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The synergistic influence of *Holothuria arenicola* extract and imidazole carboxamide on Cellosaurus cell line B16-F10

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Abstract

Skin cancer has been reported as a contemporary malignant cancer. Here, anti-cancer effects of sea cucumber extract (SCE) from *Holothuria arenicola* have been examined on melanoma cells and compared with imidazole carboxamide (Dacarbazine) as a chemotherapy medication against melanoma and Hodgkin's lymphoma. MTT assay and morphological analysis were performed to evaluate cytotoxic effects of *H. arenicola* extract. Also, several methods were exerted to detect cell dying by SCE and imidazole carboxamide. The MTT assay showed that B16F10 cells proliferation was blocked by SCE (IC_{50}=31µg ml^{-1}) and imidazole carboxamide (IC_{50}=1600 µg ml^{-1}) in a dose and time dependent manner. Apoptosis induction yield treatment occurred at IC_{50} concentration of SCE and imidazole carboxamide using DAPI staining, Acridine orange/Propodium iodide, PI flow cytometry and annexin/PI assay. The caspase colorimetric kit indicated that SCE and imidazole carboxamide could induce apoptosis through an intrinsic pathway. Collectively, our findings suggested that the methanolic SCE has more efficient cytotoxicity efficiency compared to imidazole carboxamide. Therefore, SCE may be considered as a futuristic marine natural product regarding prevention or treatment of melanoma malignancy.

Keywords: Holothuroidea, *Holothuria arenicola*, Skin cancer, Imidazole carboxamide, Marine, Caspase

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Introduction

Cancer is one of the most dramatic health disorders and is categorized as the third main cause of human mortality after cardiovascular disease and stroke (Saunders and Wallace, 2010). Melanoma, a malignant skin cancer (Park and Ki, 2010), is well known as an aggressive chemotherapeutic resistant cancer (Tabolacci *et al*., 2010; Emtyazjoo *et al*., 2012), which has showed a steady increased incidence (Hamm *et al*., 2008). Although, the cause of increasing incidences of melanoma has remained unknown, it has been speculated that prolonged ultraviolet (UV) radiation exposure plays an important role in melanoma cancer development (Pretto and Francesca, 2013). Several risk factors have been recognized for melanoma formation including UV exposure, increased number of nevi or the presence of dysplastic nevi depends on skin type (Shackleton and Quintana, 2010). Surgery and chemotherapy have been known as the conventional therapeutic strategies that are limited to use despite their high side effects and low response rates (Park and Ki, 2010). Vemurafenib and Ipilimumab were introduced as monoclonal antibodies in 2010 that are able to distinguish cancer cells during standard chemotherapeutic processes in patients with metastatic melanoma. On the other hand, high-dose Interleukin-2 (HD IL-2) and Interferon-α (IFN-α), which are used in immunotherapy, as well as Dacarbazine (DTIC), have been approved as non-targeting therapeutic agents (Pretto and Francesca ., 2013), which usually result in tumor recurrence because of drug resistance (Han *et al*., 2013). Therefore, an attempt to discover novel cures seems necessary to fight melanoma (Scolyer *et al*., 2011).

Natural products are secondary bioactive metabolites (Demain and Vaishnav, 2011) with obvious biological effects, which makes them appropriate candidates in anti-tumor drug discovery (Sarker and Gray, 2006). There is an enhancing need for drug discovery from natural ecosystems (Siu, 2011), which seems the result of drug-resistance to the available drugs (Haefner, 2003). Oceans cover more than seventy percent of our planet's surface. There are many bioactive metabolites isolated from marine organisms, which have already been used as sources of pharmacological products (Natarajan *et al*., 2010). So, it is not surprising that scientists, who are interested in natural products, have focused on marine biodiversity (Li, 2010). During the last decade, marine invertebrates became an interesting subject to study (Abdallah and Ibrahim, 2012); and have also been known as a source of natural compounds with biomedical properties (Popov *et al*., 2013).

Sea cucumbers (order Holothurian) are benthic echinoderms (phylum Echinoderma) living on the floor of the coastal and abyssal marine environments (Esmat *et al*., 2013). Consuming sea cucumbers as nutrients or using their extracted components has been recognized to be valuable in pharmacology, which is mostly because of various bioactive components

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accumulated in their epidermis (Bordbar *et al*., 2011). The existence of triterpenoid glycosides, chondroitin sulfates, glycosaminoglycan (GAGs), sulfated polysaccharides, sterols (glycosides and sulfates), phenol compounds and peptides have been reported from different parts of sea cucumber indicating pharmaceutical and medicinal advantages of the organism (Esmat *et al*., 2013). With regard to extracting bioactive compounds, with cytotoxic and anti-cancer properties, this study has been carried out to examine anti-proliferative and cytotoxic activities of the extract of sea cucumber (*Holothuria arenicola*) on B16F10 melanoma cell line and compare the results with those belonging to imidazole carboxamide (Dacarbazine).

Materials and methods

List of laboratory materials

RPMI-1640, FBS, penicillin/streptomycin, PBS, PI, AnnexinV-FITC kit and Caspase-3 and Caspase-9 kit, DAPI, MTT ,Acridine orange/propodium iodide were purchased from Bioidea (Iran), GIBCO (USA), PAA (Austria), Sigma (USA), abcam (UK), Applicam (USA) , Sigma (USA), respectively.

Preparation of sea cucumber extract

The specimens of sea cucumber (*H. arenicola*) were collected from the coastal waters of Qeshm Island in the northeastern Persian Gulf (Keshavarz *et al*., 2012). The specimens were washed and stored at −80 °C until laboratory examinations. Sea cucumber extract (SCE) was obtained from the epidermis. For this purpose about 30 g of tissue was dried, split and minced with 300 mL methanol and placed on a stirrer at room temperature for 72h. Finally, the solution was filtered and concentrated under a vacuum evaporator (Wijesinghe *et al*., 2013). The concentration of the crude extract was 50 mg; the stock solution was dissolved in 100 µL DMSO and kept at –20 ºC. The concentrations of 1-250 µg mL^{-1} were then prepared by diluting with complete media, so that the final concentrations of DMSO did not exceed 0.5%.

Cell cultivation

The B16F10 Cell line was obtained from the Pasteur Institute of Iran cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotic and incubated in a CO_2 incubator at 37°C.

MTT assay for assessment cell viability

B16F10 cells (2×10^4) were seeded in 96- well plates. Treatments were conducted using different concentrations of SCE (15, 31, 62, 125, 250 µg mL^{-1}) and imidazole carboxamide (Dacarbazine) (1000, 1200, 1400, 1600, 1800, 2000 µg mL^{-1}) in three time periods (24, 48 and 72 h). Then MTT assay was performed. Briefly; after the treatment period, MTT (30 µL) was added to each well for 3 h, then 80 µL of DMSO was added to dissolve formazan crystals. The absorbance was measured at a wavelength of 560 nm (Shahbazzadeh *et al*., 2011; Thangam *et al*., 2014) and the cell viability inhibition was

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considered according to the following equation:

Cell viability (%) =Absorbance in test wells/ Absorbance in control wells×100

Analysis of apoptosis by DAPI staining

DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) staining was utilized to assess the changed morphology of the nuclei after the treatment. The cells ($1\text{-}5\times10^5$) were seeded on the cover slip in the 6-well plate. 24 h after treatment with different concentrations of SCE and imidazole carboxamide drug and a combination of both, the cells were stained with DAPI and were incubated for 10 min in the dark. Finally the cells were suspended in 1000 µL of methanol and the morphology of the nuclei was observed under fluorescence microscopy (Wang *et al*., 2010).

Detection of apoptosis by dual Acridine orange/ propodium iodide staining

The B16F10 Cells ($1\text{-}5\times10^5$) were treated with SCE, the drug and a combination of both for 24 h. Then, the cells were observed using a fluorescence microscope for the morphological changes with Acridine orange/Propodium iodide dye. Both control and treated cells were stained with Acridine orange (100 µg mL^{-1}) and Propodium iodide (100 µg mL^{-1}) (1:1). The cells were immediately moved on to slides and observed under a fluorescence microscope for the evaluation of the cells undergoing apoptosis.

Flow cytometry based apoptosis revealing

Twenty four hours after the treatment, B16F10 cells (1×10^6 cells mL^{-1}), untreated and treated cells were resuspended in 1 mL of binding buffer, and 5 µl Annexin V- FITC and 5 µl PI was added, then kept for 15 min in the dark and analyzed by flow cytometry (Massaoka *et al*., 2012; Lu *et al*., 2013)

Measuring cell death by PI

After the duration of the treatment, 700µl of PI solution was added to the cells in each plate and placed into the incubator for 20 minutes in the dark. Then the cells were transferred into separate micro tubes and analyzed by flow cytometry (Lu *et al*., 2013).

Caspas-3 and Caspas-9 activity assays

The assay was performed using a Caspase-3 or Caspase-9, Apoptosis Detection, Colorimetric BioAssay Kit (abcam-UK) based on the protocol of the company. B16F10 cells (2×10^6) were treated with two concentrations of SCE including, 31 and 62 µg mL^{-1}, for 24 h. The control and treated cells were resuspended in 50 µL of cell lysis buffer (supplied with the kit) and incubated on ice for 10 min. Supernatants (cytosolic extract) were centrifuged and moved to new tubes and kept on ice. The caspase-9 assay was done according to the supplied kit protocol. 50 µL of 2X reaction buffer (containing 10 mM DTT) was added to each sample. 5 µL of LEHD-pNA substrate (4 mM) (200 µM final concentration) was added and incubation was done at 37 °C for 1-2 h.

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Absorbance was read at 400 nm and calculations were thereby done (Roy *et al*., 2008; Chen *et al*., 2013).

Statistics

Data analyses were performed using one-way analysis of variance (ANOVA) to determine the significance among the groups. All tests were assumed statistically significant at $p \leq 0.05$.

Results

Cell morphology observations

Morphological evaluation of melanoma cells after 24 h exposure to methanolic SCE, Dacarbazine, and a combination of both showed some changes in morphology of melanoma cells including cell shrinkage, reduced cell volume, plasma membrane istortion, nuclear condensation and apoptotic bodies all of which are known as apoptotic characteristics (Fig.1).

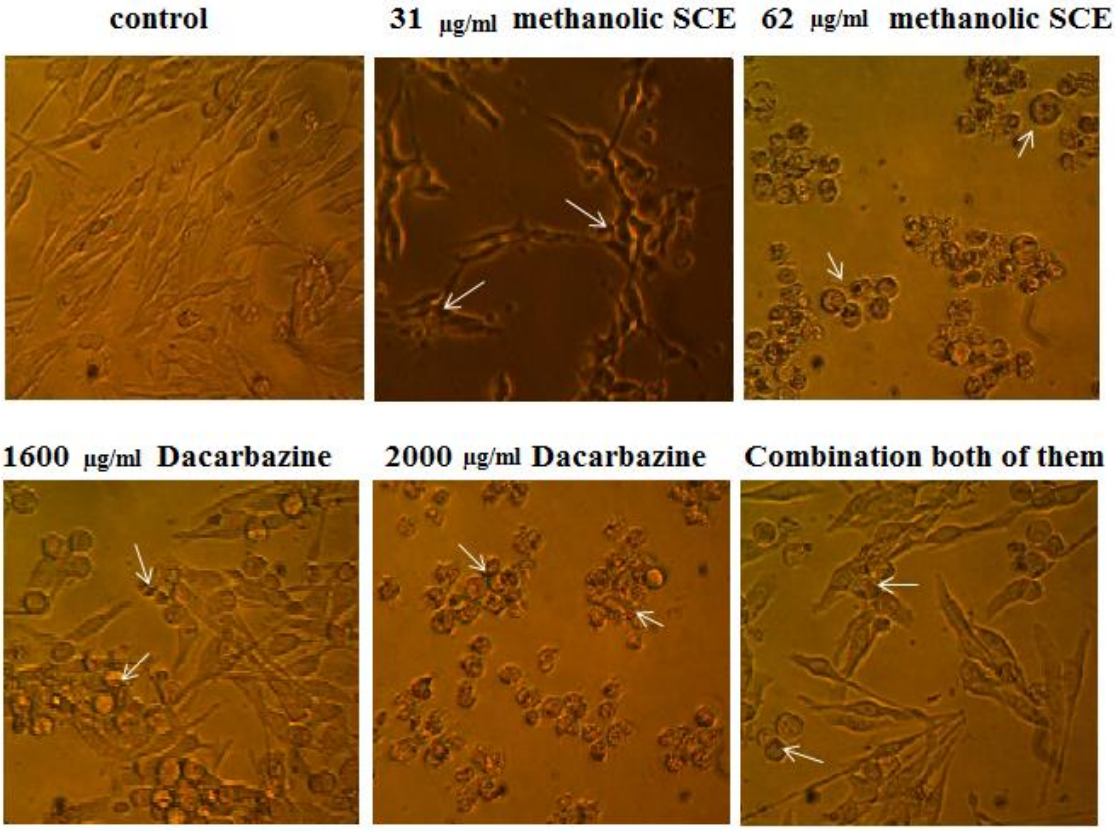

Figure 1: Influences of methanolic SCE and Dacarbazine on B16F10 cell morphology. Cancer cell line B16F10 was treated with different concentrations of sea cucumber extract, imidazole carboxamide and both of them (15µg mL^{-1} methanolic SCE and 1400 µg mL^{-1} dacarbazine) (Magnification × 200).

Evaluation of cytotoxic potential

The dose-dependent inhibitory effects of the extract, of Dacarbazine drug, and both of them were designed against the percentage of cell viability in order to determine the inhibitory concentrations required to reduce 50% of cell viability (IC_{50}) compared to the untreated control (Table 1).

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Table 1: Inhibitory effects of *Holothuria arenicola* extracts and imidazole carboxamide on growth of cancer cell line B16F10. *Data represent IC_{50}, the inhibitory concentrations of the extract, of dacarbazine and of the combination required to reduce 50% of cell viability by using MTT assay, mean±SD (*n*=3).

Groups	B16F10 IC_{50} after 24 h (µg mL^{-1})*
Methanolic extract of sea cucumber	31 µg mL^{-1}
Dacarbazine	1600 µg mL^{-1}
Methanolic extract of sea cucumber and Dacarbazine	15 µg mL^{-1}+1400 µg mL^{-1}

The IC_{50} inhibitory concentrations of SCE and Dacarbazine obtained were 31 and 1600 µg mL^{-1} (p<0.05), respectively. However, the results showed that the combination was more cytotoxic compared to either Dacarbazine or SCE (IC_{50}=15 µg mL^{-1} of extract+1400 µg mL^{-1} of Dacarbazine (p<0.05) (Fig. 2).

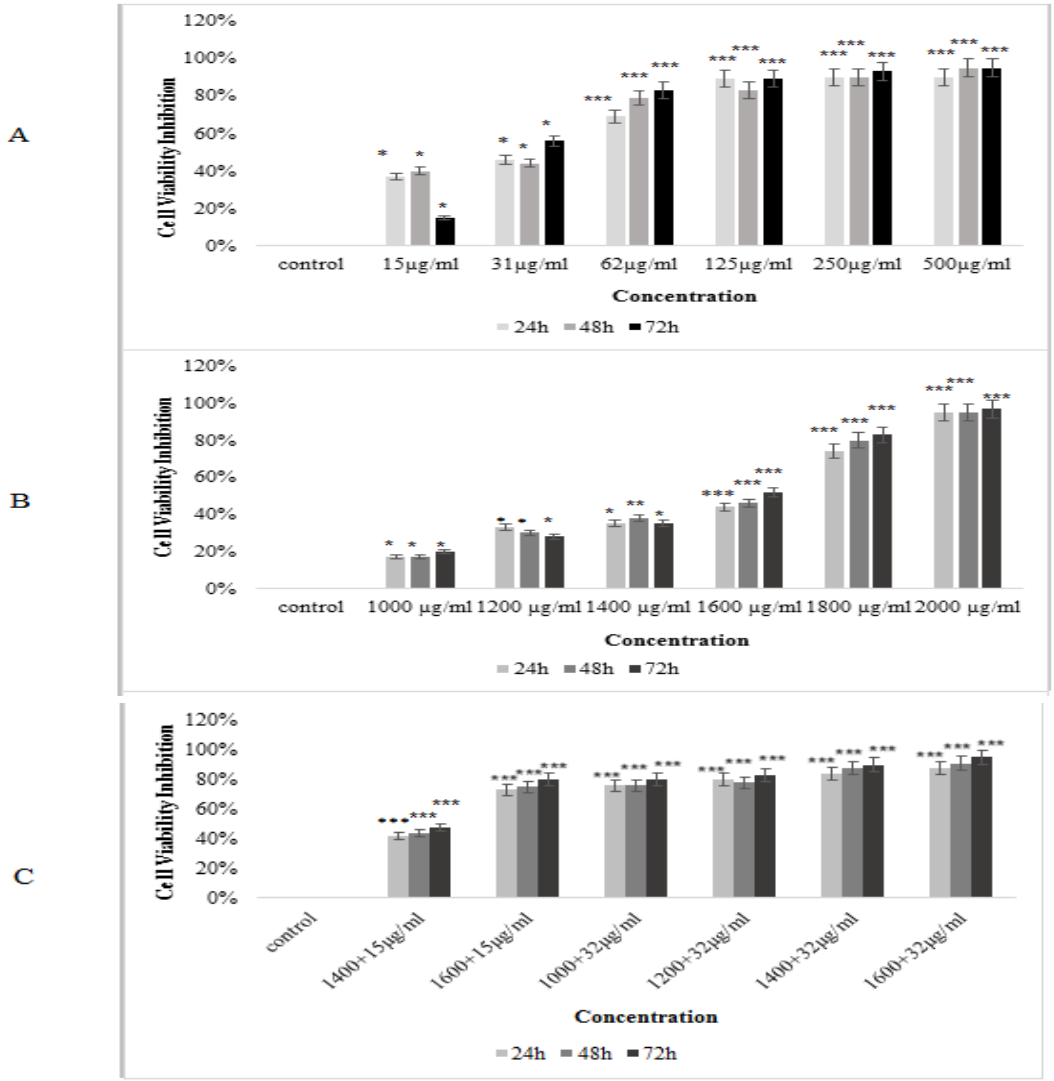

Figure 2: Percentages of cell viability inhibition of melanoma cell line B16F10 treated with different concentrations of methanolic SCE and Dacarbazine after 24, 48 and 72 h based alone or in combination by MTT assay. The data were represented as mean±SD and **p*<0.05, ***p*<0.005 and ****p*<0.001 were considered to be significant. (A: methanolic SCE, B: dacarbazine drug and C: combination of both of them).

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Apoptosis evaluation by DAPI staining

DAPI was applied as a fluorescence stain to investigate nuclear apoptosis. The output of DAPI staining showed that chromatin condensation observed in 31 µg mL^{-1} (particularly in 62 µg mL^{-1}) of SCE as well as in 1600, 1800 and 2000 µg mL^{-1} of Dacarbazine and the synergistic treatments with lower IC_{50} concentration of each one (IC_{50}=31 µg mL^{-1} for extract and IC_{50}=1600 µg mL^{-1} for dacarbazine) (Fig. 3A).

Assessment of apoptosis by AO/PI double staining

This method is utilized to recognize live, apoptosis and necrosis cells, which are categorized by their color (green, stained bright green, stained orange and stained red for live, apoptotic, early apoptotic, late apoptotic and necrotic cells, respectively. Significant differences in apoptosis induction observed between the control and treatment cells after treating with methanolic SCE, Dacarbazine and a combination of the two for 24 h. (Fig. 3B).

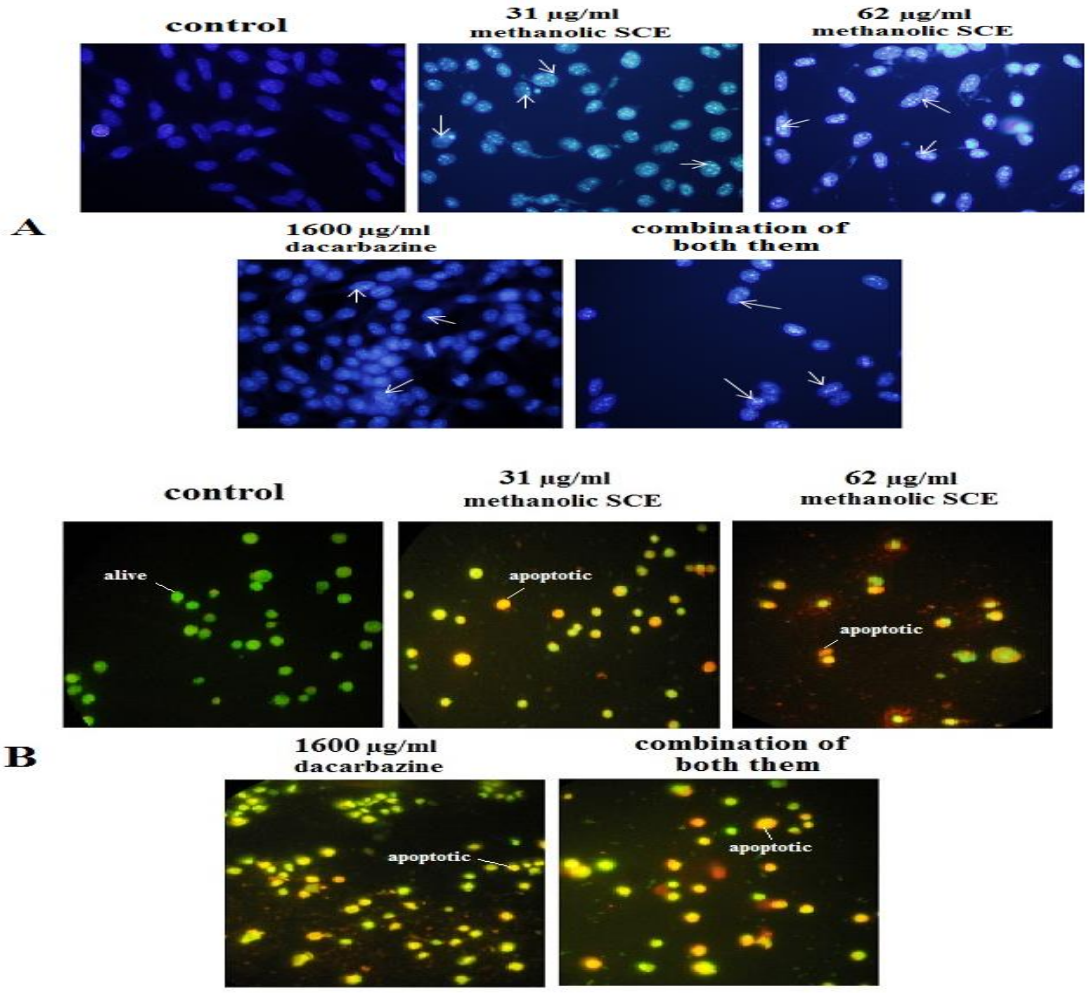

Figure 3: The fluorescence micrograph of melanoma cancer cell line B16F10 treated with different concentrations of sea cucumber extract, Dacarbazine and the combination of both of them after 24 h of treatment with DAPI assay (A) and acridine orange/ propidium iodide assay (B).(combination of both them=15 µg mL^{-1} methanolic SCE+1400 µg mL^{-1} dacarbazine).

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Cytometry analysis for apoptosis detection

Fig. 4 shows the results of SCE, Dacarbazine and synergism effects on cell cycle phases of B16F10 cells during 24 hours of treatment. The sub-G1 peak (apoptotic peak) observed in 31 µg mL^{-1} and 62 µg mL^{-1} of methanolic SCE, 1600, 1800 and 2000 µg mL^{-1} of imidazole carboxamide and 15 µg mL^{-1} SCE plus 1400 µg mL^{-1} of imidazole carboxamide. These results showed that arresting cell cycle in Sub-G1 phase mediates reducing B16F10 cells proliferation (Fig. 4).

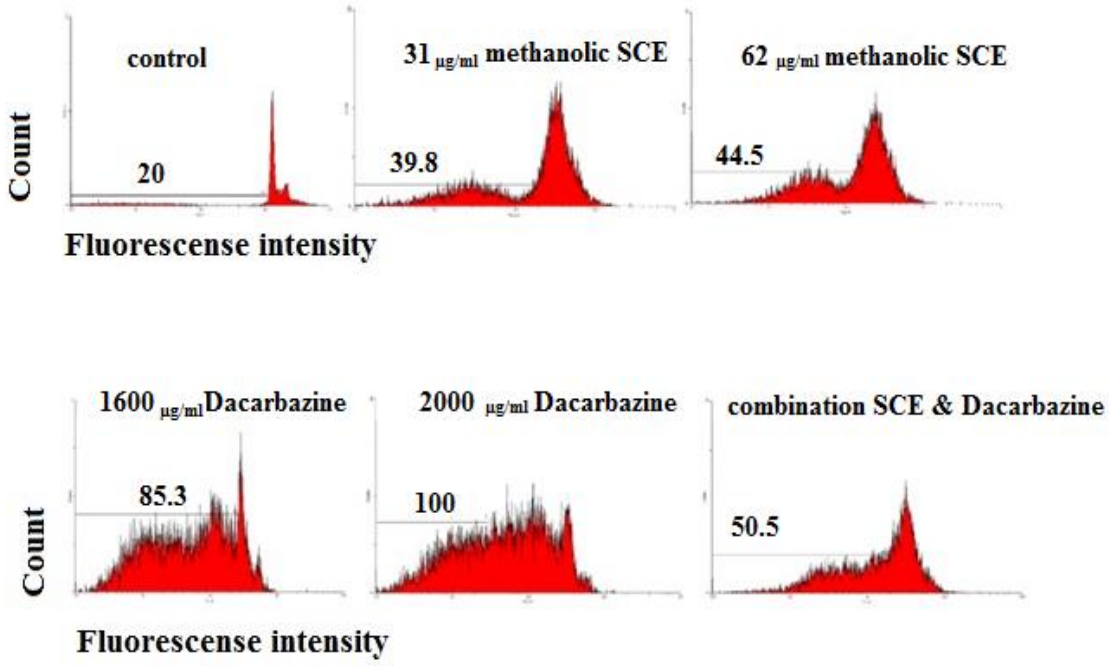

Figure 4: Cell cycle phase determination of melanoma cell line B16F10, which were induced by different concentrations of methanolic SCE, Dacarbazine and the combination of both of them after 24 h by flow cytometry.

Induced apoptosis in B16F10 using SCE, imidazole carboxamide and the combination

Annexin V-FITC is a recombined protein that specifically binds to phosphatidylserine and remains with high affinity for separating apoptosis from necrosis. Investigating the effects of different SCE concentrations on B16F10 cells, using annexin V-FITC/PI staining, indicated that in 31 µg mL^{-1} and 62 µg mL^{-1} of methanolic SCE the apoptotic ratio increased in treated B16F10 cells compared to untreated cells. These results demonstrated that SCE, like Dacarbazine, inhibited B16F10 cell proliferation by inducing apoptosis. (Fig.5)

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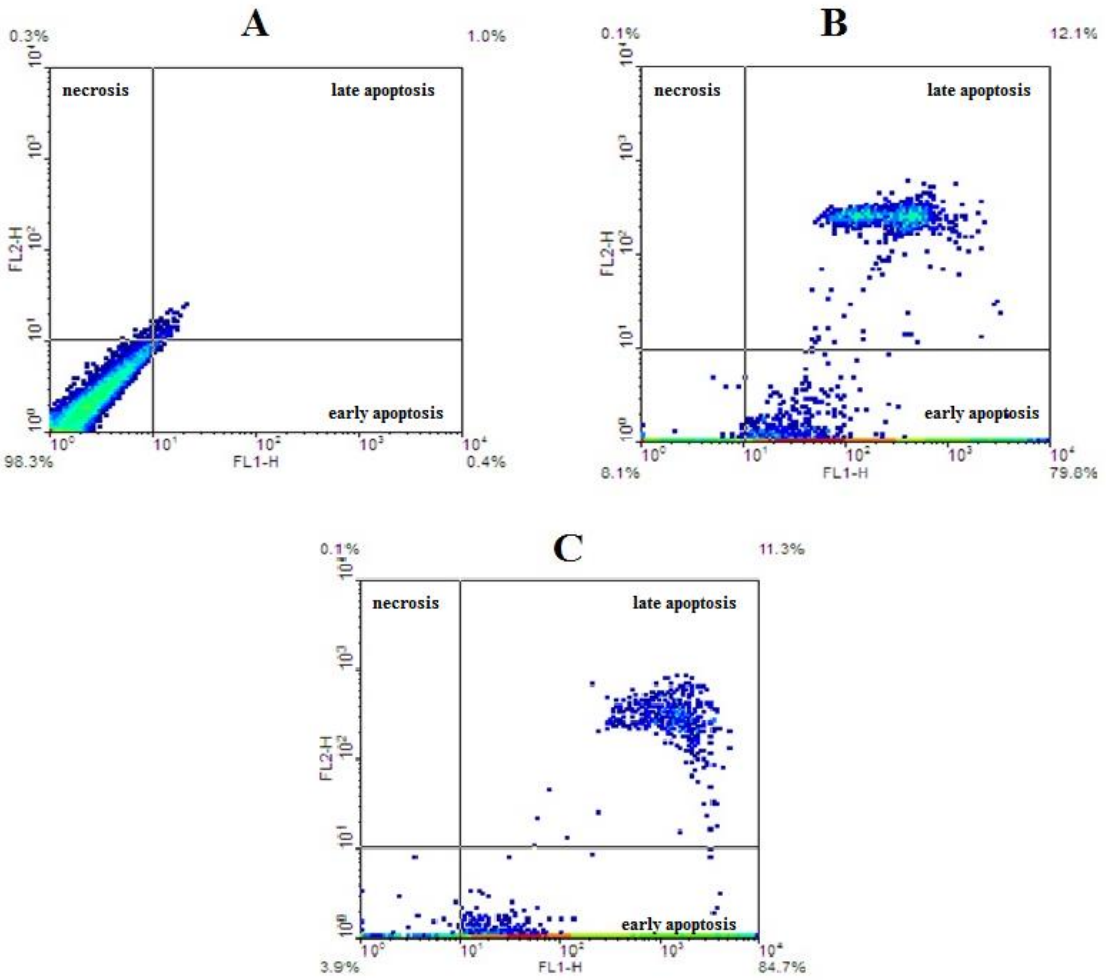

Figure 5: Determination of apoptosis from necrosis on melanoma cell line B16F10, which were treated using different concentrations of sea cucumber extract, after 24h based on Annexin/PI assay. (A: control, B: 31 µg mL^{-1} of methanolic SCE and C: 62 µg mL^{-1} of methanolic SCE).

Inducing caspase 3 and caspase 9 activity in B16F10 cells by SCE, dacarbazine and synergism

The results showed that enzymatic activity of caspase 3 (as apoptosis executioner) and caspase 9 (as apoptosis effector) increased among the groups treated with SCE and Dacarbazine and the combination of both at lower concentrations of IC_{50} of them after 24 h compared with the control (Figs. 6, 7).

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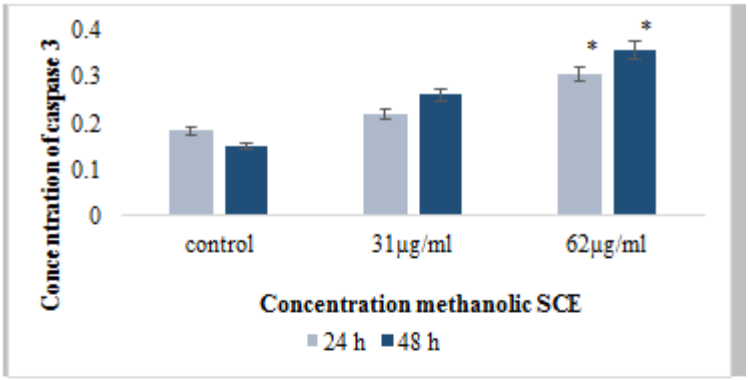

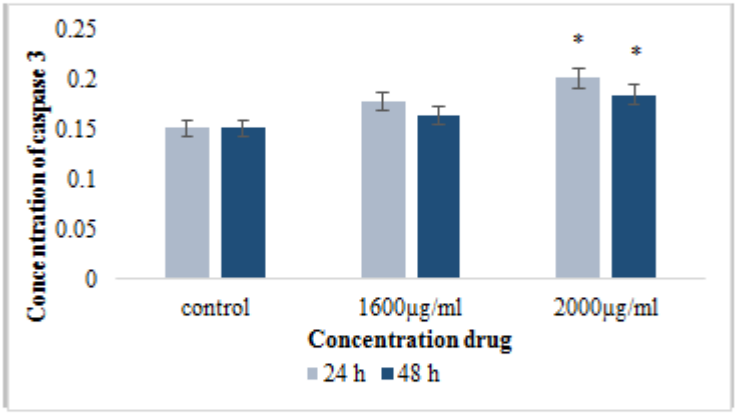

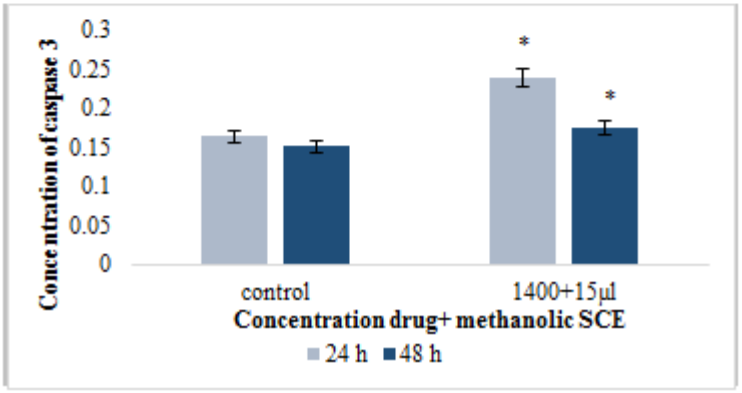

Figure 6: The melanoma cancer cell line B16F10 treated with different concentrations of methanolic SCE, Dacarbazine and the combination of them for measurement of caspase 3 activity. The data were represented as mean±SD and *p<0.05, were considered to be significant.

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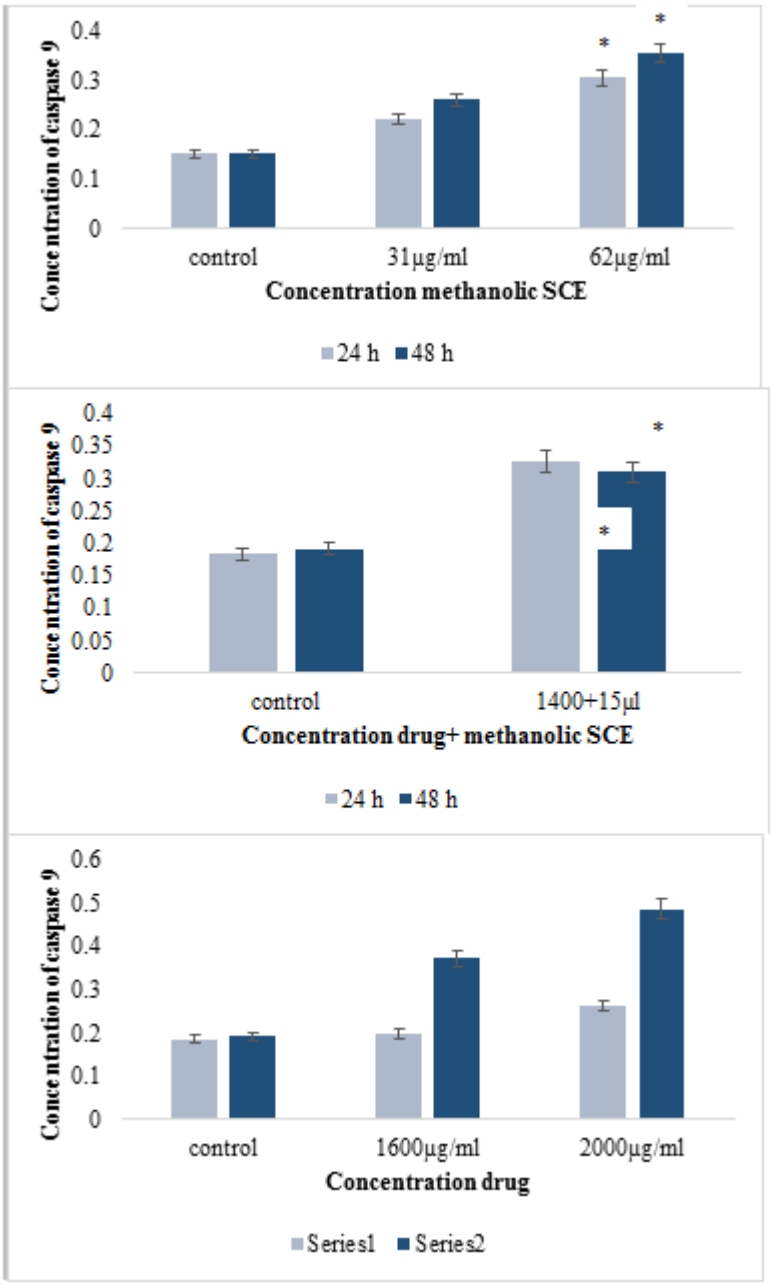

Figure 7: The melanoma cancer cell line B16F10 treated with different concentrations of sea cucumber extract, Dacarbazine and the combination of them for apoptosis pathway detection using caspase 9 enzymatic measurement. The data were represented as mean±SD and *p<0.05, were considered to be significant.

Discussion

Conventional treatments for melanoma including surgery and chemotherapy with Dacarbazine (DTIC) cause some side effects. Thus, scientists have been interested in finding more potent drugs with low side effects (Shackleton and Quintana, 2010). Therefore, we decided to examine the anti-melanoma efficacy of sea cucumber as a treatment for metastatic melanoma.

In this experiment, the cytotoxic effect of the sea cucumber *H. arenicola* methanolic extract on B16F10 cells has

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been examined and compared with imidazole carboxamide as an approved chemotherapeutic drug. The results showed that alcoholic sea cucumber extract has better anti-proliferative effects on B16F10 cells compared to Dacarbazine in a dose and time dependent manner. Further, treatment with the IC_{50} concentration and higher doses of SCE induced apoptosis in the B16F10 cell line, which seems a valuable achievement for controlling and treating melanoma.

Althunibat in 2013 found that the aqueous and organic extracts of two sea cucumber species, *Holothuria edulis* Lesson (Holothuriidae) and *Stichopus horrens* Selenka (Stichopodidae), have significant cytotoxicity against two human cancer cell lines including A549 and TE1. Further, the study showed that an organic extract of *S. horrens* has the highest cytotoxic effects against A549 and TE1 cancer cells (Althunibat, 2013). M. Gushi and colleagues examined the effects of sea cucumber (*Stichopus japonicus*) extract on the proliferation of colon adenocarcinoma Caco-2 cells and reported that sea cucumber extract inhibited cell proliferation (Gushi *et al*., 2005). However, we found that a dosage higher than 125 µg mL^{-1} of sea cucumber alcoholic extract can initiate cell lysis of B16F10 cell line. Mutee and colleagues examined the cytotoxicological activity of *Acanthaster planci* extract on MCF-7 and HCT-116, which showed that the extract has very potent cytotoxic activity, whereas the chloroform and methanol extracts showed moderate cytotoxicity (Mutee *et al*., 2012).

Enhanced inhibitory effects of methanolic extract *Ciona intestinalis* on human cell lines, $CaCO_2$, U-937 and HL-60, demonstrated that this extract suppressed cell proliferation of different origins of human cell lines with apoptotic inducement events such as caspase-3 activation and inter nucleosomal DNA degradation, hence suggests that the *Ciona intestinalis* extract possess bioactive compounds responsible for anticancer activity (Russo *et al*., 2008).

The results of the present study showed that 31 µL of sea cucumber (IC_{50}) and lower concentrations do not have potent cytotoxicity on melanoma cells. Ebrahimi Nigjeh and colleagues examined cytostatic effects of microalga, *Chaetocero scalcitrans* (EEC) crude ethanol extracts on human breast cell lines and found that MCF-7 is more sensitive compared to MCF-10A. Further, the study showed that EEC is more sensitive compared to Tamoxifen against MCF-7 after 24 hours incubation, which confirms our results about anti-cancer efficiency of marine echinoderm (Ebrahimi Nigjeh *et al*., 2013). To gain further insights into the mechanisms involved in cytotoxicity of sea cucumber extract on melanoma cells, we evaluated the uptake of Propodium Iodide (PI) using flow cytometry, DNA fragmentation using fluorescence observation and caspase-3, -9 assays. The results indicated that treatment with inhibitory concentrations of sea cucumber extract alone or along with Dacarbazine

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induced the sub-G1 accumulation after a 24 h treatment. In addition, DAPI and AO/PI assay demonstrated that defined concentrations of sea cucumber extract and Dacarbazine predominantly induced apoptosis in B16F10 cells and probably activated an intrinsic pathway which was verified by caspase-3 and -9 activation.

Furthermore the methanolic extract of sea cucumber had more anti-cancer efficiency compared to Dacarbazine against B16F10 melanoma cancer which proposed the presence of potent anti-cancer bioactive metabolites in sea cucumber extract against melanoma.

The findings of this study recommend Persian Gulf sea cucumber extract as a valuable source of marine anti-cancer bio agents. *H. arenicola* extract showed high anti-cancer efficiency against melanoma carcinoma cells in vitro; may be because methanolic extract of sea cucumber induces mitochondria mediated apoptosis via increment of caspase-9 and caspase-3 activation.

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