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Selective Toxicity of Standardized Extracts of Persian Gulf Sponge (Irciniamutans) on Skin Cells and Mitochondria isolated from Melanoma induced mouse

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ABSTRACT:
Melanoma is an aggressive and highly lethal cancer with poor prognosis and resistance to current treatments. Apoptosis signaling is believed to be suppressed in melanoma. Evidence suggests that compounds isolated from marine sponges have anti-cancer properties. This study was designed to evaluate the apoptotic effect of methanolic, diethyl ether, and n-hexane extracts of Irciniamutans (I.mutans) on skin mitochondria isolated from mice animal models of melanoma. Mitochondria were isolated by differential centrifugation. According to our results, methanolic, diethyl ether, and n-hexane extracts of I.mutans raised the reactive oxygen species (ROS) level only in the cancerous skin mitochondria group. Furthermore, methanolic, diethyl ether, and n-hexane extracts induced swelling in the mitochondria, decreased the mitochondrial membrane potential (MMP) and the release of cytochrome c from the mitochondria. Based on these results, the potentially bioactive compounds found in I.mutans render the sea sponge as a strong candidate for further researches in molecular identification and confirmatory in-vivo researches.

Keywords: Irciniamutans; Melanoma; Apoptosis; Mitochondria; Reactive Oxygen Species.

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1. Introduction

Melanoma is as a solid malignant tumor originated from melanocytic cells; it is considered as one of the most important aggressive and malignant skin cancers [1-4]. To date, the incidence of melanoma is rising, especially within the Caucasian population. Studies have demonstrated that incident rates in Europe, America, and Australia are rising sharply. According to the World Health Organization (WHO), nearly 132,000 new cases are detected each year worldwide [5-8]. Melanoma is the fourth most common cancer in men and the sixth in women [9, 10]. Furthermore, melanoma prognosis is very poor. [2, 11].

In recent years, despite the availability of treatment methods and strategies, such as chemotherapy,

radiotherapy, surgery and immunotherapy, the average survival rate is very low [10]. Various studies have shown that melanoma treatment is a major challenge for oncology researchers [11, 12]. Resistance to chemotherapyis one of the main reasons of melanoma treatments failures [2, 5, 6, 10]. It is well documented that a number of chemotherapy drugs via the intrinsic or mitochondrial pathway induce apoptosis signaling in the tumor cells. Studies have shown that, cells in melanoma lesions show an inherently low level of spontaneous cell death, and resistance to apoptosis through activation of proliferation and survival pathways. Therefore, the activation of apoptotic programs in melanoma cells provide a potential key requisite to overcome drug resistance and ameliorate clinical outcomes [2, 10, 13, 14].

Most anticancer medicines have a natural origin. Currently, natural compounds or lead compounds from various sources such as, marine organisms, plants, and microorganisms are used in clinic for the treatment of many chronic diseases, including cancer. Many of these natural compounds are capable of inducing apoptosis in melanoma cells [9, 15]. Marine sponges are rich sources of anti-cancer compounds. These natural compounds have different pharmacological activities [16]. Anticancer treatments (such as, alkylating agents, ionizing radiation, platinum compounds and DNA topoisomerase inhibitor) are used as targeted therapies (such as, apoptosis-inducers) to identify specific targets for tumor cells to increase treatment efficacy [17].

Our previous studies demonstrate that marine organisms (such as sea sponges, sea cucumbers and sea squirts) have cytotoxic effects on cancer cells (including, hepatocellular carcinoma, leukemia and melanoma) [18-21]. Research has shown that certain changes in mitochondria occur during melanoma, and these changes consequently affect the treatment [22, 23]. In this study, we investigate the cytotoxicity effect of (I.mutans) (sea sponge found in the Persian Gulf) extracts on skin mitochondria isolated from a typical animal model of melanoma.

2. Materials & Methods

Sampling and extraction of Sponge Samples:

Sponge samples (I.mutans) were collected from the Bandar-e-lengehcoast, south of Iran. The (I.mutans) samples were transported to the laboratory via iced boxes. The samples were washed with cold water, weighed, and evaluated. Bioactive agents were extracted using the method described by Mamelona et al., [18]. The (I.mutans) 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,the powdered samples were kept in n-hexane about 24 hours to produce non-polar and polar extractions, then the solution was filtered and n-hexane was evaporated to dryness, at low pressure at 35- 40ºC by using Rota-vapors. For diethyl ether extract the sample was put in diethyl ether about 48 hours, the solution was filtered and diethyl ether was evaporated to dryness, at low pressure at 35- 40ºC by using Rota-vapors. Finally the sample wassoaked in methanol for 72 hours, after filter and dryness the polar extrac was produced. Finally, the powdered extracts of (I.mutans) werestored at -20 ºC.

Standardization of extracts by GC-MS analysis:

The n-hexane, diethyl ether and methanolic extracts of the sea cucumber (I.mutans) were analyzed by GC-MS (Agilent7000 series Triple Quad GC/MS Main Frame). The GC column dimension was: 30 mm, 0.25 mm, 0.5 mm AB-35MS fused silica capillary column. The GC conditions were as follows: injector temperature 250°C column temp isothermal at 100°C then programmed to rise up to 250°C at 6°C/min and held at this temperature for 10 minutes. The ion source temperature was 200°C and the interface temperature was 250°C. Helium gas was engaged for carrier gas at the rate of 1ml/min. Spectra was obtained in the EI mode with 70eV ionization energy. The compounds were identified by comparison with the standards. GC-MS Analysis of the n-hexane, diethyl ether, and methanolic extracts of (I.mutans) showed the fallowing information (table 1).

Animals

The animal model for melanoma was acquired from Pasteur Institute. All experimentations were conducted according to the ethical standards and protocols approved by the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Isolation of Mitochondria From mouse melanoma cells

The preparation of isolated mouse skin cells was completed using the two-step collagenase skin perfusion technique. To assess cellular integrity (or viability), the trypan blue exclusion test was performed [24, 25]. Mitochondria were prepared from cancerous cells in accordance with our previous published work.

Determination of succinate dehydrogenase (SDH) activity

The SDH activity was measured using a MTT dye. At first, 100 µl of mitochondrial suspension from both groups were incubated with various concentrations (125, 250, 500, 1000, and 1500 µg/ml) of aqueous, methanol, diethyl ether, and n-hexane extracts of I.mutansat 37°C for 1 hour. Furthermore, 0.4% of MTT (MTT dye plus succinate dehydrogenase) was added to the medium and then incubated at 37°C for 30 minutes. Finally, the product of formazancrystals was dissolved in 100 µl DMSO and the absorbance (λ=570 nm) was measured using an ELISA reader (Tecan, Rainbow Thermo, Austria) [18, 19].

Determination of ROS formation

The ROS generation was done using dichlorodihydro-fluoresceindiacetate (DCFH-DA) as a fluorescent probe. Isolated mitochondria from both groupswere placed in a respiration buffer. Subsequently, DCFH-DA at a concentration of 10 µM was added to the mitochondrial suspension. Finally, the fluorescence intensity of dichlorofluorescein (DCF) as a ROS generation was assayed using the Shimadzu RF-5000 U fluorescence spectrophotometer (λ Ex = 488 nm, and λ Em= 527 nm) [18, 19].

Determination of MMP collapse

Rhodamine 123, Rh 123, at a final concentration of 10 µM was added to the mitochondrial suspension from both groups in the MMP assay buffer. The cytosolic Rh 123 fluorescence intensity as a MMP collapse was determined using the Shimadzu RF-5000U fluorescence spectrophotometer (Ex = 490 nm, and λ Em= 535nm) [18, 19].

Mitochondrial Swelling assay

Isolated mitochondria from both groups were suspended in swelling buffer and incubated at 30°C with several concentrations (250, 500, and 1000 µg/ml) of three extracts (methanol, diethyl ether, and n-hexane) of I.mutans. The absorbance was then measured at 549 nm using an ELISA reader (Tecan, Rainbow Thermo, Austria). It should be noted that the decrease in absorbance is indicative of swelling in the mitochondria [18, 19].

Cytochrome c release:

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

Apoptosis assay:

Melanoma cells exposed to 500 µg/ml (IC50) concentration ofmethanolic,diethyl etherand n-hexane extracts of I.mutans for 6h.following the exposure, we Collected 1–5 x 105 cells by centrifugation and re-suspended cells in 500 µL of 1X binding buffer, then Added 5 µL of annexin V-FITC and 5 µL of propidium iodide (PI, optional), Incubated at room temperature for 5 min in the dark. Finally, the cells were analyzed with flowcytometry (BD) Equipped with a 605nm argon ion lase, r and supplied with the flowing software1.2.5 and the signals were obtained using a 530nm band pass filter (FL-3 channel). Each determination is based on the mean fluorescence intensity of 10,000 counts.

Table 1.GC–MS analysis of the n-hexane, diethyl ether and methanolic extracts of I.mutans

No	Name of the compound	Molecular formula	Chemical Structure	MW	%
1	Colecalciferol	C27H44O	Vitamin D3	384.64	23.98
2	Digitoxin	C41H64O13	phytosteroid	764.939	21.36
3	Eicosane	C20H42	alkane	10.49	
Diethyl ether extract					
1	oleyl alcohol	C18H36O unsaturated fatty alcohol		268.478	9.22
2	Phenol 2,4-bis (1,1- imethylethyl)	C14H22O alkylatedphenol		206.324	1.55
3	phthalate	C6H4(C8H17COO)2	esters of phthalic acid	390.56	1.93
4	Ricinoleic acid	C18H34O3	fatty acid	298.461	20.75

Statistical Analysis

The results are presented as mean±SD. The sssays 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 posthoc-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 Bonferoniepost 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.

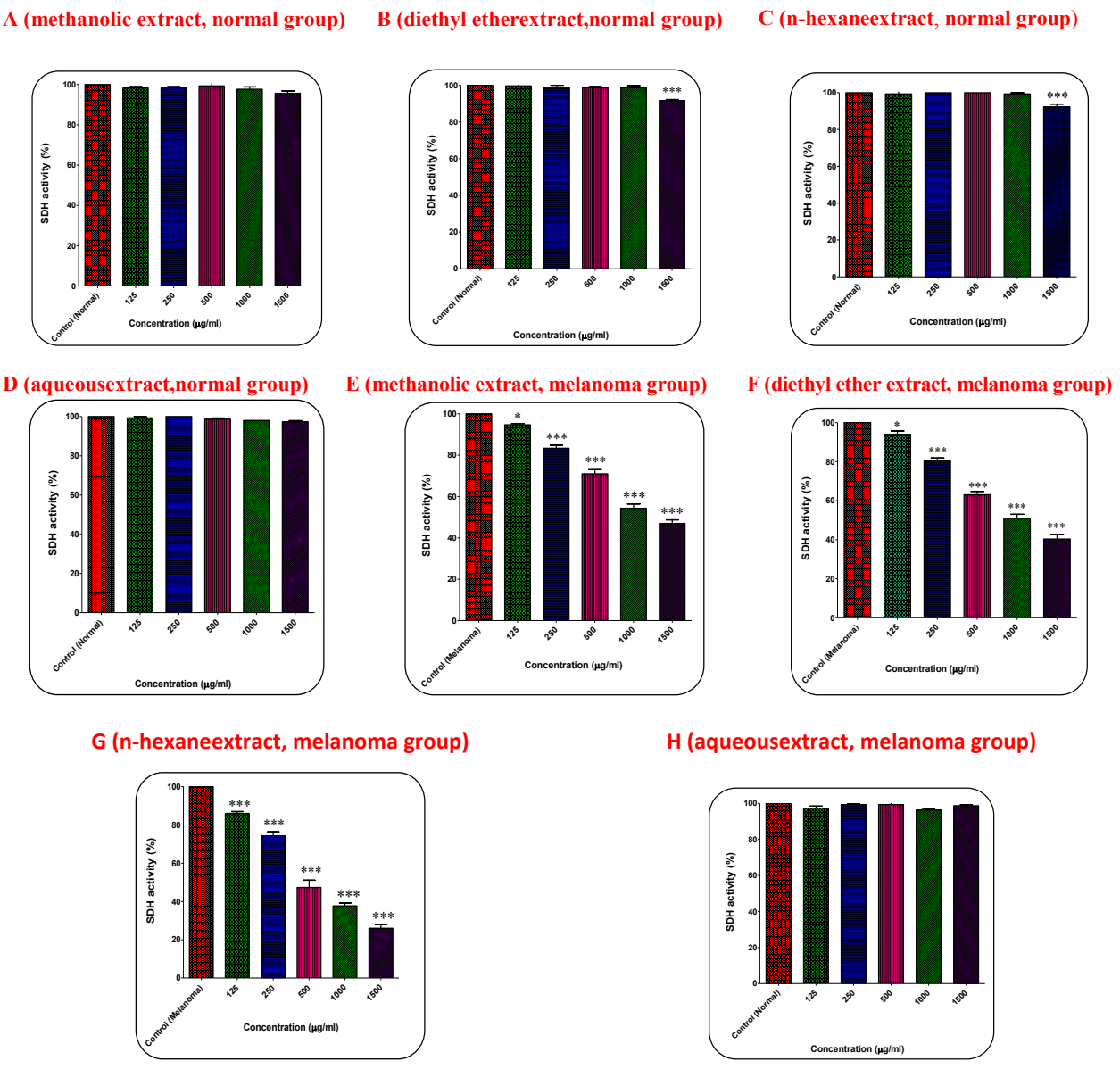


Figure 1. The effect of *I.mutans*extracts on SDH activity. (A) The effect of methanolic (B), diethyl ether, (C) n-hexane and (D) aqueous extracts of*I.mutans*on SDH activity on the normal group. The effect of methanolic (E), diethyl ether, (F) n-hexane and (G) aqueous extracts (H) of *I.mutans*on SDH activity on the cancerous group. Values are reported as mean ± SD (n = 3). *and***aresignificantly different compared to corresponding control group ($p<0.05$ and $p<0.001$).

3. Results and discussion

Effect of I.mutans extracts on mitochondrial SDH activity:

As shown in Figure 1E-G, our results indicate that different concentrations (125, 250, 500, 1000 and 1500 µg/ml) of methanolic extract (containing polar and non-polar compounds) and n-hexane and diethyl ether extracts (containing non-polar compounds) significantly ($P<0.05$) decrease SDH activity only in the cancerous group. On the other hand, only diethyl ether and n-hexane extracts at the highest concentration (1500 µg/ml) could affect SDH activity in the normal group (Figure 1B and C). Aqueous extracts (containing polar compounds) at all mentioned concentrations did not significantly affect SDH activity in both groups (Figure 1-D and H).

I.mutans extracts induced ROS generation:

Our results indicate that all the applied concentrations (250,500 and 1000µg/ml) of methanolic, diethyl ether and n-hexane extracts of I.mutansinduced significant ROS generation in a time and concentration dependent manner only in the skin mitochondria isolated from the cancerous group (Figure 2A-C). It should be noted that the increase in fluorescent intensity (DCF) is indicative of ROS generation in the mitochondria.

I.mutans declined Mitochondria Membrane Potential (MMP):

In this study, MMP was assayed using Rh 123.Three extracts of I.mutans (250, 500, and 1000 µg/ml) significantly induced the collapse of MMP in a time and concentration dependent manner in the skin mitochondria isolated from the cancerous group (Figure 3A-C).

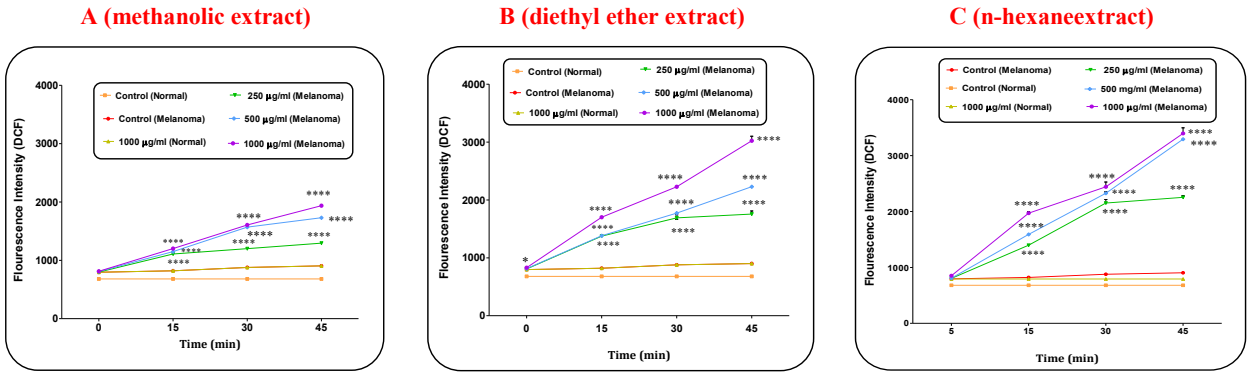


Figure 2. The Effect of *I.mutans*extracts on ROS level. (A) The effect of methanolic (B), diethyl ether and (C) n-hexane extracts of the *I.mutans*with various concentrations (250, 500 and 1000 µg/ml) on mitochondrial ROS formation. Values are reported as mean ± SD (n = 3). **** is significantly different compared to corresponding control group ($p<0.0001$).

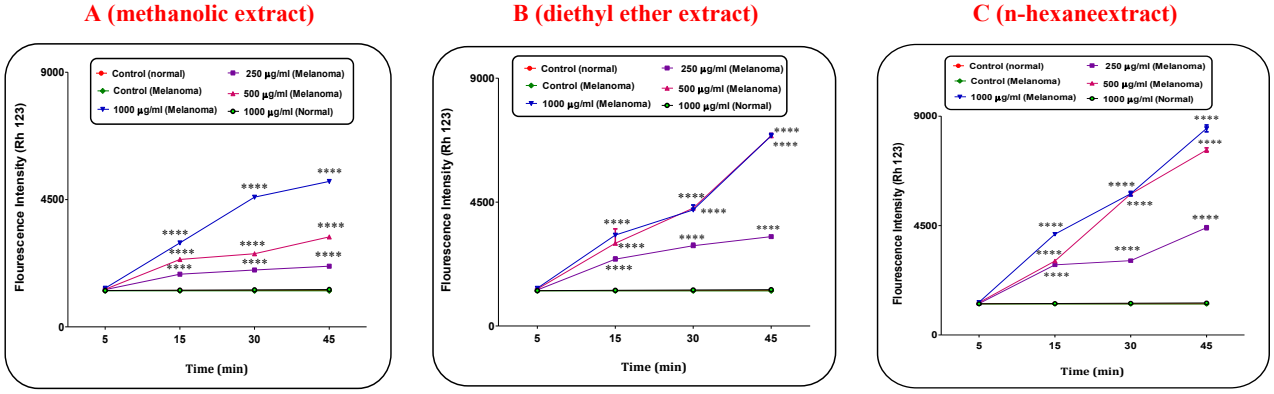


Figure 3. The Effect of *I.mutans*extracts on the MMP collapse. (A) The effect of methanolic (B), diethyl ether and (C) n-hexane extracts of the *I.mutans*with various concentrations (250, 500 and 1000 µg/ml) on MMP collapse. Values are reported as mean ± SD (n = 3). **** Significantly different compared to corresponding control group ($p<0.0001$).

I.mutans increased mitochondrial swelling:

Mitochondrial swelling as an indicator of the mitochondrial permeability transition (MPT) was assayed. As shown in Figure 4A-C, we showed that the addition of various concentration extracts (250, 500 and 1000 µg/ml) of I.mutanssignificantly increased mitochondrial swelling only in the skin of mitochondria isolated from the cancerous group.

I.mutans increased cytochrome c release:

Our results show that I.mutansat concentrations of 500 µg/ml induced a significant ($P<0.05$) release of cytochrome c only in the cancerous group (Figure 5A-C). Furthermore, we show that pretreatment of methanolic, diethyl ether, and n-hexane extracts (500 µg/ml)-treated cancerous mitochondria by cyclosporine A (CsA) and butylated hydroxyl toluene (BHT) significantly decreased cytochrome c release.

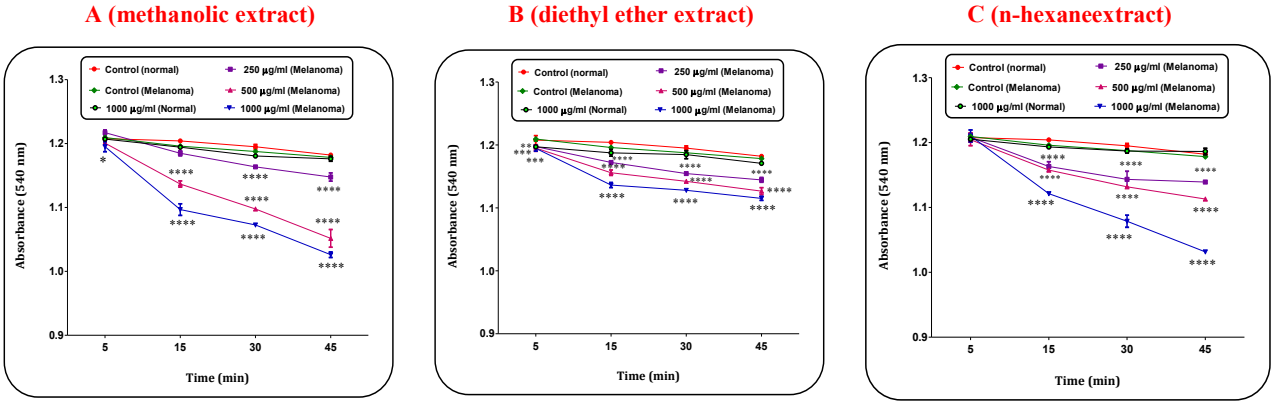


Figure 4. The Effect of *I.mutans*extracts on Mitochondrial Swelling. (A) The effect of methanolic (B), diethyl ether and (C) n-hexane extracts of *I.mutans*with various concentrations (250, 500, and 1000 µg/ml) on mitochondria swelling. Values are reported as mean ± SD (n = 3). *, **, *** and **** are significantly different from the corresponding control group ($p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$, respectively).

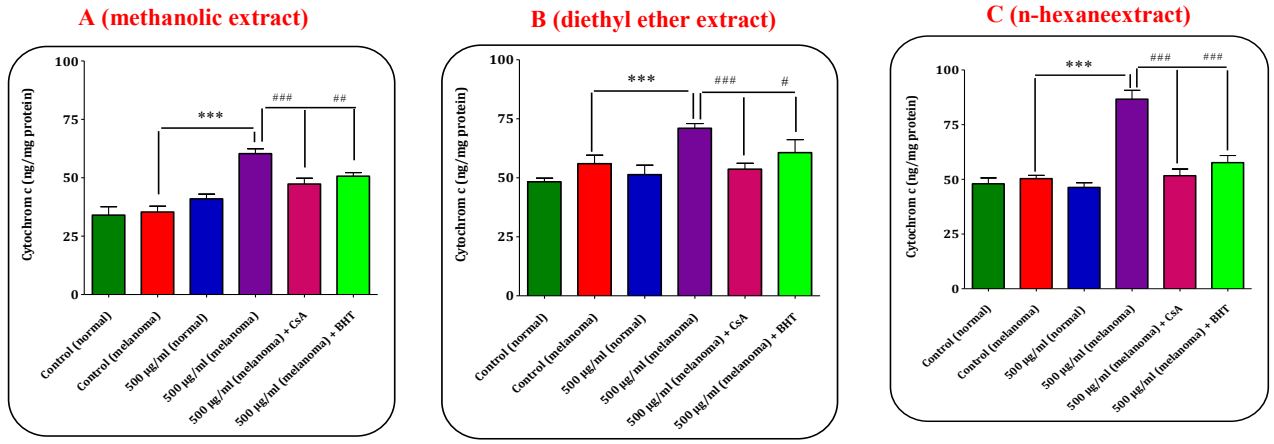


Figure 5 . Determination of cytochrome c release Cytochrome c release was determined by using corresponding rat/mouse cytochrome c ELISA kit. Cytochrome c expulsion increases after addition of (A) methanolic (B), diethyl ether and (C) n-hexane extracts of *I.mutans*(500 µg/ml) on the mitochondria obtained from the skin. Values are reported as mean ± SD (n = 3). The one-way ANOVA test was performed. *** is significantly different from the corresponding control ($p<0.001$). $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ are Significant difference in comparison with three extracts of *I.mutans*(500µg/ml) -treated mitochondria ($p<0.05$ $p<0.01$ and $p<0.001$, respectively).

I.mutans induced apoptosis signaling

To determine if apoptosis is involved in the cytotoxic effects of n-hexane, diethyl ether and methanolic extracts of I.mutans, apoptotic cells were determined by annexin V/PI staining of DNA fragments by flowcytometry. The cells were exposed to 500 µg/ml (IC50) concentration of non-polar (n-hexane and diethyl ether) and both polar and non-polar (methanolic) extracts of I.mutans for 6h. The order of efficacy of I.mutans extracts at inducing apoptosis was: n-hexane extract > diethyl ether extract > methanolic extract in the melanoma cell group (Figure 6A-C). Methanolic, diethyl ether and n-hexane extracts of I.mutansat concentrations of 500 µg/ml did not induce apoptosis in the skin cell isolated from the normal group (Figure 6D-F).

4. Discussion and Conclusion

Melanoma is one of the most invasive skin cancers with a well-established resistance to common treatments. Furthermore, the incidence of this cancer has increased sharply over the pastdecades [10, 15]. Previous studies have shown that the inhibition of apoptosis signaling (both within the intrinsic and extrinsic pathway) is a key mechanism of resistance towards treatments [2, 13]. Therefore, the identification of novel anticancer compounds capable of inducing apoptosisis vital in the treatment of melanoma. This study was designed to evaluate the selective toxicity and apoptotic effect of extracts obtained from I.mutanson skin mitochondria isolated from a typical animal model of melanoma.

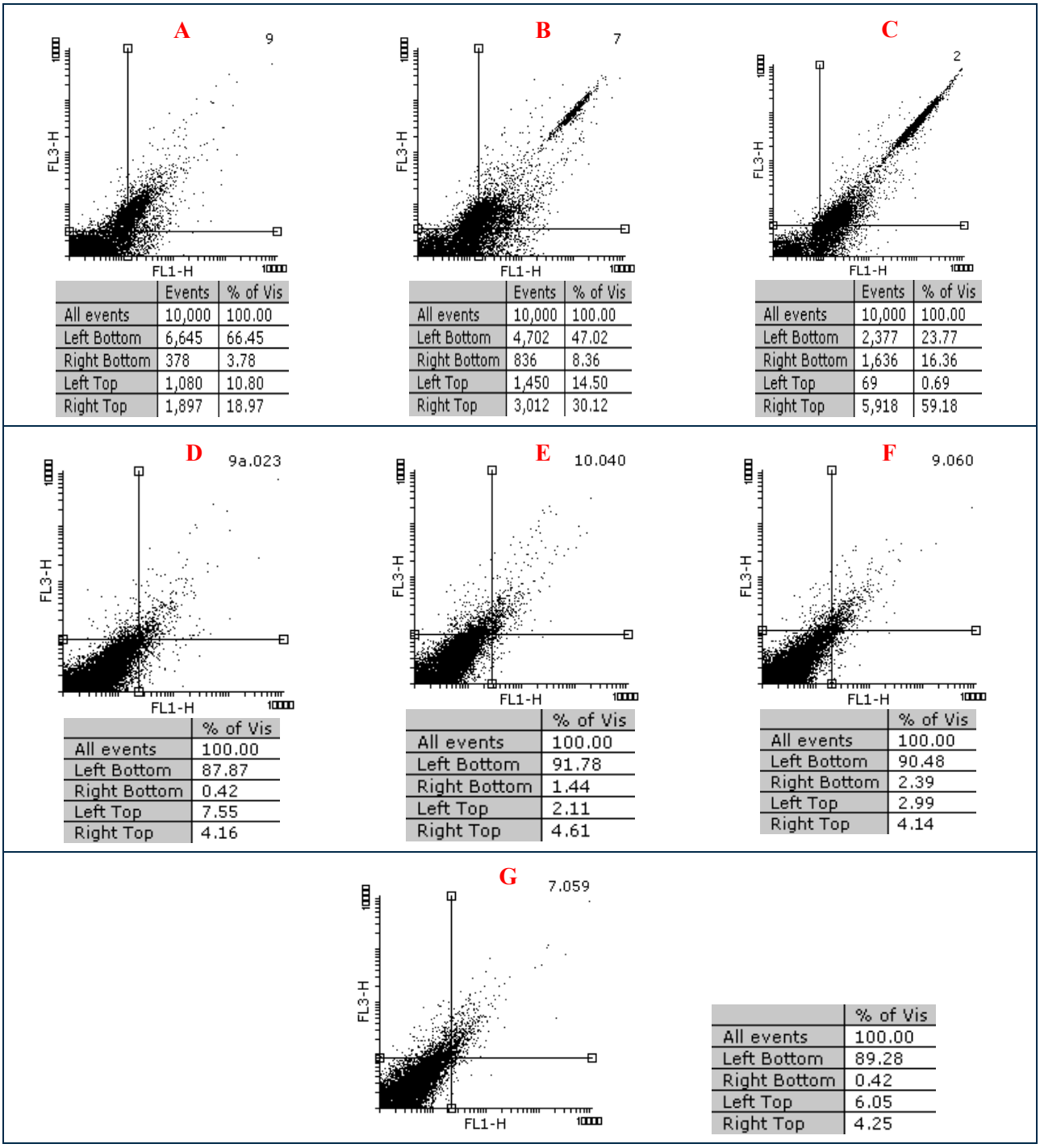
Marine plants and animals have various promising pharmacological properties and have therefore, attracted the attention of researchers. The anticancer activity of marine organisms is one of its most important properties [26]. Additionally, the bioactive compounds isolated from marine animals are effective in the treatment of various cancers [27, 28]. It has been shown that several cytotoxic compounds isolated from marine sponges, exert their toxic effects via inducing apoptosis signaling [29]. At first, our results show that all extracts, except aqueous extracts, in a time-dependent manner have been able to reduce SDH (mitochondrial complex II) activity. Our result is also in agreement with our previous studies [18, 19, 21]. These results suggest that the active compounds fromI. mutansthat potentially have cytotoxic effects do not have a polar nature.

One of the characteristics of cancer cells is their escape from apoptosis. It has been shown that this phenomenon is strongly associated with the mitochondria. During the process of apoptosis, the changes in the mitochondria membrane, cytochrome c explosion from mitochondria into the cytosol and subsequent activation of caspases (the executors of programmed apoptosis) occur [30]. Several differences exist in the shape, size, genome, and function of mitochondria in healthy versus cancerous cells. Due to these differences, researchers are actively exploring the mitochondria for potential cancer treatments [18, 21].

Our results demonstrate thatmethanolic, diethyl ether, and n-hexane extracts of I.mutansincrease ROS level only in the skin mitochondria obtained from cancerous groups. Our resultsare also in agreement with our previous study [18]. Evidences suggest that numerous marine-derived extracts and compounds (including compounds derived from marine sponges) are able to increase ROS generation and subsequently induce apoptosis on different cancer cells [31]. Furthermore, cancer cells are more vulnerable to ROS than normal cells, and this phenomenon can be used to kill cancer cells [18, 32].

In the following, we evaluated the MMP changes. Our results indicated collapse of the MMP only on cancerous mitochondria after addition of all applied concentration of methanolic, diethyl ether, and n-hexane extracts of I.mutans. Also, the changes in MMP and its collapse is an important factor in the induction of apoptosis. After the collapse of MMP, swelling in the mitochondria and release of cytochrome c from mitochondria through the all three extracts was also observed. These events are indicative of the onset of apoptosis signaling [33, 34]. Also, our results indicated that cytochrome c release by all three extracts was inhibited by cyclosporine A and BHT.

Finally, we suggest that the effect of methanolic, diethyl ether, and n-hexane extracts of I.mutans on skin mitochondria isolated from normal and melanoma may be attributed to the presence of numerous arrays of bioactive compounds, such as alkaloids, steroids, terpenes, pep¬tides, macrolides, and polyketides. This study provesthe evidence that mitochondrial targeting is the critical mechanism by which Persian Gulf sponge (I.mutans) extracts could potentially and selectively induce apoptosis in skin mitochondria isolated from melanoma cells.



	Events	% of Vis
All events	10,000	100.00
Left Bottom	6,645	66.45
Right Bottom	378	3.78
Left Top	1,080	10.80
Right Top	1,897	18.97

	Events	% of Vis
All events	10,000	100.00
Left Bottom	4,702	47.02
Right Bottom	836	8.36
Left Top	1,450	14.50
Right Top	3,012	30.12

	Events	% of Vis
All events	10,000	100.00
Left Bottom	2,377	23.77
Right Bottom	1,636	16.36
Left Top	69	0.69
Right Top	5,918	59.18

	% of Vis
All events	100.00
Left Bottom	87.87
Right Bottom	0.42
Left Top	7.55
Right Top	4.16

	% of Vis
All events	100.00
Left Bottom	91.78
Right Bottom	1.44
Left Top	2.11
Right Top	4.61

	% of Vis
All events	100.00
Left Bottom	90.48
Right Bottom	2.39
Left Top	2.99
Right Top	4.14

	% of Vis
All events	100.00
Left Bottom	89.28
Right Bottom	0.42
Left Top	6.05
Right Top	4.25

Figure 6. Apoptosis assay. Melanoma cells were incubated with methanolic, diethyl ether, and n-hexane extracts of the *I.mutans* (IC50:500 mg/ml) for 6 h. (A) Melanoma cells plus methanolic extract (500 mg/ml) (6 h); (B) melanoma cells plus diethyl ether extract (500 mg/ml) (6 h); (C) melanoma cells plus n-hexane extract (500 mg/ml) (6 h); (D) normal skin cells plus methanolic extract (500 mg/ml) (6 h); (E) normal skin cells plus diethyl ether extract (500 mg/ml) (6 h); (F) normal skin cells plus n-hexane extract (500 mg/ml) (6 h); (G) Untreated melanoma control (6 h). Explanation for flowcytometry reports; left bottom: %Live cells, right bottom: %Early apoptotic cells, right top: % late apoptotic cells, left top: % necrotic cells.

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Conflict of interest

The authors have no conflict of interest to declare.

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