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Cytotoxicity evaluation of extracts and fractions of five marine sponges from the Persian Gulf and HPLC fingerprint analysis of cytotoxic extracts

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ABSTRACT

Objective: To screen the cytotoxic effects of some marine sponges extracts on HeLa and PC12 cells.**Methods:** Five marine sponges including *Ircinia echinata* (*I. echinata*), *Dysidea avara*, *Axinella sinoxea*, *Haliclona tubifera* and *Haliclona violacea* were collected from the Persian Gulf (Hengam Island). The cytotoxic effect of these sponges was evaluated by using MTT assay. The metabolic high performance liquid chromatography fingerprint of *I. echinata* was also carried out at two wavelengths (254 and 280 nm).**Results:** Among the sponges tested in this study, the extracts of *I. echinata* and *Dysidea avara* possessed the cytotoxic effect on HeLa and PC12 cells. The obtained fractions from high performance liquid chromatography were evaluated for their cytotoxic properties against the cell lines. The isolated fractions did not show significant cytotoxic properties.**Conclusions:** *I. echinata* could be considered as a potential extract for chemotherapy. Further investigation is needed to determine the accuracy of mechanism.

1. Introduction

Nowadays, cancer is a complex disease which is non-curable in most cases [1]. Cervical cancer is one of the most common cancers in Iran and the entire world. Current systemic therapies for cancers are often limited by their non-specific effects and toxicity to normal tissues [2]. Although the main goal of chemotherapy is elimination of tumor cells, in most cases, chemotherapy influences normal cells and damages them [3].

The main strategy of chemotherapy protocols is killing the cancer cells without toxic effect on the host. Resistance of cancer cells to conventional chemotherapeutic agents and nonspecific effect of drugs for treatment of tumors are a common problem. This phenomenon leads to more aggressive phenotype of the cells, which have a greater tendency to metastasize. It also emphasizes the need to identify new anti-cancer compounds; hence, it is necessary to investigate the possible mechanisms of the synergistic effects between drugs being commonly used.

Different natural compounds can improve the efficiency of chemotherapeutic agents, decrease the resistance of cancer cells to chemotherapeutic drugs and alleviate the adverse side effects of chemotherapy [4]. Nowadays, researchers attempt to find new natural compounds with anticancer properties.

Sponges are sessile animals that filter water through their porous bodies, ingest food particles and dissolve materials.

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Sponges live in all types of regions all over the world. About 99% of all sponges live in the marine environment. Sponges also produce their own toxins through normal metabolism, or in collaboration with many microbes that live inside them. Whatever the source of these toxic chemicals is, many have been found to be highly toxic to other life forms. In fact, some of the most toxic chemicals known in nature have been discovered from sponges. Researches indicated that one of the toxic natural products was marine sponges. Because toxins produced by marine sponges can immediately enter the sea, in where they are diluted. As a result, the toxins produced by these sponges must be very powerful in terms of effectiveness that leads to toxicity. Some of these chemicals have potential pharmaceutical applications, including anticancer, antimalaria and analgesics [5]. In fact, the major reason why our knowledge of sponges has escalated over the past few decades is directly due to the increasing interest in their pharmaceutical properties.

In this study, the cytotoxic effects of extracts and fractions from marine sponges were evaluated on cervical cancer (HeLa) and pheochromocytoma (PC12) cell lines.

2. Materials and methods

2.1. Reagents

MTT was purchased from Sigma. High-glucose Dulbecco's Modified Eagles Medium, penicillin–streptomycin, fetal bovine serum and 5× trypsin/ethylene diamine tetraacetic acid were purchased from Gibco. HeLa and PC12 cells were obtained from Pasteur Institute, Tehran, Iran. Paclitaxel manufactured by EBWE Company was purchased from market.

2.2. Sponge material

The sponges [*Ircinia echinata* (*I. echinata*), *Dysidea avara* (*D. avara*), *Axinella sinoxea* (*A. sinoxea*), *Haliclona tubifera* (*H. tubifera*) and *Haliclona violacea* (*H. violacea*)] were collected from the depths of 15–17, 10, 7–15, 15–20 and 15–20 m in the Persian Gulf (Hengam Island), respectively, and identified by Melika Nazemi.

2.3. Preparation of alcoholic extracts

The sponges were dried by freeze drier and powdered. About 50 g of powder was separated and extracted with methanol–dichloromethane (1:1) at room temperature for 48 h. Then, the solution was filtered. This procedure was repeated three times and the resulting extracts were combined. The combined extracts were concentrated in vacuum and the residue was dried at 40 °C in incubator. This dried sample was stored at 4 °C until use.

2.4. Cell culture

HeLa and PC12 cells were cultured as monolayers in 75 cm² culture flasks in high-glucose Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum. The medium was changed every 48 h and sub-cultured with 5× trypsin/ethylene diamine tetraacetic acid. Cells were maintained at 37 °C under a humidified atmosphere containing 5% CO₂.

2.5. Cell viability

Cytotoxicity was assessed using the colorimetric MTT assay. Briefly, the cells were seeded in 96 well tissue culture plates and incubated with 50 µg/mL of each extract of sponges for 72 h. Then in another 96 well tissue culture plates, the cells were seeded and incubated with increasing concentrations of extract of *I. echinata* (1, 10, 25 and 50 µg/mL) for 72 h. About 20 µL of MTT in phosphate-buffered saline (5 mg/mL) was then added to each well. Following 4 h incubation at 37 °C, the medium/MTT mixtures were removed and the blue insoluble formazan product formed by living cells was dissolved in dimethyl sulfoxide (DMSO) (100 µL/well). The optical density of the wells was then measured with a microplate reader at 570 nm. All tests were performed in triplicate and cytotoxicity was expressed as IC₅₀, which was calculated using GraphPad Prism 5 software and presented as mean ± SEM of six independent experiments with three replicates for each concentration. Paclitaxel was used as a positive control.

2.6. Preliminary fractionations and metabolic fingerprinting using high performance liquid chromatography (HPLC)–UV

2.6.1. Instruments

Chromatographic measurements were carried out using a Knauer HPLC system (Berlin, Germany) equipped with a K-1001 HPLC Pump and a UV detector K-2800 which was set at 254 and 280 nm. The other HPLC equipments included a Knauer K-1500 solvent organizer and a Knauer K-500 degasser. Adjustments of pH of solutions were determined by a 3030 Jenway pH meter (Leeds, UK). The column used was a C18 (250 mm × 21.2 mm, 5 µm) from Capital (Broxburn, UK). The polyamide solid-phase extraction (SPE) cartridge (Chromabond PA) (3 mL/500 mg) was obtained from Macherey–Nagel (Düren, Germany). The system was equipped with Chromgate HPLC software, version 3.3.

2.6.2. Sample preparation and SPE

Sponges were directly collected from Persian Gulf without any commercial manipulation in order to avoid potential changes during handling, storage and processing. The SPE polyamide cartridge was sequentially conditioned with 5 mL of *n*-hexane, 5 mL of methanol and 10 mL of double distilled deionized water without allowing the cartridge to be dried. The filtrate was passed through the cartridge, washed by 8 mL water/methanol (80:20 v/v, adjusted at pH = 3 with concentrated HCl) to remove interferences and eluted with 4 mL HPLC grade methanol. The elution was dried by blowing N₂ stream and dissolved in 1 mL of acetonitrile, filtered through a 0.45 µm syringe filter and injected into the HPLC system.

2.6.3. Chromatographic conditions

Fractionation of *I. echinata* extract was performed using reversed-phase HPLC with a linear gradient mobile phase of 20%–100% methyl alcohol in H₂O including 0.05% trifluoroacetic acid with a flow rate of 9 mL/min at room temperature. About 300 mg of *I. echinata* was dissolved in 2 mL DMSO. The sample was loaded on an ACE 5 C18 column (5 µmol/L, 250 mm × 21.2 mm) (Advanced Chromatography Technologies

Limited, Aberdeen, Scotland) and injection volume was 1 mL and analyses were monitored at 254 and 280 nm. To validate the gradient method, a standard sample containing uracil, methyl *p*-hydroxybenzoate, ethyl *p*-hydroxybenzoate and benzophenone was injected repeatedly until four thoroughly separated peaks were observed. To separate the fractions, we injected 1 mL of the prepared extract of *I. echinata* into the HPLC.

2.7. Statistical analysis

All the data were analyzed using One-way ANOVA followed by Tukey–Kramer's multiple comparison test. *P* values < 0.05 were considered significant.

3. Results

3.1. Effect of marine sponges extracts on cell viability in HeLa

As shown in Figure 1, *I. echinata* incubated at 50 µg/mL significantly decreased cell viability to 47% of control (*P* < 0.001), and *D. avara* at 50 µg/mL significantly decreased

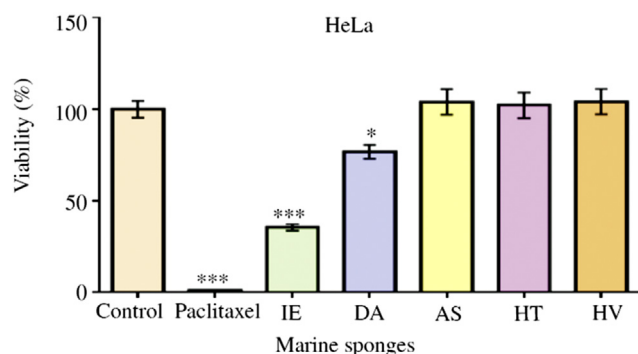


Figure 1. Effect of alcoholic extracts of marine sponges on cell viability in HeLa cells.

Cells were incubated with 50 µg/mL sponge extract for 72 h. Viabilities were determined by MTT assay. Results were expressed as mean ± SEM of six independent experiments. IE: *I. echinata*; DA: *D. avara*; AS: *A. sinoxea*; HT: *H. tubifera*; HV: *H. violacea*; *: *P* < 0.05; ***: *P* < 0.001 compared to control.

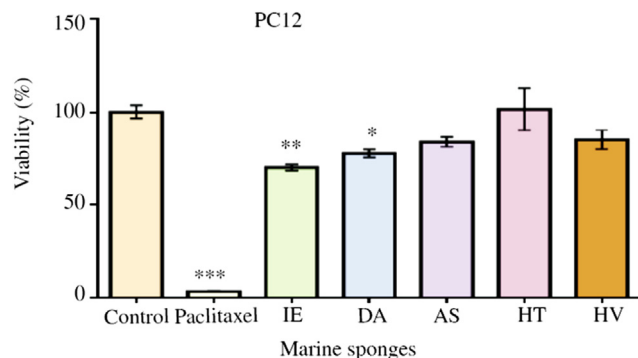


Figure 2. Effect of alcoholic extracts of marine sponges on cell viability in PC12 cells.

Cells were incubated with 50 µg/mL sponges extracts for 72 h. Viabilities were determined by MTT assay. Results were expressed as mean ± SEM of six independent experiments. *: *P* < 0.05; **: *P* < 0.01; ***: *P* < 0.001 compared to control.

cell viability to 76.64% of control (*P* < 0.05). However, the other extracts of sponges were not able to reduce cell growth significantly as compared with negative control including cancer cells and medium without drug. Paclitaxel as positive control showed the viability of 3.7% at a concentration of 0.6 µg/mL (*P* < 0.001).

3.2. Effect of marine sponges extracts on cell viability in PC12

As shown in Figure 2, *I. echinata* incubated at 50 µg/mL significantly decreased cell viability to 68% of control (*P* < 0.01), and *D. avara* significantly decreased cell viability to 79.03% of control (*P* < 0.05). However, the other extracts of sponges were not able to reduce cell viabilities significantly. Paclitaxel as positive control reduced the viability to 4.5% at a concentration of 0.6 µg/mL (*P* < 0.001).

3.3. Effect of *I. echinata* extract on cell viability in HeLa and PC12 cells

As shown in Figure 3, *I. echinata* significantly decreased cell viability to 47% (*P* < 0.001) and 68% (*P* < 0.01) in HeLa and PC12, respectively at 50 µg/mL, but the other concentrations did not show significant effect. Among the tested sponges, *I. echinata* showed the highest activity against HeLa and PC12, so it was further fractionated.

3.4. Fractionation of *I. echinata* extract using HPLC

It had experimentally been revealed that compounds with retention times between those of 4-hydroxymethyl benzoate and benzophenone have suitable physicochemical properties to be absorbed through gastrointestinal tract and are orally bioavailable. Peaks of compounds from marine extracts that appeared in the first 8 min in HPLC chromatograms were not typically evaluated due to high quantities of marine salts (Figures 4 and 5).

To separate the fractions, we injected 1 mL of the prepared extract of *I. echinata* into the HPLC and the fractions 1 and 2 were isolated according to chromatogram in the wavelength of 254 nm.

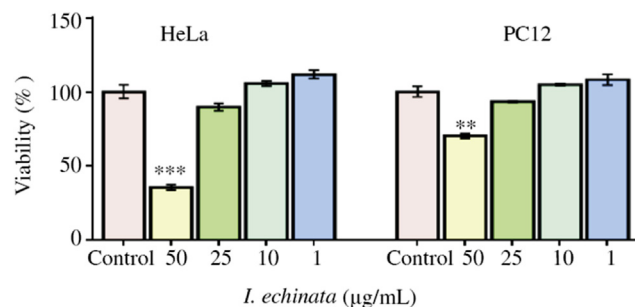


Figure 3. Effect of increasing doses of alcoholic extract of *I. echinata* on cell viability in HeLa and PC12 cells.

Cells were incubated with 1, 10, 25 and 50 µg/mL of *I. echinata* extract for 72 h. Viabilities were tested using MTT assay. Results were expressed as mean ± SEM of six independent experiments. **: *P* < 0.01; ***: *P* < 0.001 compared to control.

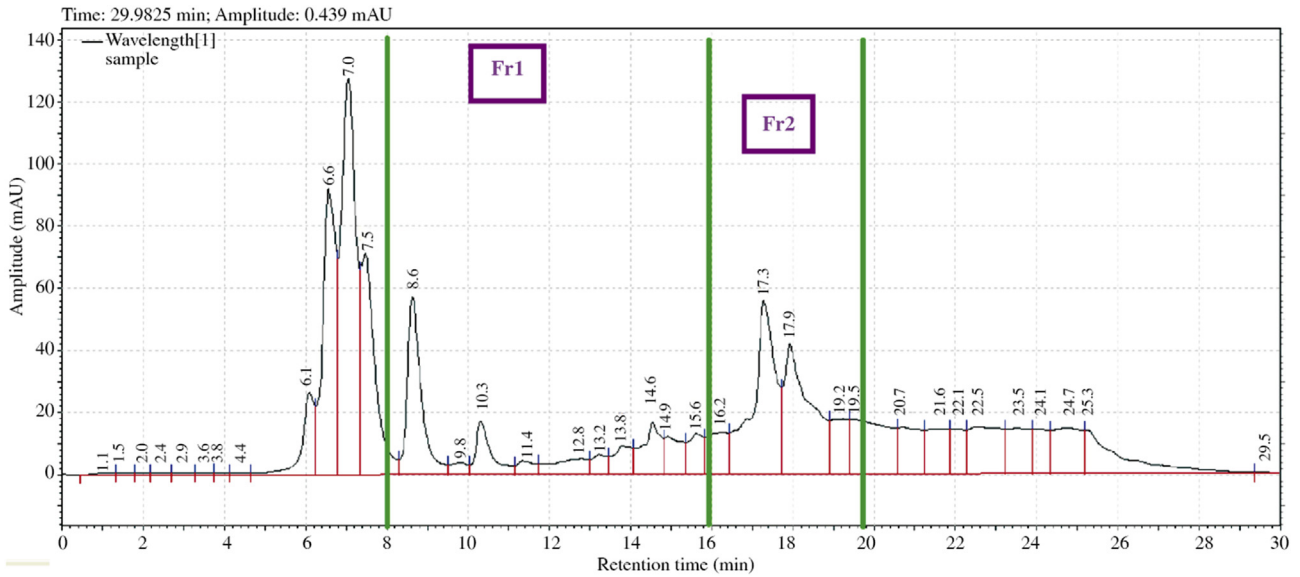


Figure 4. The HPLC fingerprint analysis of *I. echinata* extract at 254 nm for 30 min run time.

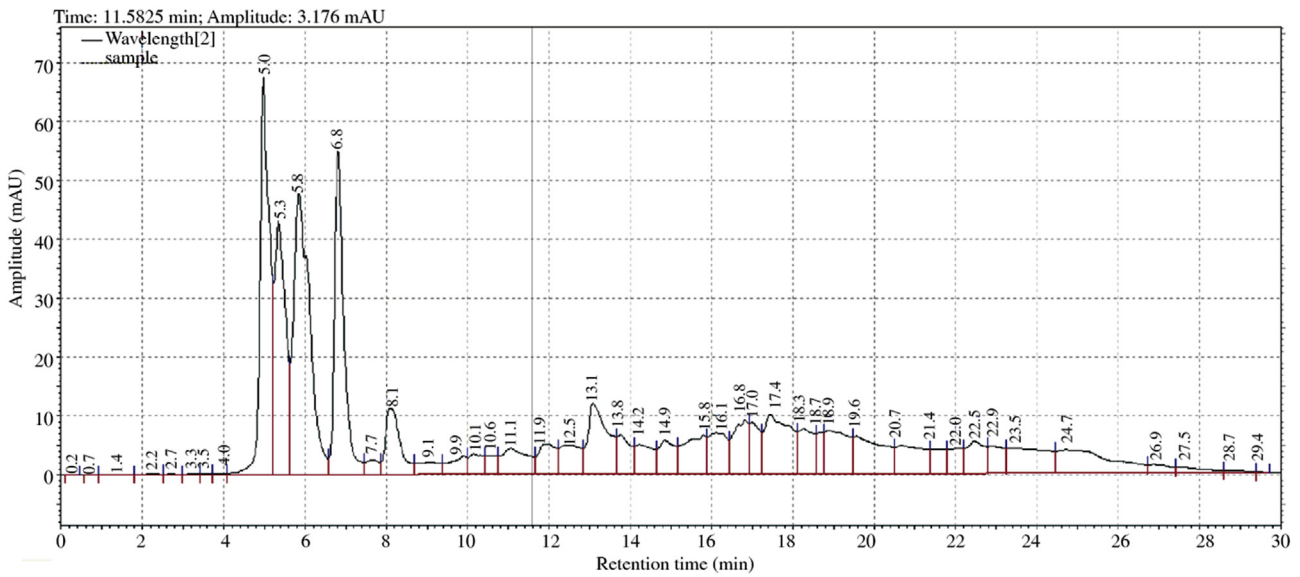


Figure 5. The HPLC fingerprint analysis of *I. echinata* extract at 280 nm for 30 min run time.

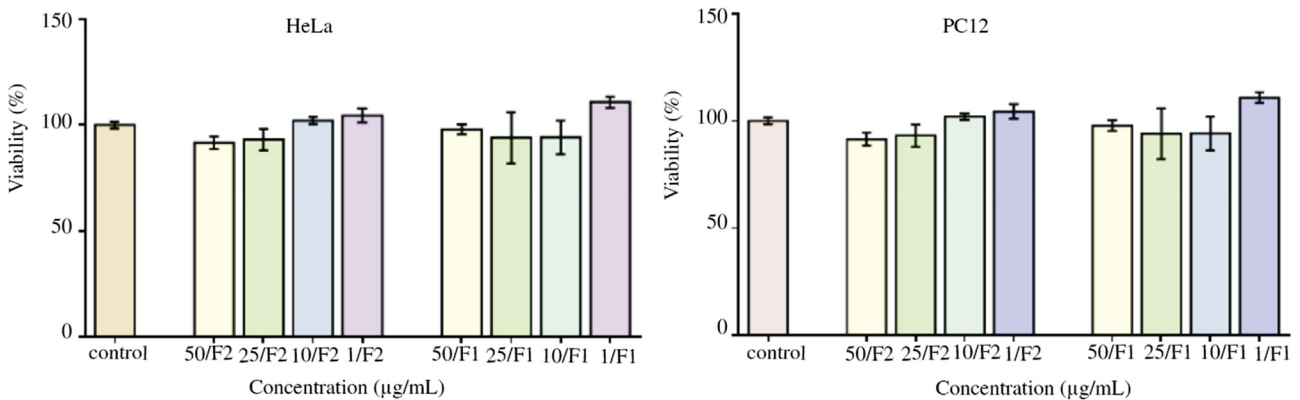


Figure 6. Effect of fractions of alcoholic extract of *I. echinata* on cell viability of HeLa and PC12 cells. F1: Fraction 1; F2: Fraction 2. Cells were incubated with 1, 10, 25 and 50 µg/mL extract of sponge for 72 h. Viabilities were tested using MTT assay. Results were expressed as mean ± SEM of six independent experiments.

3.5. Effect of fractions from *I. echinata* extract on cell viability in HeLa and PC12 cells

As shown in Figure 6, fractions from *I. echinata* extract exhibited no effect at different concentrations on HeLa and PC12 cells proliferation.

4. Discussion

Cancer is a growing health problem in both developing and developed countries. According to the recent report of World Health Organization [6], 8.2 million patients died from cancer in 2012. It has been also estimated that the number of annual cancer cases will have been increased from 14 million in 2012 to 22 million within the next two decades [6]. Cervical cancer is the second most common cancer in Iran. Unfortunately, this type of cancer is developing rapidly [6]. Currently, the main treatments for cancer are chemotherapy, radiotherapy and surgery. Some of the most used chemotherapy drugs include antimetabolites (e.g. methotrexate), DNA-interactive agents (e.g. cisplatin, doxorubicin), antitubulin agents (taxanes), hormones and molecular targeting agencisplatin [7]. However, clinical uses of these drugs are accompanied by several unwanted effects such as hair loss, suppression of bone marrow, drug resistance, gastrointestinal lesions, neurologic dysfunction and cardiac toxicity [7–9]. Therefore, the search for new anticancer agents with better efficacy and less side effects has been continued.

Natural compounds are good sources for the development of new remedies for different diseases. These compounds have been applied for treating and preventing human disease such as infection, cancer, pain and immune disease.

The ocean occupies about 70% of the earth and contains a huge number of marine organisms. Although collecting and identifying marine organisms were difficult to researchers, but marine resources are still attractive for pharmaceutical industries. Among marine resources, marine sponges are known to have about 15000 species worldwide. This study describes a screening for cytotoxicity of extracts of five marine sponges collected from Persian Gulf. Our findings revealed that among the extracts of five sponges, the extracts of *I. echinata* and *D. avara* inhibited cell growth at dose of 50 µg/mL with different potency. In our study, we separated fractions on the basis of quadruple standard including uracil, 4-hydroxymethyl benzoate, 4-hydroxy ethyl benzoate and benzophenone. According to the retention times of these compounds, we were able to identify and isolate compounds with drug-like properties and avoid isolating very polar compounds including salts that are commonly present in marine extracts. Taken together, the isolated fractions did not show significant cytotoxic properties. But it should be investigated whether fractions from HPLC columns in the first 8 min might contain cytotoxic compounds. One may suppose that cell growth can be affected by change in osmolality due to high amount of salts in total extracts. It should be pointed out, however, the low concentration 50 µg/mL of total extract that we used in cytotoxicity assay is not able to affect osmolality of cell culture. Therefore we expect that cytotoxic effects observed for *I. echinata* could be due to its cytotoxic compounds.

Recent years, marine organisms have been recognized as a resource for novel bioactive compounds. Marines have been used for medicinal purposes in India, China and the Near East. Marine is composed of biomolecules such as peptides, enzymes, enzyme

inhibitors and lipids which have the potential for the prevention and treatment of cancer. Purified peptides from this source have been shown to have cytotoxic effects on several human cancers such as pancreatic, breast, bladder and lung cancers. Different studies have reported cytotoxicity effects of sponges on cell lines. *Hyrtios* spp. increased cell death in colorectal carcinoma RKO cell line by the induction of p53 and p21 proteins [10]. In another study, the marine sponge *Aurora globostellata* exhibited strong cytotoxic activities against a panel of cancer cells [11]. The anticancer properties of marine sponges might be due to the presence of the active secondary metabolites such as alkaloids and quinine derivatives [12]. Alkaloids are microtubule intermediary agents which can bind with beta-tubulin, thus preventing the cell from making the mitotic spindle fibers which is necessary to move the chromosome around when the cell divides [13], inhibiting topoisomerase [14], mitochondrial damage and inducing the release of cytochrome C and apoptosis inducing factors. Moreover, quinine derivatives can interfere the DNA and RNA replication and mitochondrial oxidative pathways or the formation of super oxide, peroxide and hydroxide radicals as toxic products in the cell line [11]. Triterpenoids are the most abundant secondary metabolite present in marine sponges. A large number of triterpenoids are known to exhibit cytotoxicity against a variety of tumor cells as well as anticancer efficacy in preclinical animal models [15].

Ultimately, further investigation is needed to determine the mechanism of action for cytotoxic activity of *I. echinata* as well as characterization of active compounds in its extract.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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