RESEARCH ARTICLE

Selective Toxicity of Non Polar Bioactive Compounds of Persian Gulf Sea Squirt *Phallusia Nigra* on Skin Mitochondria Isolated from Rat Model of Melanoma

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Abstract

Background: Skin cancer is the most prevalent cancer and one of the major causes of mortality worldwide. Marin animals have attracted much attention in recent years as useful substances having application in medicine. It was shown that Phallusia nigra (P. nigra) known as sea squirt could play an important role in cancer therapy. **Methods:** This study was designed to figure out the probable selective toxicity of n-hexane, diethyl ether, methanolic and aqueous extracts of P. nigra on cancerous mitochondria isolated from the skin of melanoma induced rats. In our study, mitochondria were isolated from the skin tissue of both melanoma induced and normal healthyrats. Different concentrations of four different extracts of P. nigra (250, 500 and 1000 µg/ml) were added to mitochondrial samples obtained from both groups, separately. **Results:** Our results showed that n-hexane, diethyl ether and methanolic extracts (but not aqueous extract) of P. nigra in all concentrations applied (250, 500 and 1000 µg/ml) significantly induced toxic alterations only in the cancerous but not normal healthy skin mitochondria including; increased reactive oxygen species (ROS) formation, mitochondrial swelling, decreased mitochondrial membrane potential (MMP) and cytochrome c release. Flow-cytometry analysis demonstrated that n-hexane, diethyl ether and methanolic extracts of P. nigra progressively induced apoptosis and necrosis only on melanoma cells but not healthy skin cells. **Conclusions:** Our results suggest that non polar bioactive compounds in P. nigra may be hopeful candidates for further studies including molecular identification, confirmatory in vivo experiments and finally clinical trials designed for new drug treatment of melanoma skin cancer.

Keywords: *Phullusia Nigra*- Melanoma-Mitochondria- ROS

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Introduction

Skin cancer is the most prevalent cancer and one of the major causes of mortality worldwide (Wang et al., 2012, Chinembiri et al., 2014). In recent decades, the incidence of skin cancer has increased. According to Canadian cancer research, between the years 1970 to 2007, melanoma was the second leading cause of death by cancer. It is estimated that skin cancer is the most common form of cancer in the United States of America (USA). In Asia, the incidence of this cancer is also growing (Wang et al., 2012). Depending on the basal and squamous cell carcinoma (BCC, SCC), there are two types of skin cancer including melanoma and non-melanoma. BCC is the more common type of tumor than melanoma, while melanoma is the most lethal. Previous studies have shown that skin tumor is characterized by an imbalance toward too little apoptosis signaling, or too much cell proliferation and survival in the epidermis (Wang et al., 2012). Although, UV radiation is the major cause of skin cancer, evidence strongly suggests that various agents such as viruses, mutagens in food, genetic, dietary and lifestyle factors increase susceptibility (Wang et al., 2012, Chinembiri et al., 2014).Different treatment methods exists for skin tumors, however, selection of treatment options become difficult given the various characteristics of patients and physician opinions (Wang et al., 2012).

Today, there has been an increasing trend in the use of natural products such as complementary and alternative medicine. Globally, there are many natural compounds that remain to be exploited for therapeutic use (Wang et al., 2012). Phallusia nigra (P.nigra) is a solitary marine tunicate of the simple ascidian class. This marine animal is found in tropical seas surrounding

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the world(Gopalakrishnan et al., 2011). In recent years, interest in the potential of marine organisms for purpose of anti-cancer, anti-viral or anti-inflammatory use are growing (Zovko et al., 2014). The high anti-tumor potency of P.nigra reflects its strong potential and feasibility as a source of anti-cancer drugs (Wang et al., 2012). Although, many marine-derived compounds are at different stages of development (clinical testing), only four anti-cancer drugs of marine origin extent in clinical use.

Disruption of apoptosis signaling is a key mechanism of resistance within melanoma disorders. (Grossman and Altieri, 2001, Hussein et al., 2003, Eberle et al., 2007). Previous research has demonstrated the vital role mitochondria play in apoptosis. Differences exist between the cells of malignant and healthy mitochondria. Such differences can be genomic or structural. Consequently, the mitochondria is viewed as a target for cancer therapy (Talari et al., 2014, Seydi et al., 2015). Previous studies have shown that some marine animals such as sea sponges and sea cucumbers possesses a cytotoxic effect on cancer cells (leukemia and liver cancers) (Seydi et al., 2015, Salimi et al., 2016).It has been reported that, the total extract of P. nigra has anti-proliferative properties (Meenakshi et al., 2013).There is a significant lack of information about the toxicity of P. nigra on melanoma. Hence, the authors of this paper decided to study the cytotoxicity effects of P. nigra extracts on skin mitochondria isolated from a typical animal model of melanoma. Given that all mitochondrial pathways may involve cytotoxicity induced by P. nigra and due to the vital role played by the mitochondria, this research was designed to study mitochondrial agents iso¬lated from an animal model of melanoma to recognize the novel anti-cancer potential in Iranian Persian Gulf flora and fauna.

Materials and Methods

Tunicate samples

Tunicate samples (P.nigra) were collected during low tide from the Bandar-e Lengeh coast in southern Iran. They were kept in iced boxes, transported to the laboratory and washed with cold water, weighed and then measured.

Preparation extraction of Phallusia nigra

The bioactive compounds were extracted based on their polarity, using water and organic solvents according to the method described by Sarhadizadeh et al (Sarhadizadeh et al., 2014). The samples from the gonad (G), respiration tree (RT), Cuvieri an organ (CO), and body wall (BW) were defrosted before use. The recovered body wall was cut into small pieces and the samples were homogenized with a blender and then suspended. This was followed by successive extractions with methanol (50%) by percolation (72 h for each solvent) at room temperature. After filtration and centrifugation at 30,000 ×g for 15 minutes in 4°C, the extracts were evaporated under a vacuum at 45°C with a rotary evaporator. Finally, the powdered extracts of each sample were obtained with a freeze dryer and stored at -20°C.

Extraction, fractionation, and isolation procedure of Phallusia nigra

Briefly, fresh samples of P.nigra (2.0 kg) were cut into small pieces and extracted with methanol (4 × 4 l) at room temperature. The combined extract was filtered, and then concentrated into a viscous mass (45.0 g) under reduced pressure, below 45°C, in a rota vapor. The animal residue was further extracted with 50% methanol-chloroform (4 × 4l) and the combined extract was filtered and concentrated under reduced pressure as described above, into a green viscous mass (35.0 g). The remaining residue was rejected. The dried residue was stored at -20°C, to be used in mitochondria parameters assay.

For standardization of methanolic extracts, total phenolic (TP) determination was performed as follows: 2.5 g of oil samples were diluted with 2.5 ml of n-hexane, and extracted three times via centrifugation at 5000 rpm for 5 minutes in 4°C with CH3OH/H2O (80:20 v/v) extract. Then, 2.5 ml of Folin-Ciocalteu reagent and 5 ml of Na2CO3 (7.5%) was added to extract in a 50 ml volume flask, reaching the final volume with deionized water. The samples were stored overnight and the spectrophotometric analysis was performed at λ = 765 nm. The methanolic extracts of P.nigra consisted of 1045 ± 73 mg/g and 785 ± 42 mg/g of TPs, respectively.

Animals

The melanoma animal models were purchased from Pasteur Institute. The study was performed on 25 male Albino\vistar rats with the age of 6-7 weeks and weights of 220-250 grams In order to induce tumoral cells; tumor was inoculated intra dermally from another mouse with F10 melanoma cells. After anesthesia, a small incision in posterior-lateral part of the body was made and a part of the tumor was extracted and divided into small parts (about 2mm each). Using a scalpel, they were then placed underneath the skin, and after all these parts were sutured. The tumor size was measured every 3 days with a digital caliper. Their volume calculated based on O'reilly et al.

V = (tumor weights) 2 (tumor length) 0.52 (O'Reilly et al., 1997).

Isolation of mitochondria from rat melanoma cells

The preparation of isolated rat skin cells is typically performed using the two-step collagenase skin perfusion technique (Seglen, 1976, Talari et al., 2014). In order to evaluate cellular integrity (or viability), the tryp a blue exclusion test was performed (Pourahmad et al., 2010, Pourahmad et al., 2011). The two-step collagenase skin perfusion technique resulted in a viability of 90%normal/ melanoma cells. Then, the mitochondria were prepared from melanoma cells (Barogi et al., 1995). Subsequently, the cells were pelleted by centrifugation at 300 g for 3 minutes. In the next step, the supernatant was kept while the pellet was homogenized for 10 minutes, followed by centrifugation at 760 g for 5 minutes. The supernatants from previous steps were combined and centrifuged for 20 minutes at 8,000 g. With the exception of the mitochondria used to assess ROS production, MMP, and swelling, the final mitochondrial pellets were suspended in Tris buffer (0.05 M of 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.

Succinate dehydrogenase (SDH) activity

The activity of SDH was assayed by measuring the reduction of MTT (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide). Briefly, 100 µl of mitochondrial suspension was incubated with different concentrations of diethyl ether, methanol, n-hexane and aqueous extracts of P. nigra (125 - 1500 µg/ml) at 37°C for 60 minutes. Then 0.4% of MTT was added to the medium and incubated at 37°C for 30 minutes. The product of formazancrystals were dissolved in 100 µl DMSO and the absorbance at 570 nm was measured with an ELISA reader (Tecan, Rainbow Thermo, Austria) (Zhao et al., 2010).

Determination of ROS levels

ROS measurements were performed using the fluorescent probe dichlorodihydro fluorescein diacetate (DCFH-DA). Briefly, isolated mitochondria from normal and melanoma groups were placed in respiration buffer, and then DCFH-DA was added (final concentration, 10 µM) to the mitochondria, which were then incubated for 10 minutes at 37°C. For the next step, the fluorescence intensity of dichlorofluorescein (DCF) was measured using the Shimadzu RF-5000 U fluorescence spectrophotometer at an excitation wavelength of 488 nm and an emission wavelength of 527 nm (Seydi et al., 2015).

Determination of MMP

Rhodamine 123 (Rh 123) (10 µM) was added to the mitochondrial suspensions (1000 µg mitochondrial protein/ml) in MMP assay buffer. The cytosolic Rh 123 fluorescence intensity, which represents the redistribution of the dye from the mitochondria into the cytosol, was determined using the Shimadzu RF-5,000U fluorescence spectrophotometer at an excitation wavelength of 490 nm and an emission wavelength of 535 nm (Talari et al., 2014).

Determination of mitochondrial swelling

The isolated mitochondria from the normal and melanoma groups were suspended in swelling buffer and incubated at 30°C with 250, 500 and 1000 µg/ml of diethyl ether, methanol and n-hexane extracts. The absorbance was then measured at 540 nm at 10-min intervals with an ELISA reader (Tecan, Rainbow Thermo, Austria). A decrease in absorbance indicates an increase in mitochondrial swelling (Shaki et al., 2012, Hosseini et al., 2013).

Cytochrome c release

The concentration of cytochrome c was measured by using the Quantikine Human Cytochrome c Immunoassay kit provided by R and D Systems, Inc, (Minneapolis, USA). In brief, according to the protocol of the manufacturer, a monoclonal antibody specific for cytochrome c was pre coated onto the microplate. 75 microliters of conjugate containing of monoclonal antibody specific for cytochrome c conjugated to horseradish peroxidase and 50 µL of positive control and standard were added to each well of the microplate. One microgram of protein from each supernatant fraction was added to the sample wells. All of the standards, controls, and samples were added to two wells of the microplate. After 2 h of incubation, 100 µL of substrate was added to each well and then incubated for 30 min. After adding100 µl of the stop solution to each well, the optical density of each well was monitored by the spectrophotometer set to 450 nm.

Quantification of apoptosis

Analysis of apoptosis by propidium iodide staining is a rapid and simple flow cytometry method for measuring apoptosis in cells. Fundamentally, in this method propidium iodide (PI), a fluorogenic compound used for nuclear staining by binding to nucleic acids and fluorescence emission induced of 488-nm laser beam, is proportional to the DNA content of a cell. Briefly, following the exposure with n-hexane, diethyl ether and methanolic extracts of P.nigra (12 h), we suspended cells at 1×106 cells per 1 ml of PBS and Centrifuged at 200g for 5 min at room temperature. After that cell pellet was resuspended in 1 ml of fluorochrome hypotonic solution containing 0.1% sodium citrate (wt/v), 0.1% Triton X-100 (v/v), 50 mg/ ml PI in deionized/distilled water. Prior to flowcytometric analysis cells were maintained in the dark room at 4°C, for at least 1 hour. Finally cells were analyzed by flow cytometry Useing 488-nm laser beam for excitation.

Statistical Analysis

Results are presented as a mean ± SD. All statistical analyses were performed using SPSS software, version 20. The assays were performed three times, 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 some experiments, the two-way ANOVA test followed by the post hoc Bonferroni test was also performed. Statistical significance was set at $P < 0.05$.For quantification of apoptosis, Samples analyzed with flowcytometry (BD), supplied with the flowing software1.2.5.

Results

Effect of Phallusia nigra extracts on mitochondrial SDH activity

The activity of mitochondrial SDH obtained from normal and melanoma groups was assessed using the MTT test after 1 h incubation in the presence of different concentrations of P. nigra extracts(125, 250, 500, 1000 and 1500 µg/ml). Our results show that all applied concentrations of non-polar extracts of P. nigra, suchasn-hexane, diethyle the randm ethanolic extracts which contain both polar and non-polar compounds, induced a selectively significant ($P<0.05$) concentration-dependent decrease in SDH activity on skin mitochondria obtained from the melanoma group (Figure 1E-H).The remaining extracts did not affect SDH activity on the mitochondria from the normal group; the exception is diethyl ether extract at the highest concentration (Figure 1B). The aqueous extract, which consists of only polar

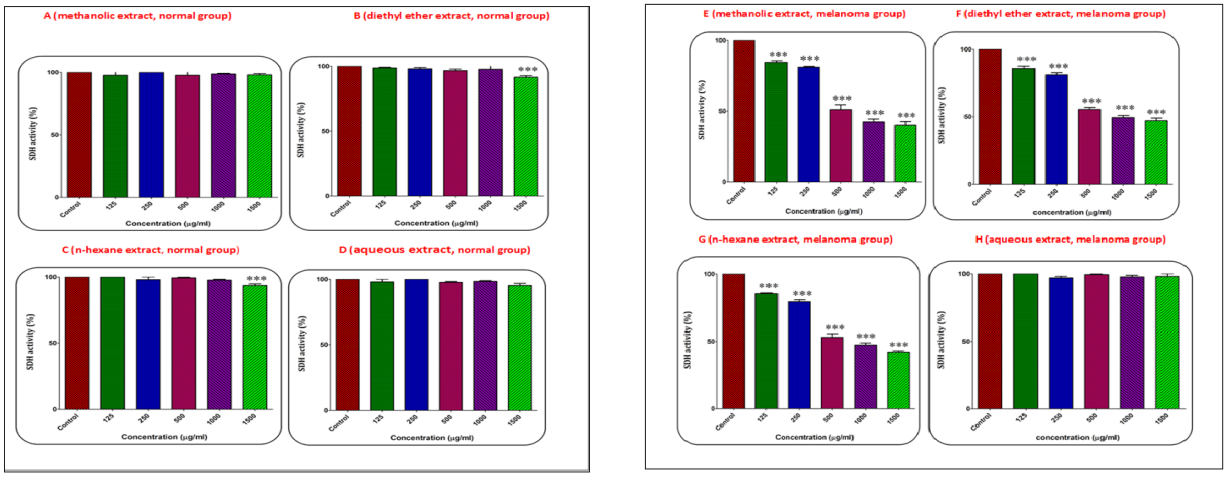

Figure 1. The Effect of P. Nigra Concentrations Extracts on the SDH Activity. (A) The effect of methanolic (B), diethyl ether, (C) n-hexane and (D) aqueous extracts of the P. nigra concentrations on SDH activity on skin mitochondria obtained from the normal group. The effect of methanolic (E), diethyl ether, (F) n-hexane and (G) aqueous extracts (H) of the P. nigra concentrations on SDH activity on skin mitochondria obtained from the melanoma group. Values are presented as mean ± SD (n = 3). The one-way ANOVA test was performed. *** Significantly different from the corresponding control (p<0.001).

compounds at all applied concentrations (125, 250, 500, 1000 and 1500 µg/ml), did not induce any significant decrease in SDH activity on either normal or cancerous melanoma mitochondria (Figure 1-D and H).The authors of this paper believe that the active compounds with potential anti-tumor properties do not have a polar nature.

Phallusia nigra extracts induced ROS generation

As shown in Figure 2A-C, different concentrations of n-hexane, diethyl ether and methanolic extracts of P. nigra (250,500 and 1000µg/ml) induced a significant (P<0.05) ROS generation (demonstrated as fluorescence intensity units emitted from highly fluorescent DCF) in the skin mitochondria obtained from melanoma group but not of the control group. This activity occurred in a time and concentration-dependent manner.

Also, n-hexane, diethyl ether and methanolic extracts of P. nigra at all applied concentrations (250, 500 and 1000 µg/ml) did not significantly affect ROS generation in the skin mitochondria from the normal group (Figure 2A-C).

Phallusia nigra declined Mitochondria Membrane Potential (MMP)

As shown in Figure3A-C,all used extracts of P. nigra at concentrations of 250, 500 and 1000 µg/ml significantly (P < 0.05) declined the MMP (demonstrated as fluorescence intensity units emitted from Rh 123, redistributed from damaged mitochondria into the cytosol) in a time and concentration-dependent manner in the skin mitochondria obtained from the melanoma group.

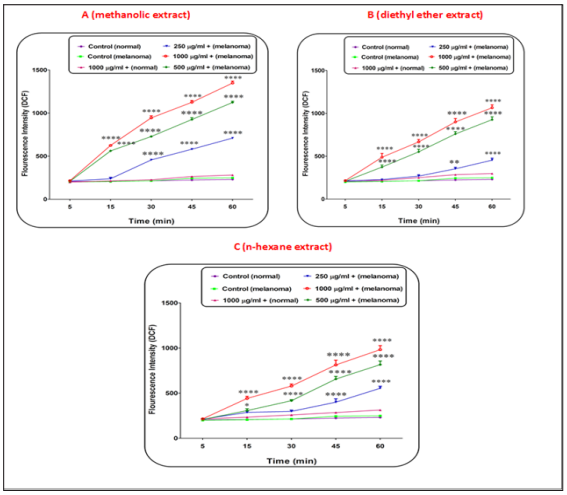

Figure 2. The Effect of P. Nigra Concentrations on ROS Generation. (A) The effect of methanolic (B), diethyl ether and (C) n-hexane extracts of the P. nigra with different concentrations (250, 500 and 1000 µg/ml) on mitochondrial ROS formation. Values are presented as mean ± SD (n = 3). The two-way ANOVA test was performed. *,** and **** significantly different from the corresponding control (p<0.05, p<0.01 and p<0.0001, respectively)

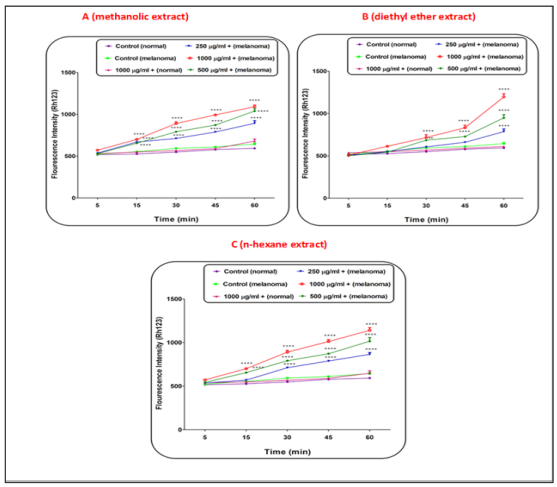

Figure 3. The Effect of P. Nigra Concentrations on the MMP Collapse. (A) The effect of methanolic (B), diethyl ether and (C) n-hexane extracts of the P. nigra with different concentrations (250, 500 and 1000 µg/ml) on MMP. Values are presented as mean ± SD (n = 3). The two-way ANOVA test was performed. **and**** significantly different from the corresponding control (p<0.01 and p<0.0001, respectively)

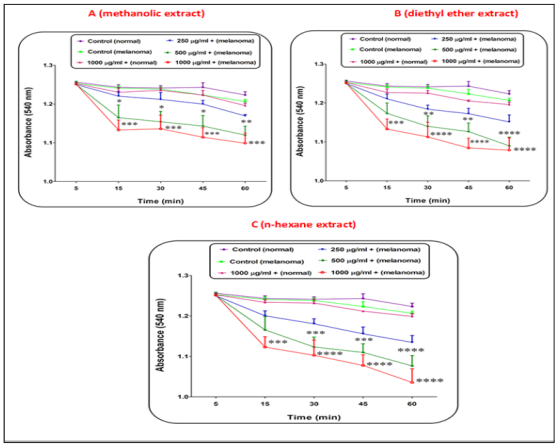

Figure 4. The Effect of P. Nigra Concentrations on Mitochondrial Swelling. (A) The effect of methanolic (B), diethyl ether and (C) n-hexane extracts of the P. nigra with different concentrations (500, and 1000 µg/ml) on the mitochondria swelling. Values are presented as mean ± SD (n = 3). The two-way ANOVA test was performed. *,** , *** and **** significantly different from the corresponding control ($p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively)

Also, n-hexane, diethyl ether and methanolic extracts at all applied concentrations (250, 500 and 1000 µg/ml) did not significantly affect the MMP on the skin mitochondria from normal group (Figure 3A-C).

Phallusia nigra increased mitochondrial swelling

We measured the decrease of absorbance on mitochondrial samples at 540 nm in order to assay mitochondrial swelling, another indicator of the

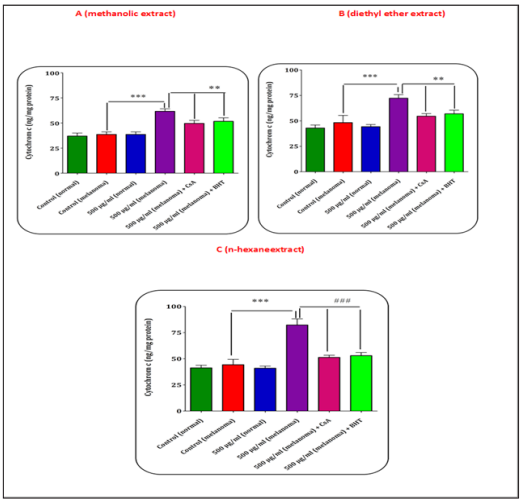

Figure 5. Cytochrome c Expulsion Assay. Cytochrome c Expulsion was Determined by Using Corresponding rat/mouse Cytochrome c ELISA kit. The Cytochrome c Expulsion is Increased after Addition of (A) methanolic (B), diethyl ether and (C) n-hexane extracts of the P. nigra (500µg/ml) on the mitochondria obtained from the skin. Data represented as mean ± SD of data determined from three separate experiments. Values are presented as mean ± SD (n = 3). The one-way ANOVA test was performed. *** Significantly different from the corresponding control ($p<0.001$). ** and### Significant difference in comparison with three extracts of P. nigra (500µg/ml) - treated mitochondria $p<0.01$ and $p<0.001$, respectively.

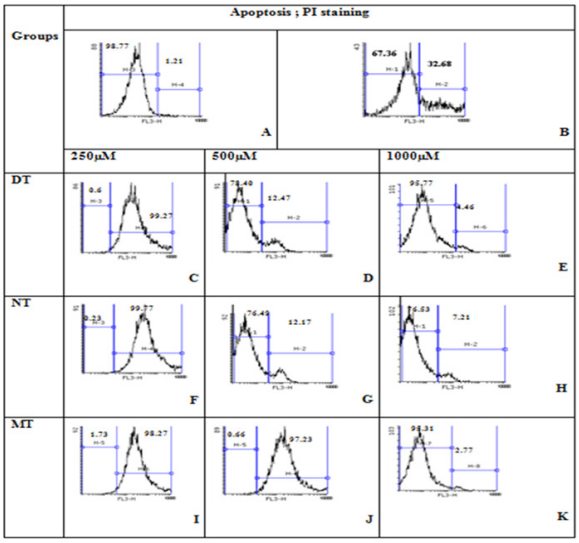

Figure 6. Apoptosisassay. Melanoma Cells were Incubated with Methanolic, Diethyl Ether and n-Hexane Extracts of the P. Nigra with Different Concentrations (250, 500 and 1000 µg/ml) for 6 Hours. A) Untreated control (6h); B) Melanoma control (6h); C) Melanoma control plus diethyl ether extract (250 µg/ml) (6h); D) Melanoma control plus diethyl ether extract (500 µg/ml) (6 h); E) Melanoma control plus diethyl ether extract (1000 µg/ml) (6h);F) Melanoma control plus n-hexane extract (250 µg/ml) (6h); G) Melanoma control plus n-hexane extract (500 µg/ml) (6 h); H) Melanoma control plus n-hexane extract (1000 µg/ml) (6h); I) Melanoma control plus methanolic extract (250 µg/ml) (6h); J) Melanoma control plus methanolic extract (500 µg/ml) (6 h); K) Melanoma control plus methanolic extract (1000 µg/ml) (6h).

mitochondrial permeability transition (MPT). Our results show that the addition of different concentrations extracts of P. nigra (500 and 1000 µg/ml) significantly ($P < 0.05$) increased the mitochondrial swelling in the skin mitochondria obtained from the melanoma group. Also, n-hexane, diethyl ether and methanolic extracts of P. nigra at concentrations of 250 µg/ml did not significantly affect mitochondrial swelling.

Also, n-hexane, diethyl ether and methanolic extracts at all applied concentrations (250, 500 and 1000 µg/ml) did not significantly affect mitochondrial swelling in the skin mitochondria from the normal group (Figure 4A-C).

Phallusia nigra increased cytochrome c release

As shown in Figure 5A-C, our results show that the addition of extracts of P. nigra (500 µg/ml) induced a significant ($P<0.05$) release of cytochrome c only in the melanoma mitochondria. Furthermore, our results show that pretreatment of n-hexane, diethyl ether and methanolic extracts (500 µg/ml) on melanoma mitochondria by cyclosporine A (CsA) and antioxidants such as butylated hydroxyl toluene (BHT) significantly inhibited cytochrome c release.

Phallusia nigra induced apoptosis signaling

To determine if apoptosis is involved in the cytotoxic effects of n-hexane, diethyl ether and methanolic extracts of P. nigra, apoptotic cells were determined by PI staining

of DNA fragments by flow-cytometry (sub-G1 peak). Cells were exposed to different concentrations (250, 500 and 1000 µg/ml) of non-polar (n-hexane and diethyl ether) and both polar and non-polar (methanolic) extracts of P. nigra for 6h. Non-polar extracts (n-hexane and diethyl ether) treatments of the melanoma cells significantly increased the sub-G1 peak with a concomitant decrease in G1 phase at concentrations of 500 and 1000µg/ml but not at 250µg/ml. Both polar and non-polar compounds extract of P. nigra (methanolic) treatment of the melanoma cells significantly increased the sub-G1 peak with a concomitant decrease in G1 phase at a concentration of 1000 µg/ml but not at 250 and 500 µg/ml (Figure 6).

Discussion

The incidence of skin cancer has risen sharply over the past decades, rendering it as one of the most common tumors in Caucasians, worldwide (Wang et al., 2012, Chinembiri et al., 2014). Presently the standard chemotherapy in the treatment of un-respectable/ metastatic melanoma is largely palliative (Zovko et al., 2014). Recently, several new targeted treatments and immunotherapies have been approved for progressed melanoma (Badaboina et al., 2013, Leiros et al., 2014, Firoozinia et al., 2015).In spite of new advances seen in the treatment of melanoma, treatment for most melanomas is still elusive and patients have to undergo various lines of treatment alone or in combination to maximize their alters of survival (Badaboina et al., 2013, Leiros et al., 2014, Firoozinia et al., 2015). It has been reported that melanoma cells are resistant to anti-cancer drugs and are able to bypass their effects. Hence, the quest for new molecules that could enhance current treatments is still open and of relevance from a clinical perspective (Leiros et al., 2014, Zovko et al., 2014).

It was reported that significant variation in mitochondrial content and function exists in melanoma, often controlled via microopthalmia transcription factor (MITF) and Peroxisome proliferator-activated receptor Gamma Coactivator- 1 (PGC-1). In melanocytes, the expression of Bcl2 (as an anti- apoptotic protein) is higher in comparison to other tissues. Furthermore, reported that mitochondria play an important role in the in the generation of ROS in the melanoma cells (Theodosakis et al., 2014). Today, an abundance of natural compounds are known globally for their medicinal properties. Several of these compounds are yet to be exploited for potential application in the pharmaceutical industry. Over 70% of anti-cancer agents originate from natural sources. In particular, natural products provide a variety of lead compounds used or are currently investigated for their anti-melanoma properties and other cancer activities (Hosseini et al., 2013, Zovko et al., 2014).

Previous studies have shown that marine animals (such as P.nigra) con¬tain several numbers of biological compounds with strong anticancer effects. Therapeutic effects of marine animals are related to the presence of functional biological compounds which can potentially explain the considerable suppression of tumor viability (Shen et al., 2003, Meenakshi et al., 2013). Other studies have shown that P. nigra contains natural compounds (Ren et al., 2003, Meenakshi et al., 2012). This study was designed to identify the selective toxicity of n-hexane, diethyl ether, methanolic and aqueous extracts of P. nigra on the skin mitochondria isolated from a rat melanoma model. Since the aqueous extract did not affect the SDH activity, which is the absolute marker of mitochondrial functionality, and it didn't induce any effect on other mitochondrial parameters (data not shown) in both tumor and non-tumor cells, we suggest that the active compounds with potentially anti-tumor effects do not have a polar nature.

As indicated by previous literature, the major distinctive function and characteristic of mitochondria would be observed between cancer and normal cells. The different size, number, and shape of the mitochondria and also, different structure, genomic mitochon¬drial alterations have also been shown in cancer cells in comparison to normal cells (Toogood, 2008, Liao et al., 2014, Talari et al., 2014).

The level of ROS as a mitochondrial parameter toxicity was evaluated. It is well documented that the mitochondria is the specific source of ROS. Approximately, 1-2% of total oxygen consumed by the mitochondria is converted to O2by several components of the mitochondrial respiratory chain. Superoxide anions generated in the mitochondria are rapidly converted to H2O2 by the mitochondrial superoxide dismutase (MnSOD). Also, mitochondrial H2O2 generation represents between 60 to 80% of the cellular generation rate. The addition of only three extracts of P.nigra into skin mitochondria obtained from the melanoma group induced the generation of H2O2 formation. Indeed, ROS induces irreversible damage to the cellular constituents that impair cellular activities. Also, raising levels of ROS could influence the apoptosis and proliferation pathway (Essack et al., 2011). Several research have established these results and have shown that treated rats with marine animals result in a decrease of SDH activity and raise ROS levels (Seydi et al., 2015, Salimi et al., 2016).

Our findings indicate that the addition of n-hexane, diethyl ether and methanolic extracts induce a collapse of MMP only in the skin mitochondria isolated from the melanoma group. Our data is also in agreement with our previous published work, which showed that the treatment of marine species on leukemia and liver cancer cells led to a collapse in the MMP (Seydi et al., 2015, Salimi et al., 2016).To correctly shows that the mitochondria plays a vital role in the apoptosis process through the redistribution of inter-membranous mitochondrial (IMM) pro-apoptotic proteins (such as cytochrome c). The collapse of MMP causes an opening of the MPT pore, which has been hypothesized to be a permanent point towards cell death (Ko et al., 2005).

Two mitochondrial toxicity parameters, mitochondrial swelling and cytochrome c expulsion, as subsequent events after mitochondrial permeability transition (PT) were also determined. In addition, P.nigra (250, 500 and 1000 µg/ml) caused selective effects only on mitochondria obtained from skin of melanoma rats.

Apoptosis play an important role in regulating cell

number in numerous developmental and physiological and pathological conditions(Sadeghnia et al., 2014). Defected cell apoptosis is a critical etiology for most malignant tumors. Investigating effective drugs to cause tumor cell apoptosis has become a focus in anti-cancer drug development. Mitochondrial targeting is the main gateway for design and development of anti-cancer drugs causing tumor cell apoptosis. Our results show thaton polar extracts of P. nigra in concentrations of 500 and 1000 µg/ml induces apoptosis in melanoma cell group. Methanolic extract by both polar and non-polar compounds can induce apoptosis only in the highest concentration (1000µg/ml). The induction of apoptosis is subsequent to the decrease of MMP and cytochrome c expulsion. The order of efficacy of tunicate extracts at inducing apoptosis was: (B), diethyl ether extract>n-hexane extract>methanolic extract in the melanoma cell group.

In conclusion, we suggest that the effect of all extracts of P. nigra on mitochondria and melanoma cells isolated from melanoma skin may be attributed to the presence of numerous arrays of bioactive compounds. As the results have shown, non-polar extracts in comparisonto polar extracts could be causing changes in mitochondrial parameters. Potential bioactive compounds exist in non-polar extracts. Finally, we suggest that non-polar extracts of P. Nigra as new anti-melanoma drug candidates through the changes caused in mitochondrial and cellular parameters. This study provides evidence that mitochondrial targeting is avital mecha-nism for the Persian Gulf sea squirt extract.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

Yalda Arast: She contributed to this research in carrying it out, analyzing the data and writing the article. Other authors: He and she contributed to this research in analyzing the data and writing the article. Jalal Pourahmad: He contributed to this research in formulating the research question(s), designing the study, carrying it out, analyzing the data and writing the article. This research was financially supported by Iran National Science Foundation

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