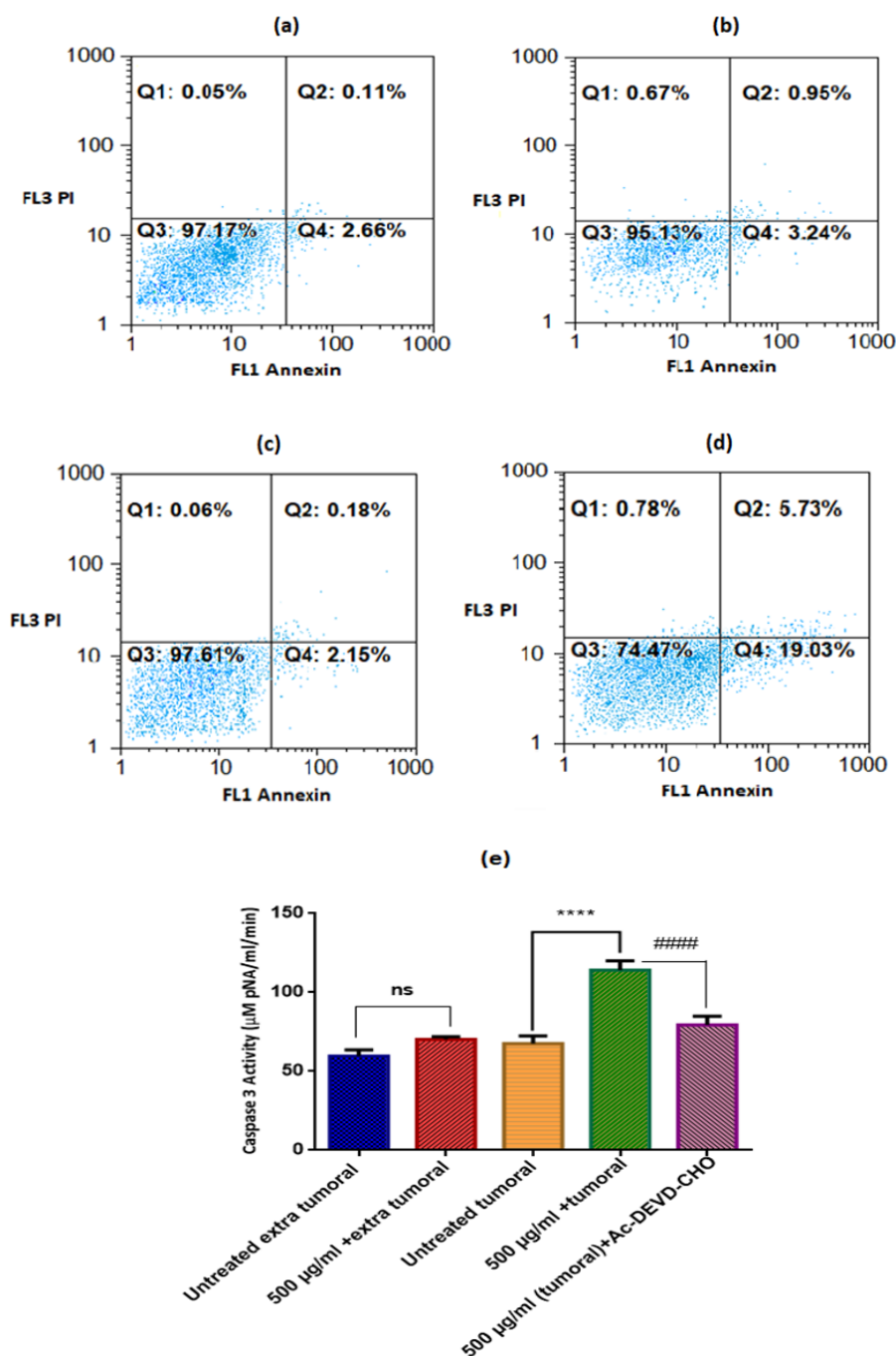


Figure 9. Analysis for Annexin-V and PI Staining of Extra Tumoral and Melanoma Cells Incubated with F1 Extract (500 µg/ml) for 12 h. Only Annexin V, positive (+) and PI, negative (-) cells were defined as apoptotic. A (untreated extra tumoral cells, Annexin V+/PI- = 2.66%), B (extra tumoral cells after 12h exposure of F1 fraction, Annexin V+/PI- = 3.24%), C (untreated melanoma cells, Annexin V+/PI- = 2.15%), D (melanoma cells after 12h exposure of F1 fraction, Annexin V+/PI- = 19.03%). Q1: % necrotic cells, Q2: % late apoptotic cells, Q3: %Live cells, Q4: %early apoptotic cells. The summarized apoptotic data (early & later) was demonstrated at graph (E). Results are expressed as means \pm SD (n=5), **** p<0.0001 vs. untreated extra tumoral cells.

Bax expression levels. Therefore, it is expected that in the future, bio prospecting efforts will continue to target marine invertebrates (Ruiz-Torres et al., 2017). According to our results, crude extract and its main fraction (F1) of the Persian Gulf sea snail, *T. coronatus*, induced apoptotic cell death in a concentration-dependent manner without cytotoxicity against extra tumoral cells. According to previous researches the most ROS in cells generated by the mitochondrial respiratory chain, that under hypoxic

conditions with increasing ROS production lead to oxidative stress that it is serious in cellular signaling pathways in cancer (Wong, 2011). Several studies have suggested that high levels of ROS can stimulate an apoptotic program by activating pro-apoptotic factors such as Bax (Ghobrial et al., 2005; Wong, 2011). Our results showed that *T. coronatus* crude extract and its F1 fraction increased ROS levels in mitochondria isolated from cancerous cells but not extra tumoral cells. Oxidative

stress induced through ROS is assumed to play a vital role in the melanoma carcinogenesis (Ayed et al., 2011; Morabito et al., 2012). In one study on leukemia cells, it was found, the ethyl acetate fraction of Meretrix lusoria extract, a marine mollusk increases ROS level (Pan et al., 2006). Accordingly, in this study, our findings showed that *T. coronatus* crude extract and its fraction 1 (F1) significantly ($P < 0.05$) increased ROS production in a time and concentration related manner only in mitochondria isolated from melanoma cells but not extra tumoral healthy cells compared to their corresponding untreated mitochondria.

Following to ROS production, mitochondrial membrane potential (MMP) is disrupted via formation of permeability transition pores that leads to induction of mitochondrial permeability transition (MPT) (Wong, 2011; Theodosakis et al., 2014). The later causes mitochondrial swelling and release of several pro-apoptotic proteins such as cytochrome c, Smac/DIABLO and the apoptosis-inducing factor (AIF) from mitochondria into cytosol (Scatena, 2012; Theodosakis et al., 2014). Although both *T. coronatus* crude extract and its F1 fraction induced the MMP collapse in the mitochondria from the melanoma tissues, but F1 fraction collapsed MMP in lower concentrations in comparison to the crude extract. Increased mitochondrial swelling as an indicator of MPT was also monitored during our study. *T. coronatus* crude extract and its F1 fraction caused significant mitochondrial swelling again, only in the mitochondria obtained from cancerous but, not extra tumoral tissue along with cytochrome c release into cytosol which is also a consequence of MPT pore-opening. MPT is associated with apoptotic protease activating factor 1 (APAF-1) and formation of a complex with pro-caspase-9, followed by activation of downstream caspases (Scatena, 2012). Moreover, pretreatment with both Cs.A as an inhibitor of MPT pore and BHT as a ROS scavenger completely blocked the release of cytochrome c from the mitochondria obtained from cancerous tissues, which proves our assumption that apoptosis induction by *T. coronatus* extract crude extract and its F1 fraction are due to the oxidative stress and relies on the opening of the MPT pore.

The caspase-3 plays a terminative role of the apoptosis signaling as an important executioner. When activated, it cleaves a series of substrates, orchestrating apoptosis (Wong, 2011; Mirshamsi et al., 2017). In this study, our results showed that F1 fraction induced a significant increase of caspase-3 activity in the animal melanoma cells and caused apoptosis enhancement. Therefore, we concluded that fraction 1 (F1) of *T. coronatus* could directly and selectively target mitochondria and induce apoptosis in melanoma cells and inhibits tumor growth. F1 fraction can therefore be a potential candidate as a novel anti-tumor agent against melanoma cells.

Statement conflict of Interest

No potential conflict of interest was reported by the authors.

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