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Putative mechanism for apoptosis-inducing properties of crude saponin isolated from sea cucumber (*Holothuria leucospilota*) as an antioxidant compound

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ABSTRACT

Objective(s): Marine organisms are known as a potential source of natural products, which contain bioactive substances with therapeutic properties. Sea cucumbers are prominent among marine organisms because of their dietary and therapeutic applications. In addition, they have capacity of synthesizing saponins molecules and other metabolites with therapeutic properties such as antitumor, antimicrobial, anti-inflammatory and antioxidant activities. The aim of this study was to evaluate the antioxidant and pro-apoptotic effects of sea cucumber saponins (SCS) isolated from *Holothuria leucospilota* species.

Materials and Methods: Evaluation of antioxidant activity of SCS was carried out by DPPH (1, 1-diphenyl-2-picrylhydrazyl), ABTS (azino-bis-3-ethylbenzothiazoline-6-sulfonic acid), power reducing and total antioxidant assays. The anti-proliferative effect was studied by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Mechanisms leading to apoptosis were also evaluated by fluorescence microscopy, flow cytometry and real time PCR.

Results: The results showed that the DPPH and ABTS activities increased in a dose dependent manner. The reducing capacity enhanced with increasing concentration of the saponin extract (0 to 2 mg/ml). The SCS exhibited moderate total antioxidant activity. Evaluation of anti-proliferative effect revealed that SCS with IC_{50} of about 6 µg/ml, can display a good cytotoxic activity in a dose dependent manner. Further apoptosis induction was confirmed by fluorescence microscopy and flow cytometry. Sea cucumber saponin was also found to exert a pro-apoptotic effect by increasing the expression of Bax and decreasing the expression of Bcl2.

Conclusion: These results indicate that the SCS may act as a natural antioxidant and antitumor agent.

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Introduction

Cancer is one of the deadliest diseases and causes millions of human deaths every year. An estimation showed that approximately 180 million people have died by cancer between 2005 and 2015 (1). There are many therapeutics to treat cancer, but most of them are limited due to their side effects. As a consequence, attentions have been focused on the identification of novel compounds with fewer side effects to treat cancer. Recently, efforts have been focused on the identification of natural products in order to identify anti-cancer compounds (2). Natural products contain many compounds that may help to regulate the effects produced by oxidation substrates in biological systems. In other words, these compounds are able to modulate the action of some of the oxidants on the tissues and can be an important factor in controlling angiogenesis and apoptosis in pathological condition (3). Preventing tumor growth must be objective the multiple physiological and biochemical pathways that lead to tumor development. A good correlation exists between antioxidant intakes and reduces cancer risk. Therefore, the antioxidant supplements as part of diet often are used to prevent cancer (4). Apoptosis is one of the important targets in cancer therapy and many drugs exert their effect through apoptosis. Apoptosis in normal conditions is a controlled way to remove old, excess and damaged cells. However, this pathway can lead to the elimination of harmful cells (5).

In the recent years attentions have been focused on the relationship between apoptosis and cancer, and the results indicate that this process is one of the effective ways to eliminate preneoplastic and neoplastic cells. Mechanisms that cause cells to enter

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apoptosis process are highly complex. Bax (pro-apoptotic) and Bcl2 (anti-apoptotic) genes are the main determinants of this event, which their expression level determines the fate of cells (6).

Marine environment is rich of unique effective natural products. Due to the specific characteristics of the marine environment (physical and chemical conditions), marine organisms contain bioactive molecules with unique properties. In recent decades, numerous bioactive substances with medicinal and therapeutic properties have been extracted from marine organisms (7). Sea cucumbers have been known as the most important marine organisms, that are used traditionally in the treatment of some diseases (8). In many countries, sea cucumbers are used as food supplies. The health benefits of these animals are often related to the presence of some bioactive compounds such as saponins in their bodies (9). Saponins were identified first in plants (10) Subsequent studies showed that these compounds exist in some animals such as sea cucumbers, starfishes and sponges (11). Saponins are composed of aglycones and sugar side chains and divided into two groups according to their aglycone structure (12). Their biological properties are determined based on these chemical structures (13). Various studies have showed that the saponins have different biological effects such as haemolytic (9), antifungal (14), anti-proliferative (11), antimicrobial and antitumor activities (15).

Induction of apoptosis is an important strategy for inhibition of tumor growth and metastasis. So far, many investigations have been done in association with oxidative stress and use of antioxidants in order to prevent some diseases such as cancer. Recently, attentions have been focused on compounds from natural products, which contain several simultaneous functions. Some of these compounds, in addition to the antioxidant benefits are also effective on apoptosis (16). In the current study, we have investigated the antioxidant properties of SCS and its ability to induce apoptosis in a human breast cancer cell line.

Materials and Methods

Chemicals

All of the chemicals (HP-20 resin, Quillaja saponin, MTT, ABTS, DPPH, ...) were purchased from Sigma- Aldrich Co. (St. Louis, MO, USA) and all solvents (ethanol, n-butanol, dichloromethane, ascetic acid) were purchased from Merck (Darmstadt, Germany). Cell cultured reagents were obtained from Gibco-USA. RNA isolation kit from Roche Company was purchased. cDNA synthesis kit and RT-PCR kit were purchased from Thermo Scientific Company.

Preparation of saponin from sea cucumber

Crude saponins were isolated from the sea cucumber, *Holothuria leucospilota* according to the method used by Hu *et al*, 2010. The body wall of sea cucumber was dried, powdered and extracted five times with refluxing ethanol. The extracted materials were evaporated in vacuum (Heidolph, Germany) and then, were defatted with dichloromethan/water. The water layer was extracted with n-butanol and the organic layer was evaporated in vacuum to get the n-butanol extract. The n-butanol extract were concentrated and dissolved in water. This fraction was loaded on Diaion HP20 resin column and then was eluted with water, 80% ethanol and 100% ethanol in sequence. Initially with water the inorganic salts and polar impurities were eluted and then the fraction eluted with 80% ethanol and collected by evaporation, which was gaineed crude saponin (17, 18).

In vitro antioxidant activity

DPPH radical scavenging assay

Free radical scavenging activity of SCS was evaluated by using its ability to trap the DPPH free radicals. For this purpose, DPPH working solutions were prepared by dissolving 1 mg DPPH in 10ml ethanol. Stock solution of saponin was prepared using distilled water in various concentrations (0-2 mg/ml). The reaction mixture was contained 1 ml DPPH working solution and 1ml saponin in various concentrations. After 30 min incubation at room temperature absorbance of sample was read at 517 nm. Butylated hydroxyanisole (BHA) was used as a standard compound (19).

ABTS radical scavenging assay

ABTS free radical scavenging activity was measured according to the method described by Li, *et al* 2011 (20) with moderate modifications. Briefly, ABTS**·+** stock solution was prepared by mixing 7 mM of ABTS and 2.45 mM of potassium persulfate and incubation at room temperature for 12-16 h. The ABTS**·+** working solution was prepared by dilution of the ABTS**·+** stock solution and distilled water to gain a 0.70±0.02 absorbance at 734 nm. The reaction mixture was prepared by mixing 1 ml of the working solution in 1ml of various concentrations of SCS. After incubation for 1 h at room temperature in dark, absorbance was taken at 734 nm.

Reducing power assay

The reducing power of SCS and BHA were determined according to the method of Chang *et al* 2007 (21). Various concentrations of SCS (0-4.0 mg/ml) and BHA (0- 4.0 mg/ml) were mixed with phosphate buffer (0.2 M, pH 6.6) and 2% potassium ferricyanide [K3Fe (CN) 6]. Then the trichloroacetic acid (w/v) 10% was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer (0.5 ml) was mixed with distilled water (0.5 ml) and FeCl3 (0.1 ml, 0.1%) for 10 min, and then the absorbance was measured at 700 nm by spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing

power. BHA was used as a standard.

Total antioxidant capacity

Total antioxidant capacity was measured in various concentrations of SCS. An aliquot of 0.2 ml of sample solution was combined with 2 ml of reagent solution included: 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. After 90 minutes incubating at 95˚C, sample was cooled in room temperature and the absorbance of the mixture was measured at 695 nm against a blank. The antioxidant activity was expressed relatively to ascorbic acid (22).

Evaluation of apoptotic effect

Cell line

MCF7 (human breast cancer cell line) was purchased from Pasture Institute of Tehran, Iran maintained in RPMI medium supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were incubated at 37˚C with 5% CO_2, 95% air in high humidity.

MTT assay

Cytotoxic effect of the SCS against MCF7 cell line was evaluated by MTT assay. For this purpose, the cells were seeded ($1x10^5$ cells/ml) in a 96-well plate. After 24 hr, the cells were treated with different concentrations of the SCS (2-10 µg/ml) for 24, 48, 72 hr. After treatment period, cells viability was evaluated by MTT assay and the absorbance was measured at 570 nm by spectrophotometer.

Assessment of MCF7 cell morphology changes

For investigation the effects of SCS on cell morphology, MCF7 cells were seeded in 6 well plates (3×10^4). After 24 hr, the cells were treated with different concentrations of SCS for 48 hr. At the end of the incubation period, morphology of the cells was investigated by inverted microscope (Nikon, Japan).

DAPI staining

For the nuclear analysis, after treatment of the MCF7 cells with different concentrations of SCS for 48 hr, the cells were washed using PBS and fixed with methanol at room temperature for 10 min. Then the fixed cells were incubated with 0.5 µg/ml of DAPI for 5 min. The apoptotic nuclei were examined under fluorescent microscope.

Acridine orange (AO) and propodium iodide (PI) double staining

For determining type of cell death the MCF7 cells were stained with acridine orange (AO) and propidium iodide (PI) which were used as fluorescent probes. To achieve this purpose, after treatment period, the cell pellets were washed with PBS and then mixed with fluorescent dye (1 : 1) containing 10 µg/ml AO and 10 µg/ml PI and observed under fluorescence microscope (23).

Analysis of cell cycle by flow cytometry

The MCF7 cells were seeded and treated with different concentrations of SCS to cell cycle assessment. The cells were washed and then mixed with PI (Sigma) containing 0.1% sodium citrate and 0.1% Triton X100 and were finally incubated for 30 at 37˚C in the dark. Analysis of cell cycle was investigated by a FACScan laser flow cytometer (FACSCalibur, Becton Dickinson, USA).

Evaluation of gene expression by Real time PCR

The expression of Bax and Bcl2 mRNA was evaluated by Real time PCR. For this purpose total RNAs of treated cells were isolated using the high pure RNA isolation kit (Roche, Germany) according to the manufacturer's instructions and then stored at -80˚C. Complementary DNA was synthesized using a revertaid first strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer's protocol. Real-time experiments were conducted on a real-time PCR detection system (Bio-Rad CFX96) using SYBR Green real-time PCR master mix (Pars Tous Iran). The reaction PCR was performed in a final volume of 20 µl containing: 10 µl SYBR Green real-time PCR master mix, 2 µl cDNA, 2 µl primer (forward and reverse) and water to reach level 20 µl. The sequences of primers that used in this study are listed in Table 1. The GAPDH was used as housekeeping gene.

Statistical analysis

Statistical analyses were performed using SPSS 16 software. All data were expressed as means ± SD, Comparisons among multiple groups were performed via analysis of variance (ANOVA) and Least Significant Difference (LSD) test. Values of $P< 0.05$ were assumed significant.

Results

Antioxidant activity test

DPPH radical scavenging activity

The free radical scavenging activity of SCS was assayed by DPPH scavenging. The SCS showed a dose dependent activity and also the DPPH scavenging effect was 59% at a concentration of 2 mg/ml (Figure 1) whereas the standard BHA exhibited 80.4% inhibition of activity at 2 mg/ml. Sea

Table 1. The sequences of primers for apoptotic and antiapoptotic evaluation

Genes	Forward 5'→3'	Reverse 5'→3'
GAPDH	5□CAAGGTCATCCATGACAACTTTG3□	5□GTCCACCACCCTGTTGCTGTAG3□
BAX	5□ TTTGCTTCAGGGTTTCATCCA 3□	5□CTCCATGTTACTGTCCAGTTCGT3□
Bcl2	5□CATGTGTGTGGAGAGCGTCAAC3□	5□CAGATAGGCACCCAGGGTGAT3□

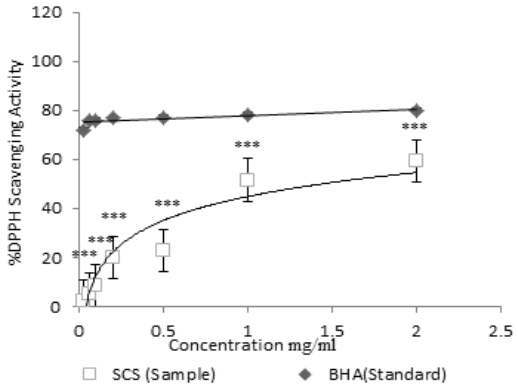

Figure 1. Scavenging activity of sea cucumber saponins on DPPH radical. The values are significantly different (P<0.05) when compared with the control (BHA) in similar concentrations

Table 2. Reducing power capacity of sea cucumber saponins. The valuesare the mean±*SD* of three parallel measurements

Concentration (mg/ml)	Absorbance (optical density) of BHA	Absorbance (optical density) of SCS
0.03	1.22±0.32	0.84±0.05
0.06	1.30±0.09	1.21±0.16
0.12	1.45±0.06	1.28±0.03
0.25	1.56±0.04	1.34±0.04
0.5	1.71±0.04	1.45±0.03
1	1.77±0.06	1.58±0.07
2	1.85±0.03	1.72±0.03
4	2.37±0.16	1.76±0.08

cucumber saponin showed moderate inhibitory effect on DPPH free radical.

ABTS radical scavenging activity

In order to evaluate the antioxidant activity of SCS, ABTS free radical scavenging activity was examined. Figure 2 shows the SCS has antiradical activity by inhibiting ABTS radical with the IC_{50} value of about 600 µg/ml. Sea cucumber saponin showed dose dependent activity and the ABTS scavenging effect has been observed 78 % at a concentration of 2 mg/ml.

Ferric reducing power (FRP)

The reducing potency of the SCS was measured using BHA as a positive control. Power reducing activity increased in a dose dependent manner. The results of the reducing power assay are given in Table 2.

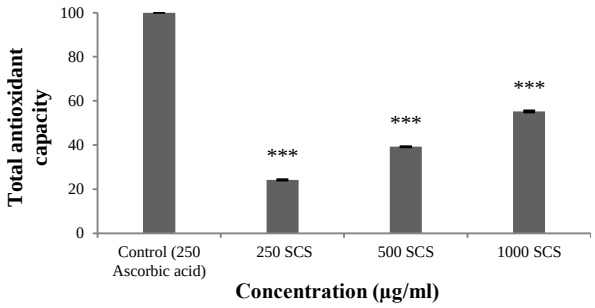

Figure 3. Total antioxidant activity of sea cucumber saponins. The values are significantly different (P<0.05) compared with ascorbic acid as a control

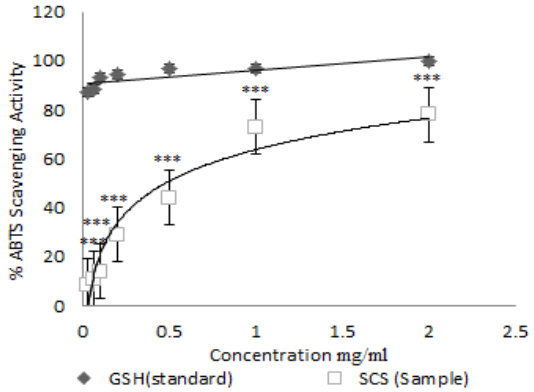

Figure 2. Scavenging activity of sea cucumber saponins on ABTS radical. The values are significantly different (P<0.05) when compared with the control (BHA) in similar concentrations

Total antioxidant capacity

Total antioxidant capacity of SCS is exhibited by the number of equivalents of ascorbic acid as a positive control. The percentage of total antioxidant activity of saponin extract was measured and the results are reported in Figure 3. The saponin extract exhibited total antioxidant activity of 55.74% at 1000 µg/ml.

Effect of SCS on cell viability of MCF-7

The results showed the decreasing percentage of MCF-7 cell viability together with increasing concentrations of SCS. Cancer cell survival in the presence of SCS was significantly suppressed in concentration about 6 µg/ml doses dependently in 24 hr, 48 hr, and 72 hr relative to the untreated groups. Thus, the detailed analysis of the results clearly suggested that SCS caused significant inhibition of MCF7 cell viability in dose dependent manner (Figure 4).

Effect of SCS on cell and nuclear morphology of MCF7 cells

The morphological analysis of MCF7 cells was performed using inverted microscope. Treated cells showed significant morphological changes in comparison with untreated cells, including cell

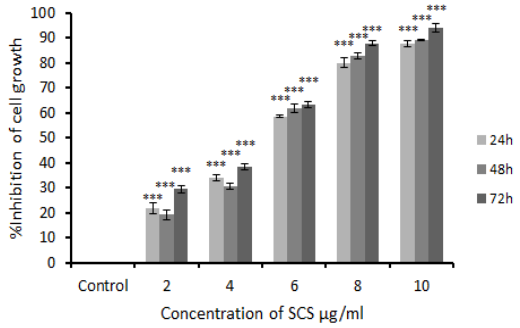

Figure 4. Cytotoxic effect of sea cucumber saponins on cell viability as compared to control group using MTT assay. Data are expressed as Mean ± SD. The values are significantly different (P<0.001)

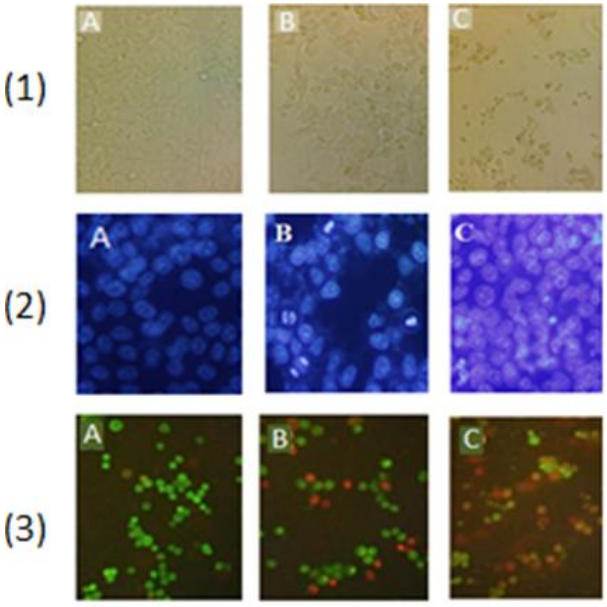

Figure 5. Effect of sea cucumber saponins on cell and nuclear morphology in MCF-7 cells. (A) Control (without treatment). (B), (C) MCF7 Cells treated with 6 and 12 µg/ml SCS for 48h, respectively. (1) Morphological investigations by inverted microscope. (2) Investigation of nuclei with DAPI staining. (3) AO/PI staining of MCF-7 cells, viewed under a fluorescence microscope

shrinkage and reduction in size, volume and density of the cells, cytoplasmic membrane blebbing and lose contact with neighboring cells (Figure 5 (1)). In investigation of MCF7 cells with DAPI staining, the results showed that in the untreated cells, the nuclei are perfectly round, while the treated cells with SCS showed some features of apoptosis such as condensed chromatin and nuclear fragmentation (Figure 5 (2)). In AO/PI staining, the green color indicated live cells, while yellow and red indicated cells that are at the early and late of apoptosis. Figure 5(3) shows cells are stained green in control group (Figure 5 (3A)), while in the treated cells yellow, orange and red staining were observed (Figure 5(3B, C)) which suggest that SCS significantly induced the apoptosis in MCF7 cells.

Effect of SCS on cell cycle

Flow cytometric analysis by propidium iodide (PI) was carried out to study the effect of SCS on cell cycle of breast cancer cells. As exhibited in Figure 6 in treated cells with 6 µg/ml of SCS sub-G1 peak was significantly increased compared with control. With increasing concentration of SCS sub diploid peak was demonstrated significant increase that this indicates the disruptive effect of SCS on breast cancer cells.

Effect of SCS on Bax and Bcl-2 expression in MCF7 cells

Gene expression analysis involved in cell death of MCF7 cells treated with various concentrations of SCS were evaluated by Real time-PCR. Further, increasing concentrations of SCS rised the expression of Bax and decreased the expression of Bcl-2 as compared with control group (Figure 7).

Discussion

ROS or RNS are different form of free radicals that are generated in several reactions in the body. Free radicals by reacting body biomolecules, can cause tissue and cell damages and ultimately lead to disease conditions including cancer. In contrast, antioxidant compounds are able to donate their electrons to free radicals, which can reduce the damage caused by oxidative stress (24, 25) Many kinds of natural products richly contain antioxidant compounds (1). Marine environment is of the richest source of natural products. Sea cucumbers, marine invertebrates belong to the class Echinodermata, mostly have bioactive molecules, which could be a result of their feeding habits according to feed phytoplankton and marine macro algae, and also absorbing these components using their body surface (26). In a previous study, Zhong *et al* (2007) found that there is a weak association between radical scavenging capacity and total phenolic content (27). So in addition to phenolic compounds, other compounds can possibly have a role in the

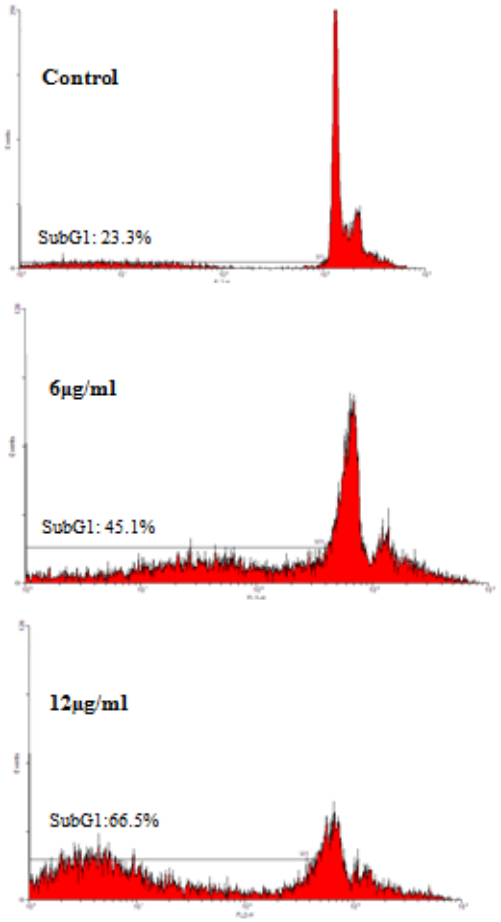

Figure 6. Effect of various concentrations of sea cucumber saponins on the cell cycle of the MCF7 cells by flow cytometry

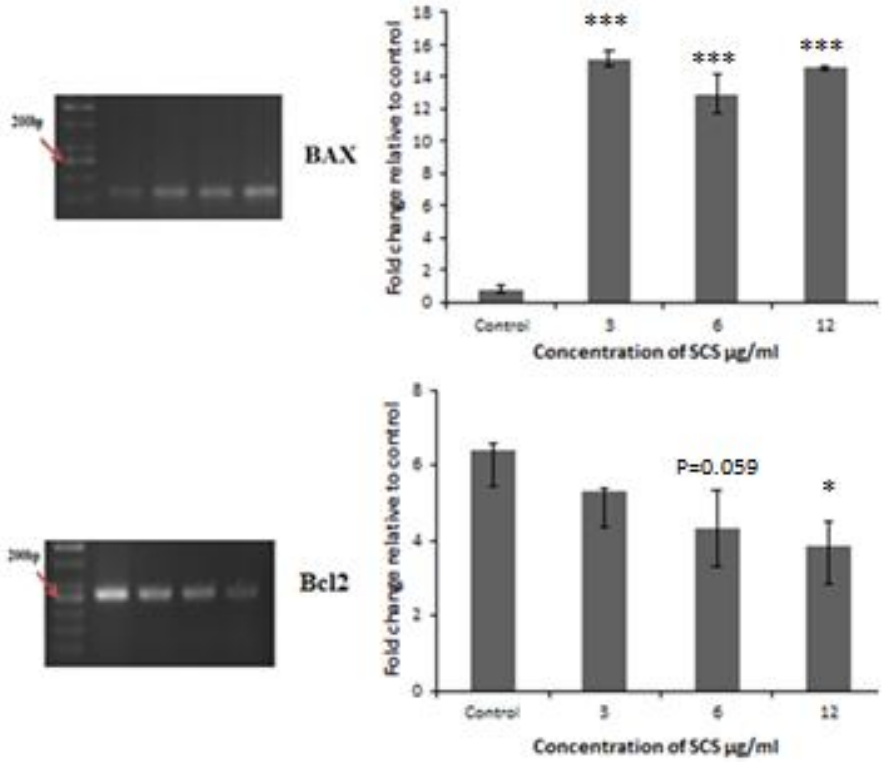

Figure 7. Bax and Bcl2 genes expression in MCF7 cells treated with various concentrations of sea cucumber saponins. Data are expressed as Mean±SD. The values are significantly different (**P*<0.05, ***P*<0.01, ****P*<0.001) compared with the control

antioxidant activity in sea cucumbers. Chenghui *et al* (2007) investigated the antioxidant properties of peptides and hydrolysates extracted from different species of sea cucumbers and found that hydrolysates have considerable antioxidant activity, which may be related to the presence of antioxidant peptides (28). Although, some studies have been done on different compounds extracted from various species of sea cucumbers (e. g. peptides, phenolic and flavonoid compounds), based on the literature, just little information exist about the antioxidant effects of saponins extracted from sea cucumber. In the present study, we investigated antioxidant and apoptotic effects of saponin extracted from *H. leucospilota.* Several procedures have been done to measure antioxidant activity of SCS in this study, due to the fact that no single method exists to evaluate the antioxidant activity of compounds. The results showed, a difference in free radical scavenging capacities for SCS. This difference may be due to the variety of the mechanisms involved in the radical-antioxidant reactions such as selectivity of the radicals or the solubility of the extracts in various systems. In the present study, SCS showed a moderate antioxidant activity in different antioxidant systems. Chen *et al* (2014) studied the antioxidant activity of saponins extracted from *Radix trichosanthis* and found that this compound may be a potential antioxidant agent (29). In 2013 Ashraf *et al* evaluated antioxidant and cytotoxicity activities of saponin and crude extracts of *Chlorophytum borivilianum* and reported total saponin fraction can display higher ferrous ion chelating than the crude extract (30). Arslana and Çelikb (2013) evaluated antioxidant and hemolytic activity of saponin rich fractions (SRPs) from Soapwort and found that some of these compounds have strong antioxidant and slight hemolytic activity (31). In an another study, Hu (2012) assessed antioxidant activity of saponins extracted from *Camellia oleifera* cake and found that saponins have a moderate antioxidant effect, with IC_{50} 3866 ± 3, 4744 ± 2 and 2389 ± 2 µg ml^{-1} for DPPH and metal chelating activity assays (32). Balance between cell proliferation and cell death can maintain tissue homeostasis in normal conditions. Cell death usually occurs via two mechanisms, which are including apoptosis and necrosis. Apoptosis is an important mechanism in the studies of cancer (33). So far, many studies have been conducted on the cytotoxic and apoptotic effect of natural compounds. Cytotoxic property of the saponin is due to the complex formation of saponins and membrane sterols which leads to form ion channels and pores. These pores have a role in disruption of cellular osmotic pressure and can eventually damage the cellular membrane (1). XU *et al* (2013) examined the relationship between the structure and cytotoxic effect of saponins. They found that the oleanane-type

saponins have a better cytotoxic activity than lupane-type saponins, and the length and linkage of glycolic chain that attached to C-3 of aglycon display an important effect to the potent cytotoxicity (34). In addition, Kaswandi, *et al* extracted crude saponins from sea cucumber (*S.badionotus*) and found that they have a cytotoxic effects against leukemia cells (35). Yang, *et al* studied the anti-proliferative effect of branched-chain fatty acid, 12-methyltetradecanoic acid extracted from sea cucumber and showed that this extract has a cytotoxic effect against prostate cancer cells (PC3) (36). Huang and Zou (2011) indicated a cytotoxic activity of a plant steroidal saponin on human lung cancer cells and saponin inhibits cell proliferation in a dose and time dependent manner which confirms our findings (37). Wijesinghe (2013) examined anticancer properties of sea cucumber (*Holothuria edulis*) extract on HL-60 leukemia cell line and showed that this extract has apoptotic effect against HL-60 cancer cell line. They also found that the levels of Bax and caspase-3 protein were increased while the level of Bcl-xl protein was decreased (38). Hsu *et al* were evaluated the apoptotic effect of saikosaponin on lymphocytes and they found a negative correlation between the levels of c-myc and p53 mRNA and the level of Bcl-2 (39). Du *et al* (2012) investigated the anti-tumor activities of two cerebrosides extracted from sea cucumber and starfish and found that both cerebrosides can exhibit cytotoxic effect on S180 cells. Quantitative real-time PCR analysis demonstrated that treatment with cerebrosides decreased the expression of mRNA level of Bcl-2, Bcl-xL, while increased Bax, Cytochrome c, caspase-9 and caspase-3 (40). The results of this study showed that the SCS have a moderate antioxidant activity and could induce apoptosis and inhibit the growth of MCF7 cell line in dose dependent manner.

Conclusion

This study revealed that the Iranian sea cucumber species (*Holothuria leucospilota*) contain promising levels of bioactive molecules and may act as a natural antioxidant and antitumor agent. Purification and identification of the structure of saponins of this species is recommended.

Acknowledgment

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