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The Use of Methanolic Extract of Persian Gulf Sea Cucumber, Holothuria, as Potential Anti-Cancer Agents

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Abstract

Introduction:

Hepatocellular carcinoma (HCC) is the fifth most malignant of liver cancer globally. Melanoma is also a highly aggressive and deadly cancer with a poor prognosis given its drug resistance. A defect in apoptosis pathway is one of the key mechanisms that contribute to drug resistance in cancer. An important sea marine animal is the Holothuria, also known as the sea cucumber, which has various pharmacological activities. Scientists have begun to further investigate on the natural bioactive compounds found in marine animals showing anti-inflammatory and anti-cancer properties. *H. sabra* and *H. parva*, sea cucumbers are known to show the mentioned properties.

Methods and **Results:**

This study was designed to determine to use of methanolic extract of Persian Gulf sea cucumber, Holothuria, as potential anti-cancer agents on liver and skin mitochondria isolated from cancerous induced animal.

The cancerous induced animals were applied for mitochondrial investigations. The mitochondria isolated from Hepatocytes and melanoma cells via differential centrifuges was treated with various concentrations (250, 500 and 1000 µg/mL) of metanolic extract of *H. parva and H. sabra*. Reactive oxygen species (ROS) formation, mitochondrial swelling, mitochondrial membrane potential (MMP), and cytochrome c release resulting from the selective toxicity of various dilution of methanolic extract of *H. sabra* and *H. parva* on mitochondria isolated from cancerous induced animals were determined.

Our results show that different concentrations (250, 500 and 1000µg/mL) of the methanolic extract significantly ($p<0.05$) induce reactive oxygen species (ROS) formation and mitochondrial swelling, decreased mitochondrial membrane potential (MMP) disruption and increased cytochrome c release only in HCC mitochondria and melanoma mitochondria, in a time-and concentration dependent manner (In comparison to the control group).

Conclusion:

Our study suggests that given bioactive compounds found in mentioned sea cucumbers can be potentially introduced as anti-cancer molecules. Further studies such as molecular identification, *in vivo* experiments and clinical trials can confirm these results.

Keywords: Hepatocellular carcinoma; Melanoma; Persian Gulf sea cucumbers, both polar and non-polar extract; selective toxicity

1.Introduction

Cancer is a growing public health problem, particularly in developed countries, despite advances in biomedical research and technology. In 2012, the annual cancer cases were 14 million and this number will increase to reach 22 million within the next two decades. Moreover, cancer is a leading cause of death worldwide, accounting for 8.2 million deaths in 2012.

Hepatocellular Carcinoma (HCC) is categorized among the deadliest cancers and the fifth principal cause of cancer across the world [1, 2]. Causative factors of HCC include Hepatitis C, which has

annually increased the rate of HCC up to 3.7 per 100 patients. Hepatitis B has also increased the rate to 2.2 per 100 patients per year [3]. Additional causative factors include food additives, non-alcoholic fatty liver disease, obesity, and various forms of pollutions and industrial chemical toxics found in the environment [4, 5].

Melanoma, also known as skin cancer, initiates in the pigment melanocytes. According to the past studies, the incidence of melanoma has increased in both developed and developing countries [1, 2].

In the early stages of these cancers, surgery can cure the disease. The disease has a poor prognosis given its resistance to conventional chemotherapy drugs. A number of chemotherapy drugs through the mitochondrial pathway induce apoptosis signaling and cell death in the cancer cells [3]. Studies have shown that disruption of the process of apoptosis is one of the main mechanisms of resistance. In fact, targeting apoptosis pathways is one of the mechanisms underlying treatment for cancers [4-7]. It is well documented that the mitochondria plays a pivotal role in apoptosis signaling. Furthermore, there are several differences between the mitochondria found in healthy and malignant cells. Due to these differences, the mitochondria have been considered for many researchers as a cancer therapeutic target [8-10].

Utilization of natural products as anti-cancer agents is increasing. The oceans cover approximately 70% of the Earth's surface. Marine environments are a unique reservoir for bioactive natural products, with structural features not generally found in terrestrial plant metabolites. In the last decade, more than 3000 new compounds have been discovered in marine environments, indicating that marine environments may offer a variety of novel therapeutic molecules. In recent decades, many natural compounds, including from marine species, with anticancer properties have been identified; some of these compounds are indifferent phases of clinical trials [11, 12]. Studies have shown that bioactive compounds isolated from sea cucumbers possess nutritional value, likely health benefits and serve as a potential treatment towards chronic diseases such as cancer [13, 14].

Sea cucumbers, belonging to the Holothuroids (Holothuroidea), are marine invertebrates that exist in benthic areas and deep seas [14]. The anticancer effects of sea cucumbers have been attributed to a unique combination, which includes triterpene glycosides, sulfated triterpene glycosides, alkaloids, frondoside A, and unsaturated fatty acids [13, 15, 16]. Results from our previous study showed that mitochondrial targeting is an important mechanism by which *H. parva* can potentially and selectively induce apoptosis in hepatocellular carcinoma (HCC) hepatocytes and human chronic lymphocytic leukemia (CLL) Lymphocytes [14, 17]. Furthermore, other studies have shown that compounds isolated from sea cucumbers inducing apoptosis leading to cancer cell death [15, 16]. There is a significant lack of evidence about the selective toxicity and apoptotic ability of *H. parva*, a novel potential anticancer candidate of Iranian Persian Gulf flora and fauna, on melanoma as well as *H. sabra* on HCC. Therefore, this study aimed to study the cytotoxicity effect of *H. sabra* extracts on HCC and *H. parva* on skin mitochondria isolated from animal model of melanoma.

2.Materials and Methods

2.1. Sampling and extraction of sea cucumber

Holothuria (*H. parva* and *H. sabra*) samples were collected from the Bandar-e-lengeh coast, south of Iran. *H. parva* and *H. sabra* samples were transported to the laboratory via iced boxes. The samples were washed with cold water, weighed, and evaluated. Bioactive agents were extracts using the method described by Mamelonaet al.[18]. *H. parva* and *H. sabra* samples were cut into small parts and homogenized using a blender. After filtration and centrifugation at 30,000 ×g for 15 min in 4º C, methanolic extracts of Holothuria (*H. parva* and *H. sabra*) were evaporated under vacuum at 45º C by a rotary evaporator. Finally, the powdered extracts of *H. parva* and *H. sabra* were stored at -20 ºC.

2.2. Animals

The animal model for melanoma was acquired from Pasteur Institute. All experimentations were conducted according to the ethical standards and the

protocols were approved by the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

2.3. Mitochondria isolation

❖ *Isolation of the Mitochondria from Hepatocytes*

Hepatocytes were isolated by using the four-step collagenase liver perfusion method. After that, to examine the cell viability, trypan blue exclusion test was used. For mitochondrial parameters assay, the mitochondria was prepared and extracted from hepatocytes. Briefly, the hepatocytes were suspended in 10mL of solution A (0.25M of sucrose, 0.01M of tricine, 1mM of EDTA, 10mM of NaH2 PO4 , and 2mM of MgCl2 ; pH=8) and subsequently frozen at –80°C for 10 min and centrifuged at 760 g for 5 min in order to break the plasma membrane. The supernatant was kept while the pellet was homogenized for 10 min, followed by centrifugation at 760 g for 5 min. The supernatants from the two previous steps were combined and centrifuged for 20 min at 8,000 g. The final mitochondria containing pellet was suspended in Tris buffer (0.05M of Tris-HCl, 0.25M of sucrose, 20 mM of KCl, 2.0mM of MgCl2 , and 1.0mM of Na2 HPO4 ; pH=7.4) at 4°C for further tests [1, 16-18].

❖ *Isolation of the Mitochondria from melanocytes*

Briefly**,** mitochondria from mouse melanoma cells were isolated using the perfusion-collagenase method [10, 19]. Then, after the measurement of cellular viability, the melanoma cells were pelleted using centrifugation at 300 × g for 3 min. Furthermore, to prepare the mitochondria from melanoma cells the differential centrifuges (760× g for 5 min and 8,000 ×g for 20 min, respectively) were used. Eventually, the final mitochondrial pellets used to measure the desired parameters suspended in the corresponding buffer.

2.4. Measurement of Succinate Dehydrogenase (SDH) activity

Briefly, the effect of methanolic extract of Holothuria (*H. parva* and *H. sabra*) (0, 125, 250, 500, 1000 and1500μg/mL) on SDH activity was measured by the decline of MTT (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide) to formazan using an ELISA reader (Tecan, Rainbow Thermo, Austria) at 570 nm [8, 20].

2.5. Mitochondrial Reactive Oxygen Species (ROS) level

We used the DCFH-DA as a fluorescent probe to assess the mitochondrial ROS level. The mitochondria isolated from melanoma and normal cells were placed in the respiration buffer. The fluorescent probe at concentration of 10 μM was added to mitochondria isolated both groups and incubated for 10min at 37◦C. Eventually, mitochondrial fluorescence (DCF) as an indicator of the level of ROS generation was measured by Shimadzu RF-5000U fluorescence spectrophotometer (Ex: 488 nm and EM: 527 nm) [14].

2.6.Measurement of Mitochondria Membrane Potential (MMP)

Rhodamine 123 (Rh 123) at a concentration of 10 μM was used as a cationic fluorescent probe and was added to the mitochondrial suspensions isolated from both groups of MMP assay buffer and incubated for 10min at 37◦C. Eventually, mitochondrial fluorescence (Rh 123) as an indicator of the collapse of MMP was measured by Shimadzu RF-5000U fluorescence spectrophotometer (Ex: 490 nm and EM: 535 nm) [14].

2.7.Determination of Mitochondrial Swelling

The effect of methanolic extract of Holothuria (*H. parva* and *H. sabra*) (250, 500, 1000μg/mL) on mitochondrial swelling was measured at 5, 15, 30, 45, and 60 min time intervals using an ELISA reader (Tecan, Rainbow Thermo, Austria)at 540 nm. The decrease in absorption reflects mitochondrial swelling [21].

2.8.Cytochrome c release

The cytochrome c release was determined with a Quantikine Rat/Mouse Cytochrome c Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA).

2.9.Statistical Analysis

Results are presented as mean ± SD. The assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance was determined using the one-way ANOVA test followed by the post-hoc Tukey test in mitochondrial succinate dehydrogenase assay and cytochrome c release measurement. Statistical significance was set at $P<0.05$. And other mitochondrial dysfunction parameters including ROS formation, MMP, and mitochondrial swelling were analyzed by two way ANOVA and Bonferonie post test. In all graphs, the data were expressed as mean ± SD and $P<0.05$ was considered statistically significant. The flowcytometric data were analyzed by FlowMax.

3. Results

3.1. Holothuria (H. parva and H. sabra) extracts decreased SDH activity

As shown in Figure 1 (A, B), methanolic extracts of *H. parva* (0, 125, 250, 500, 1000 and1500 µg/mL) induced a considerable decline in the SDH activity in the skin mitochondria isolated from melanoma and mitochondria obtained from HCC (Figure 1 (C, D)). The methanolic extracts (125, 250, 500 and 1000 µg/mL) did not induce any significant changes in the SDH activity in normal cell groups (Figures 1B, D).

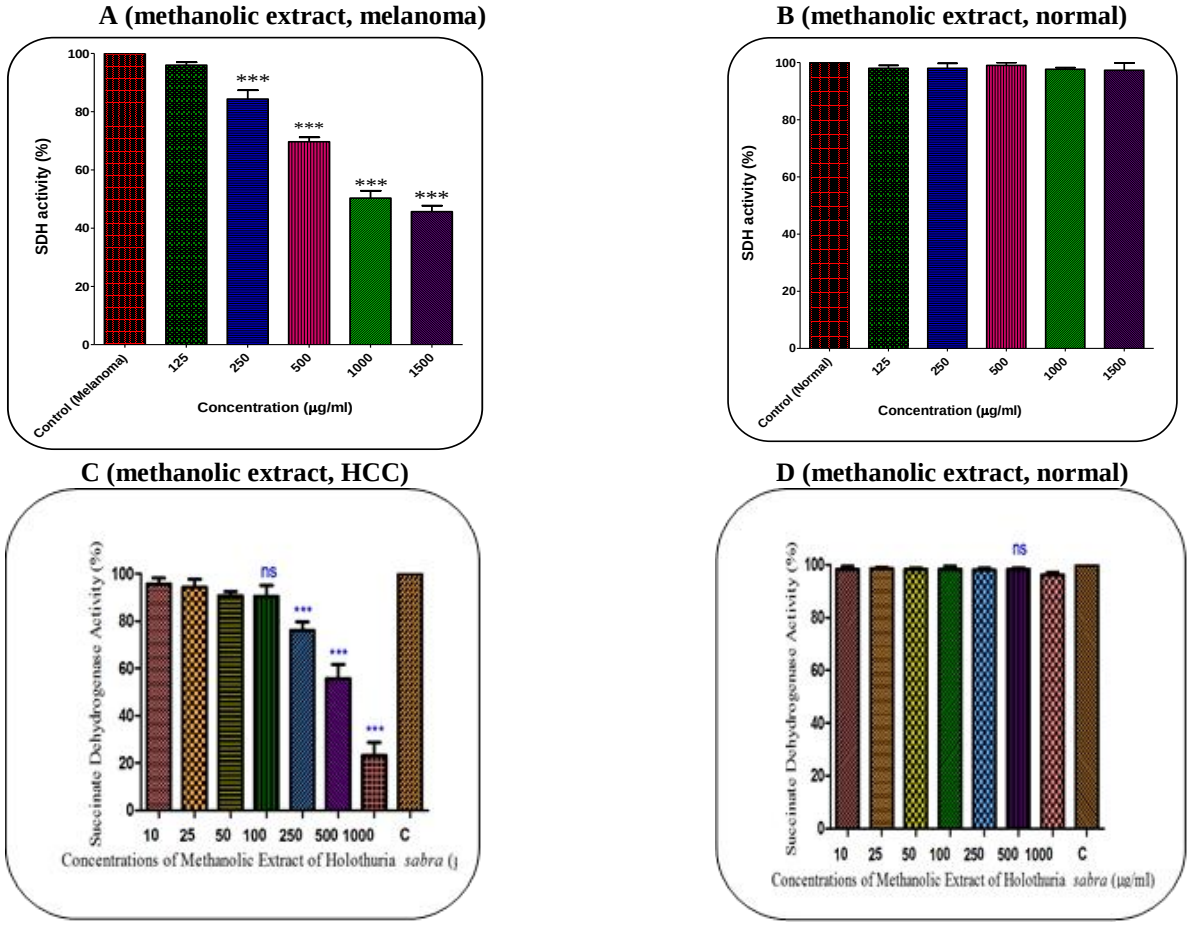
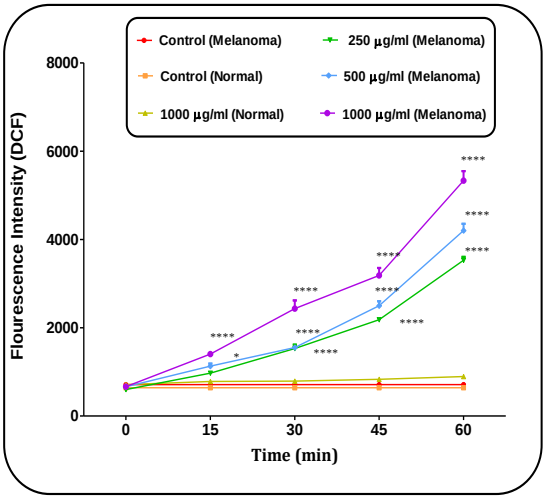


Figure 1: SDH activity. The effect of (A) methanolic extracts of the *H. parva* (0-1500 µg/mL) on SDH activity in the skin mitochondria obtained from melanoma group. (B) The Effect of methanolic extract (0-1500 µg/mL) of *H. parva* on skin mitochondria of the normal group. (C) the effect of methanolic extracts (0-1000 µg/mL) of the *H. sabra* (0-1000 µg/mL) on SDH activity in the HCC mitochondria. (D) The Effect of methanolic extracts (0-1000 µg/mL) of *H. sabra* on the SDH Activity of normal Hepatocytes. Values are presented as mean ± SD (n = 3). The one-way ANOVA test was performed. * and *** Significantly different from the corresponding control groups ($p<0.05$ and $p<0.001$, respectively).

3.2. Holothuria (H. parva and H. sabra) extracts increased Mitochondrial ROS level

Mitochondrial fluorescence (DCF) as an indicator of the level of ROS generation was measured. Methanolic extracts of *H. parva* at the concentrations of 250, 500, and 1000 μg/mL increased the level of ROS generation in a concentration and time dependent manner in the skin mitochondria isolated from melanoma cells group (Figure 2A). In the same results the methanolic extracts of *H. sabra* at the concentrations of 250, 500, and 1000 μg/mL increased the level of ROS generation in a concentration and time dependent manner in the mitochondria isolated from HCC group (Figure 2B). Our results did not show any significant changes in increasing level of ROS generation in these mitochondria isolated from the control groups (Figure 2A, B).

A



B

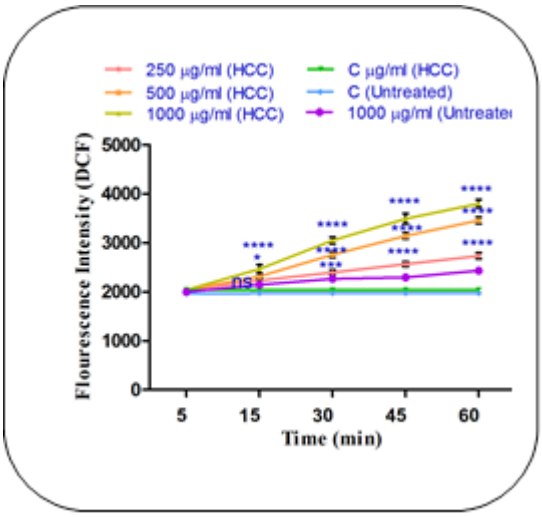


Figure 2: The mitochondrial ROS level. (A) The effect of methanolic extracts of the *H. parva* (250, 500 and 1000 µg/mL) on the level of mitochondria ROS generation in the skin mitochondria isolated from normal and melanoma cells groups. (B) The effect of methanolic extracts of the *H. sabra* (250, 500 and 1000 µg/mL) on the level of mitochondria ROS generation in the mitochondria isolated from HCC and normal cells groups. Values are presented as mean ± SD (n = 3). The two-way ANOVA test was performed. *, ***and **** significantly different from the untreated control melanoma group ($p<0.05$, $p<0.001$ and, $p<0.0001$, respectively).

3.3. Holothuria (H. parva and H. sabra) extracts declined MMP

Mitochondrial fluorescence (Rh 123) as an indicator of MMP collapse was measured. methanolic extract of *H. parva* at concentrations of 250 µg/mL (45 and 60 min), 500 µg/mL (30, 45 and 60 min), and 1000 µg/mL (15, 30, 45 and 60 min) induced collapse of MMP in the skin mitochondria isolated from melanoma cells group. (Figure 3A). Methanolic extracts of *H. parva* at (250, 500, and 1000 μg/mL) did not induce collapse of MMP in the skin mitochondria isolated from normal cells group (Figure 3A). In the same result methanolic extracts of *H. sabra* at the concentrations of 250, 500, and 1000 μg/mL increased collapse of MMP in the mitochondria isolated from the HCC group. (Figure 3 B). Our results did not show any significant changes in these mitochondria isolated from the control groups.

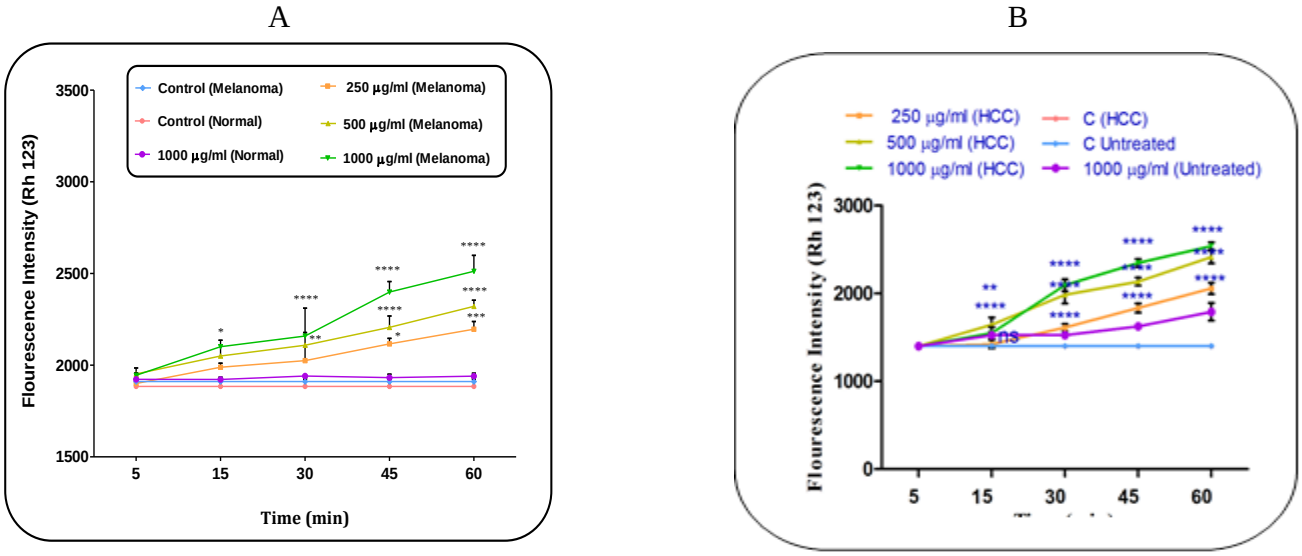


Figure 3: The MMP assay. (A) The effect of methanolic extracts of the *H. parva* (250, 500 and 1000 µg/mL) on the collapse of MMP in the skin mitochondria isolated from normal and melanoma cells group. (B) The effect of methanolic extracts of the *H. sabra* (250, 500 and 1000 µg/mL) on the collapse of MMP in the mitochondria isolated from HCC and normal cells groups. Values are presented as mean ± SD (n = 3). The two-way ANOVA test was performed. *, **, ***and **** Significantly different from the untreated control melanoma group ($p<0.05$,$p<0.01$,$p<0.001$ and, $p<0.0001$, respectively).

3.4. Holothuria (H. parva and H. sabra) extracts increased swelling in mitochondria

Mitochondrial swelling was evaluated as an indicator of the mitochondrial membrane permeability transition. In the skin mitochondria obtained from melanoma cell group, the results indicated that methanolic extracts (250, 500, and 1000 *µ*g/mL) of *H. parva* led to mitochondrial swelling (Figure 4A). In the same result in the HCC mitochondria, methanolic extracts (250, 500, and 1000 *µ*g/mL) of *H. sabra* led to an increase in mitochondrial swelling (Figure 4B). Moreover, methanolic extracts of *H. parva* and *H. sabra* at concentrations of 250, 500, and 1000 µg/mL did not induce any changes on the swelling in mitochondria in the normal groups (Figure 4A-B).

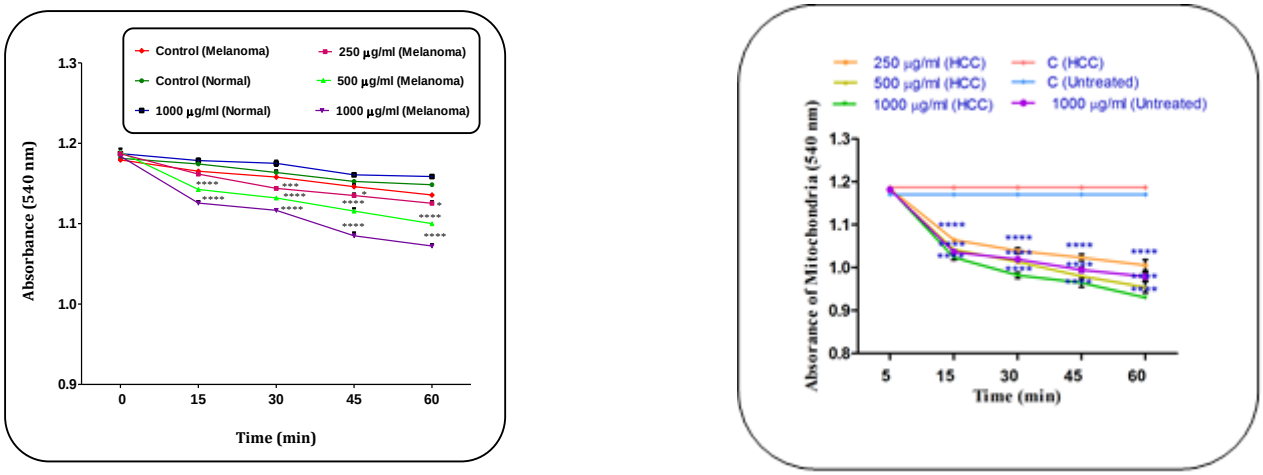


Figure 4: The mitochondrial swelling assay. The effect of (A) methanolic extracts of the *H. parva* (250, 500 and 1000 µg/mL) on the mitochondrial swelling in the skin mitochondria isolated from normal and melanoma cells group. (B) The effect of methanolic extracts of the *H. sabra* (250, 500 and 1000 µg/mL) on the mitochondrial swelling in the HCC and normal cells mitochondria. Values are presented as mean ± SD (n = 3). The two-way ANOVA test was performed. *and,***and **** Significantly different from the untreated control melanoma group ($p<0.05$,$p<0.001$ and<0.0001 , respectively).

3.5. Holothuria (H. parva and H. sabra) extracts induced cytochrome c release

Both extracts of *H. parva* and *H. sabra* at IC50 (500 µg/mL) concentrations induced the release of cytochrome c only from melanoma and HCC mitochondria into the buffer media.

Furthermore, pre-treatment of cancerous mitochondria exposed to extracts of *H. parva and H. sabra* at IC50 concentration with cyclosporine A (Cs.A) as a MPT inhibitorand butylatedhydroxyl toluene (BHT) as an antioxidant, prevented the cytochrome c release ($P < 0.05$).

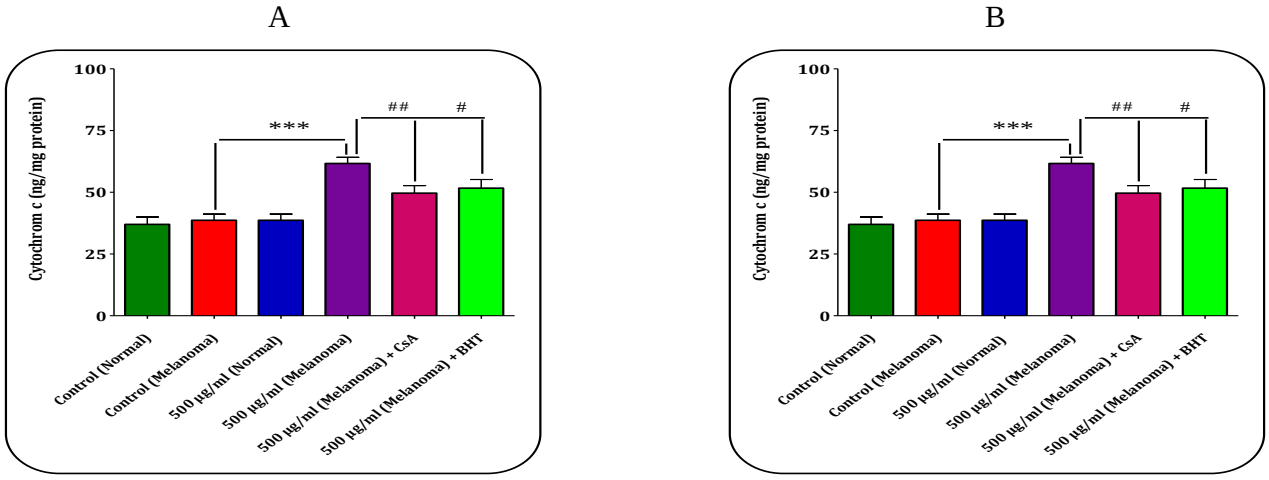


Figure 5: Cytochrome c expulsion assay. Cytochrome c expulsion was determined by using corresponding rat/mouse cytochrome c ELISA kit. The cytochrome c expulsion is increased after addition of (A) methanolic extracts of the *H. parva* (500µg/mL) on the mitochondria obtained from the melanoma induced skin. (B methanolic extracts of the *H. sabra* (500µg/mL) on the mitochondria obtained from the HCC. Values are presented as mean ± SD (n = 3). The one-way ANOVA test was performed. *** Significantly different from the untreated melanoma control mitochondria, ($p<0.001$). ##and### Significant difference in comparison with *H. parva* extract (500µg/mL) - treated melanoma mitochondria, $p<0.01$ and $p<0.001$respectively.

4. Discussion and Conclusion

Melanoma is one of the most common cancers throughout the world, more aggressive than other skin cancers and very deadly. The rise of HCC rates is also another considerable globally problem coupled with inefficient methods for HCC treatment [23].

Currently, cancer treatments are controversial [22, 23]. Several studies have shown that suppression of apoptosis is one of the reasons for the failure of cancer treatment [4-7]. During the progression of cancer, molecular alterations, such as activation of anti-apoptotic agents, inactivation of pro-apoptotic effectors, and rein for cement of survival signals are key factors that contribute to the failure of cell death control [7]. Thus, identifying novel anticancer compounds capable of inducing apoptosis are key elements in the treatment of this disease.

Studies have shown that bioactive compounds isolated from certain marine species are effective in the treatment of cancer [24, 25]. In recent years, many studies have investigated sea cucumbers given their pharmacological properties, particularly their anticancer properties. These properties are attributed to their bioactive compounds (triterpenoid glycosides, sulfated polysaccharides, sterols, etc.). Sea cucumbers are attractive due to their natural source, long history of nutrition value, and negligible toxic effects. Induction of apoptosis is one of the key anticancer mechanisms of these compounds [13, 22].

This study was designed to evaluate the selective toxicity effect of three extracts of *H. parva* on skin mitochondria isolated from atypical animal model of melanoma and three extracts of *H. sabra* on mitochondria isolated from HCC isolates from rats.

Our results show that different concentrations of *H. sabra* (250, 500 and 1000µg/ mL) significantly ($p<0.05$) decrease SDH activity in the HCC mitochondria in comparison to control rat mitochondria.

The level of ROS formation caused by the generation of H_2O_2 formation as mitochondria parameter toxicity was evaluated. In this study, three extracts of *H. sabra* at different time and concentration significantly ($p<0.05$) promoted ROS formation in the mitochondria of the HCC group compared to the control group in a time and concentration dependent manner.

The above concentrations of *H. sabra* significantly induced the collapse of MMP ($\Delta\Psi m$) in the mitochondria isolated from the HCC group in comparison to the control group. The collapse of MMP plays a vital role in subsequent processes especially in apoptosis. Mitochondrial membrane damage can cause MPT pore-opening, release of cytochrome c into the cytosol, and finally apoptosis and necrosis can be acquired [36].

In addition, Mitochondrial swelling as another indicator of MPT are significantly elevated by the given concentrations of *H. sabra* in the mitochondria obtained from HCC in comparison with the control group.

Another important result was that the mentioned extract (500µg/mL) of *H. sabra* significantly induced the release of cytochrome c only from the HCC but not from the control mitochondria.

A defected apoptosis pathway plays a critical role in most malignant tumors, and can be efficient for cancer therapies. Research has shown that apoptosis induced from marine species are due to the presence of bioactive compounds, which are considered as new anti-cancer drug [37].

Reports indicate that there are many differences (size, number, shape, genomic, and structural) between the mitochondria of normal and cancer cells [8, 9, 14]. Due to these differences, the mitochondria has been considered for many researchers as a cancer therapeutic target [8, 9, 14].

Exposing skin mitochondria isolates from melanoma cells by *H. parva* at all applied concentrations displayed a dose and time dependent inhibitory effect on SDH activity. Our result is also in agreement with our previous studies [14, 17].

Our results indicated that ROS generation increases only in the cancerous mitochondria after addition of *H. parva*. In comparison with normal and HCC cells, the mellanoma cells are more vulnerable against free radicals and subsequently oxidative stress. Several studies have shown that ROS plays an important role in the induction of apoptosis (through mitochondrial dependent pathway).

The MMP decline as one of the main triggers of the cell death (apoptosis and necrosis shows the MMP collapses only in cancerous mitochondria after addition of *H. parva*). This result is more considerable in melanoma in comparison with HCC.

Mitochondrial swelling and cytochrome c expulsion, as subsequent events after mitochondrial permeability transition (PT) were also determined. This study shows the involvements of MPT pore opening and mitochondrial swelling in cytochrome c release from only the cancerous mitochondria exposed to *H. parva*. The CsA (MPT inhibitor) significantly inhibited the cytochrome c release induced by *H. parva*. Studies have shown that release of cytochrome c from mitochondria to the cytosol is a vital event in the apoptotic process [30, 31].

In conclusion, we propose that the effect of methanolic extract of *H. parva* on skin mitochondria isolated from normal and melanoma cells and the effects of all extracts of *H. sabra* on mitochondria isolated from normal and hepatocyte cells may be attributed to the presence of numerous arrays of bioactive compounds, such as phenolic, saponins, sterols, cerebrosides, and sulfated polysaccharides. This study demonstrates that mitochondrial targeting is the vital mechanism by which the Persian Gulf sea cucumber (*H. parva* and *H. sabra*) extracts could potentially and selectively induce apoptosis pathway in skin mitochondria isolated from melanoma cells more than hepatocyte cells. Hence, this study provides evidence that sea cucumbers should be strongly

considered as a potentially new anti-cancer drug candidate.

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