

Cytotoxic Activity of Cytoplasmic Extract and Medium Culture of *Nodularia harveyana* on Melanoma Cell Line

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Abstract

Cyanobacteria produce a wide range of biologically active compounds. Among the cyanobacterial metabolites, cytotoxins are of particular interest because of their potential to kill target cancer cells. The present study was undertaken to isolate *Nodularia harveyana* from Ardakan County (Yazd Province, Iran) soil and determine the cytotoxic effect of cytoplasmic extraction and medium culture of its on the human melanoma cell line. Nitrate-free BG11 medium was used for preparing an axenic monoalgal culture of *Nodularia harveyana* ISB112. In addition, 3- to 5-week-old cytoplasmic extract and medium used for the cytotoxic study on human melanoma cell lines (B16). The cytotoxic effects of cytoplasmic extraction, and medium culture at the concentration ranging from 5 to 50 $\mu\text{g ml}^{-1}$ increased significantly in a concentration-dependent manner ($p < 0.05$). The 4- and 5-week-old cytoplasmic extract of *Nodularia harveyana* ISB112 was more effective than the 3-week-old extract and the viability percent of cells were 52.57% and 52.35%, respectively. The

results showed that the medium culture activity in the fifth-week of growth was higher than other periods and the cell's viability percent was 48.84% ($p \leq 0.05$). Overall, the results suggested that *Nodularia harveyana* ISB112 can kill tumor cells that can be used as a treatment for cancer disease.

Keywords: *Nodularia harveyana*, Cyanobacteria, Cytotoxic, Cytoplasmic extract

Introduction

Cyanobacteria, gram-negative photosynthetic prokaryotes, are a crucial component of microbial communities in varied environments and ecosystems. These organisms can grow in different habitats, especially extreme habitats, and tolerate extreme environments (Zanchett et al., 2013).

Cyanobacteria synthesize variable bioactive compounds including antifungal, anticancer, anti-HIV, and antibacterial properties (Abed et al. 2009; Gademann and Portmann, 2008; Wase and Wright, 2008).

Furthermore, it was reported that certain cyanobacterial compounds have poten-

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tial to kill tumor cells (Costa et al., 2012). The drugs used to treat cancer have many unwanted side effects, but natural products from cyanobacteria are imperative and harmless for treatment of this disease (Sithranga Boopathy and Kathiresan, 2010). The cyanobacterial natural compound's in cancer cell lines is included different mechanisms such as changing the mitochondrial membrane potential, interaction with cytoskeletal structures, or suppression of different eukaryotic enzymes (Barchi et al., 1983; Mackintosh et al., 1995; Patterson et al., 1993; Rai et al., 2018).

Cyanobacteria include the genera *Anabaena*, *Oscillatoria*, *Nostoc*, *Nodularia*, *Cylindrospermopsis*, *Lyngbya* and *Microcystis* produce cyanotoxins such as hepatotoxins, neurotoxins, cytotoxins, and dermatotoxins (Welker and von Dohren, 2006).

Recent researches indicated that *Oscillatoria boryana* and *Oscillatoria margaritifera* produce cytotoxins with cytotoxic effects against cell lines of human breast cancer and lung cancer, respectively (Nair and Bhimba, 2013; Mevers et al., 2011).

Furthermore, Gunasekera et al. (2011) studied on cocosamides A isolated from *Lyngbya majuscula* that exhibited effective cytotoxic activity against MCF7 and HT-29 cell lines. The genus *Nodularia* Mertens (Bornet and Flahault, 1888), belonging to Nostocales including filamentous and heterocystous cyanobacteria. Indeed, Nostocals consist of distinct ecological groups; planktonic types with aerotopes (gas vesicles), benthic and soil types without aerotopes (Komarek,

2013). Seven species of *Nodularia* were identified, which some of species are cytotoxic to different mammalian cell lines, making them a novel candidate for pharmaceutical research and cytotoxic agents. Indeed, some species of this genus such as *Nodularia harveyana* Thuret ex Bornet & Flahault are present in soil habitats or as a benthic form in aquatic ecosystems (Laamanen et al., 2001). The main purpose of this study was to compare in vitro cytotoxicity effects of cytoplasmic extract and medium culture of *Nodularia harveyana* ISB112 that isolated from Ardakan County (Yazd Province, Iran) soil. However, to our knowledge, little information is available on the cytotoxic effect of *Nodularia harveyana* and the accumulation of active compounds in the algal biomass or culture medium.

Material and methods

Isolation, purification and identification of species

Soil sample collected from Ardakan County (32° 17' 30" N, 54° 01' 15" E), for isolating *Nodularia harveyana* ISB112 according Rangaswamy (1966). Next, the sieved soil transferred to sterile Petri dishes containing sterile liquid nitrate-free BG-11 medium and incubated at 25 ± 2 °C under artificial illumination ($74 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) with a 12/12 h light/dark cycle for three weeks. Then, the colonies of cyanobacteria transferred to plates containing solid nitrate-free BG-11 medium for purification (Stanier et al., 1971). Indeed, the semi-permanent slides of colonies were prepared, and

the morphometric study performed by light microscopy (Olympus, Model BH-2) using identification keys (Desikachary, 1959; Prescott, 1970; Wehr et al., 2002; John et al., 2002; Komarek, 2013).

Furthermore, sequencing of the 16S ribosomal RNA (rRNA) gene is used as a molecular method to determine the cyanobacterial species. For this purpose, genomic DNA extracted from the cyanobacteria fresh mass by a Genomic DNA extraction kit (AccuPrep, Bioneer). Next, Polymerase chain reaction (PCR) amplification carried out using A2 (AGAGTTTG ATCCTGGCTCAG) and S8 (TCTACGCATTCAC CGCTAC) as primers (Ezhilarasi and Anand, 2009). Then, PCR products were sequenced using the Sanger sequencing method (Sanger and Coulson, 1975) by the Pishgam Biotech Company (Tehran, Iran).

Cyanobacterial culture

Purified *Nodularia harveyana* ISB112 was cultured under standard conditions using a nitrate-free BG-11 medium. The liquid culture of strain, was incubated in a culture chamber at 25 ± 2 °C for three, four, and five-weeks under artificial light illumination ($74 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) with a 12/12 hr light-dark cycle (Riahi et al., 2017). Next, three replications were carried out for each period. Then, biomass and culture media were separated by centrifugation ($10000 \times g$) and stored at -20 °C until the cytotoxicity experiments.

Preparation of cyanobacterial cytoplasmic extract

The cytoplasmic extract prepared according

to Ahmed et al., (2017). Next, the biomass was washed with distilled water and transferred to a tube, after that stored in liquid nitrogen (-80 °C) for 5 minutes. Then, the material was poured into warm water (50 °C) for 5 min, at the end centrifuged ($4000 \times g$) for 3 min. Finally, the supernatant separated and sterilized by passing them through a $0.22 \mu\text{m}$ filter and stored at -20 °C before use in the cytotoxicity analysis. Indeed, extraction process carried out in three replications.

Cancer cell culture

Melanoma cell line (B16) obtained from Pasteur Institute of Iran. The 1.5×10^6 cells ml^{-1} in RPMI medium supplemented with 11 mM sodium bicarbonate, 2 mM l-glutamine, 100U ml^{-1} penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin, and 5% fetal bovine serum. The cell line was plated ($200 \mu\text{l Well}^{-1}$) into 96-well microplates (Nunc, Wiesbaden, Germany); 3×10^5 cells per well and incubated for 4 h in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 (Stockert et al., 2012).

Cytotoxic assay protocol

Cytotoxic activity was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sladowski, 1993). Next, different concentrations of the cytoplasmic extract and culture medium added to triplicate wells. After 48 h incubation at 37 °C, $20 \mu\text{l}$ of MTT solution (5 mg ml^{-1} in PBS) was added to each well and incubated for 4 h at 37 °C and 5% CO_2 . Further, MTT dye was metabolized by viable cells to give a purple formazan product. After this, the medium removed gently and

100 μ l of acidic isopropanol (0.04 M HCl in isopropanol) added to dissolve the formazan crystals. Then, the absorbance rate measured at 570 nm and the percentage of cell survival calculated. Finally, the mean and standard deviation (SD) values from at least three different experiments analyzed.

Determination of cell viability under an inverted microscope

The viability of cells was determined under an inverted microscope (Zeiss Axio Vert. A1 inverted microscope), at magnification 40X after being treated by fifth-week-old (*Nodularia harveyana* ISB112) cytoplasmic extract at 50 μ g ml⁻¹ concentration for 48 h (Horobin, 1982 a, b).

Statistical analysis

All of the statistical analyses performed using the SPSS software version 16 (Package for the Social Sciences, SPSS Inc., USA). One-way analysis of variance (ANOVA) and TukeyHSD test with a significance lev-

el of 0.05 used to determine whether there were significant differences between the cytoplasmic and culture medium tests. Histograms have drawn using GraphPad Prism 8.4.2 (GraphPad Software Inc., San Diego, CA, USA).

Results

The microscopic image of *Nodularia harveyana* ISB112 used in this study show the heterocystous filaments (Fig. 1).

The cytotoxic activity of the cytoplasmic extract, and culture medium of *Nodularia harveyana* ISB112 for third-, fourth- and fifth-week-old at various concentrations (5, 10, 15, 25, and 50 μ g ml⁻¹) against melanoma cell line (B16) analyzed by commercial MTT assay.

The cytotoxic effects of cytoplasmic extract and medium culture at different concentrations are showed in Fig. 2 A, B. The results indicated that the cell-killing ability of sam-

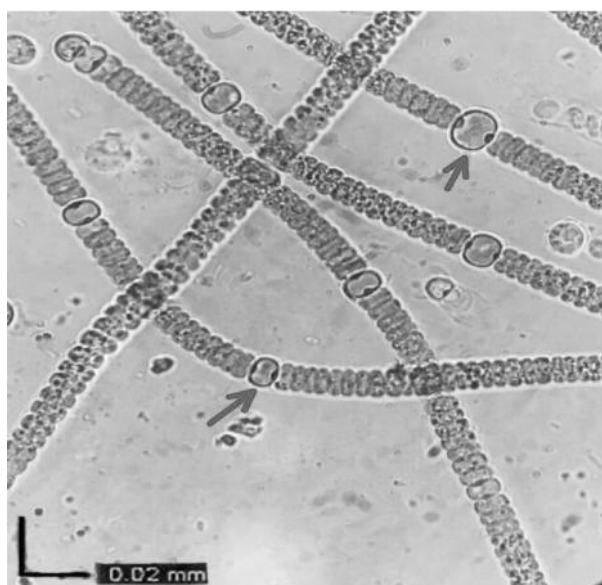


Fig. 1. *Nodularia harveyana* ISB112, the heterocysts are marked with arrows

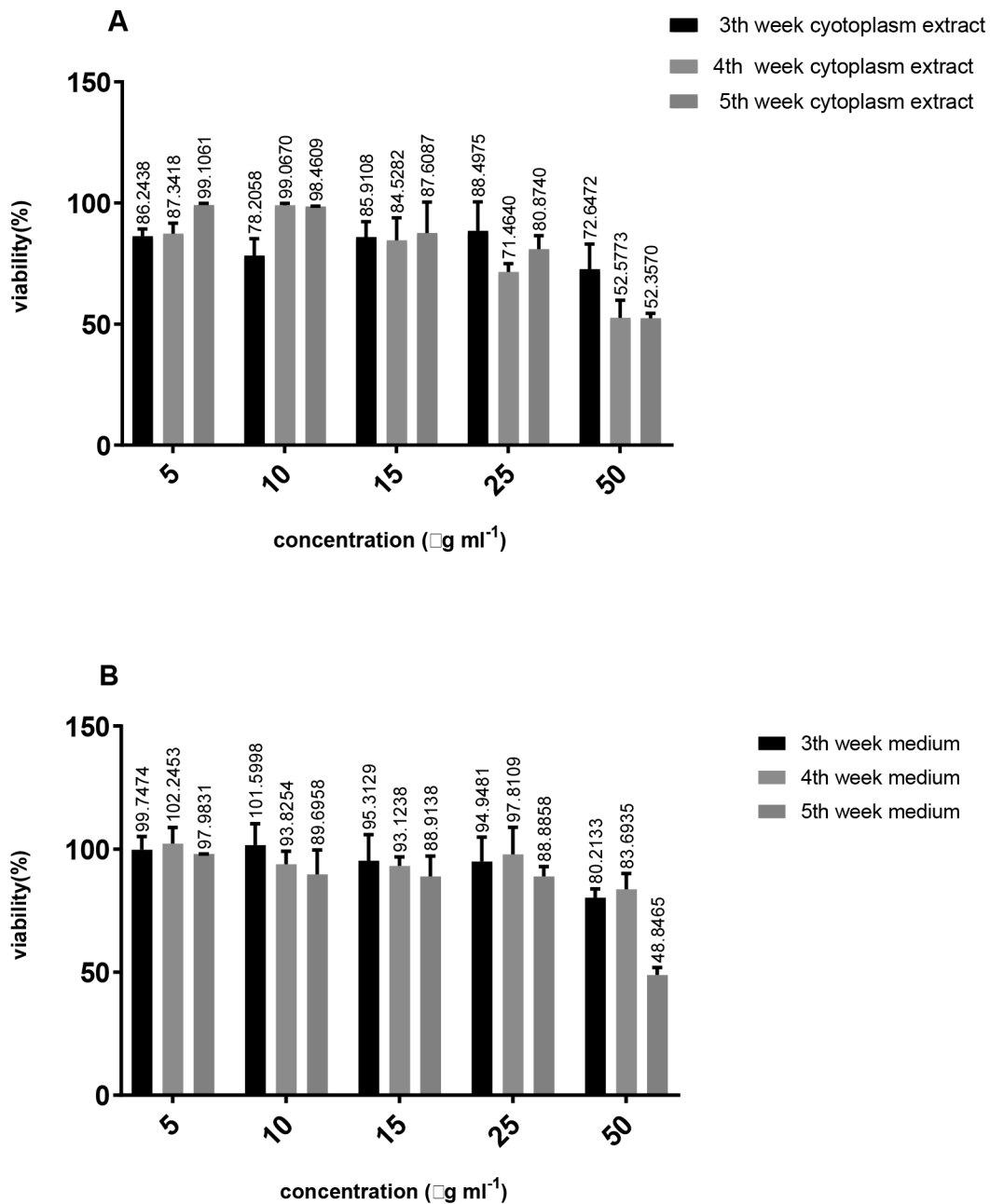


Fig. 2. Cytotoxicity of *Nodularia harveyana* ISB112 during a period of three, four, and five weeks of cyanobacteria growth to human melanoma cell line (B16) by MTT assay.

ples increased significantly at the concentration ranging from 5 to 50 µg ml⁻¹ in a concentration-dependent manner.

Indeed, the viability percent of cells treated with three weeks, four weeks and five weeks in cytoplasmic extraction, at the concentration of 50 µg ml⁻¹ were 72.64%, 52.57%,

and 52.35%, respectively. Further, the cytotoxic activity of the fourth- and fifth-week-old was higher than the third-week-old cytoplasm extraction ($p \leq 0.05$). Additionally, among culture media, at the concentration of 50 µg ml⁻¹ the viability present of cells treated by third-, fourth- and fifth-week-old were

80.21%, 83.69%, and 48.84%, respectively. The result showed that cytotoxic activity increased in older culture (fifth-week) as compared to younger one ($p \leq 0.05$). Furthermore, the results indicate that *Nodularia harveyana* ISB112 can release the metabolites that have cytotoxic effects on the culture medium.

Moreover, the effect of fifth-week-old cytoplasmic extract on melanoma cells (B16) viability and the amount of MTT formazan shows in Figure 3 is directly proportional to

the number of living cells.

Besides, melanoma cells (B16) exposed for 48 h to fifth-week-old cytoplasmic extract, indicating that these cells underwent lysis and complete degeneration after treatment with the cyanobacterial extract (Fig. 3 c). According to the amount of MTT formazan that is directly proportional to the number of living cells, melanoma cells after treatment for 48 h show lower MTT formazan crystals than control cells (Fig. 3 c, d).

The B16 cells were incubated for 48 h with

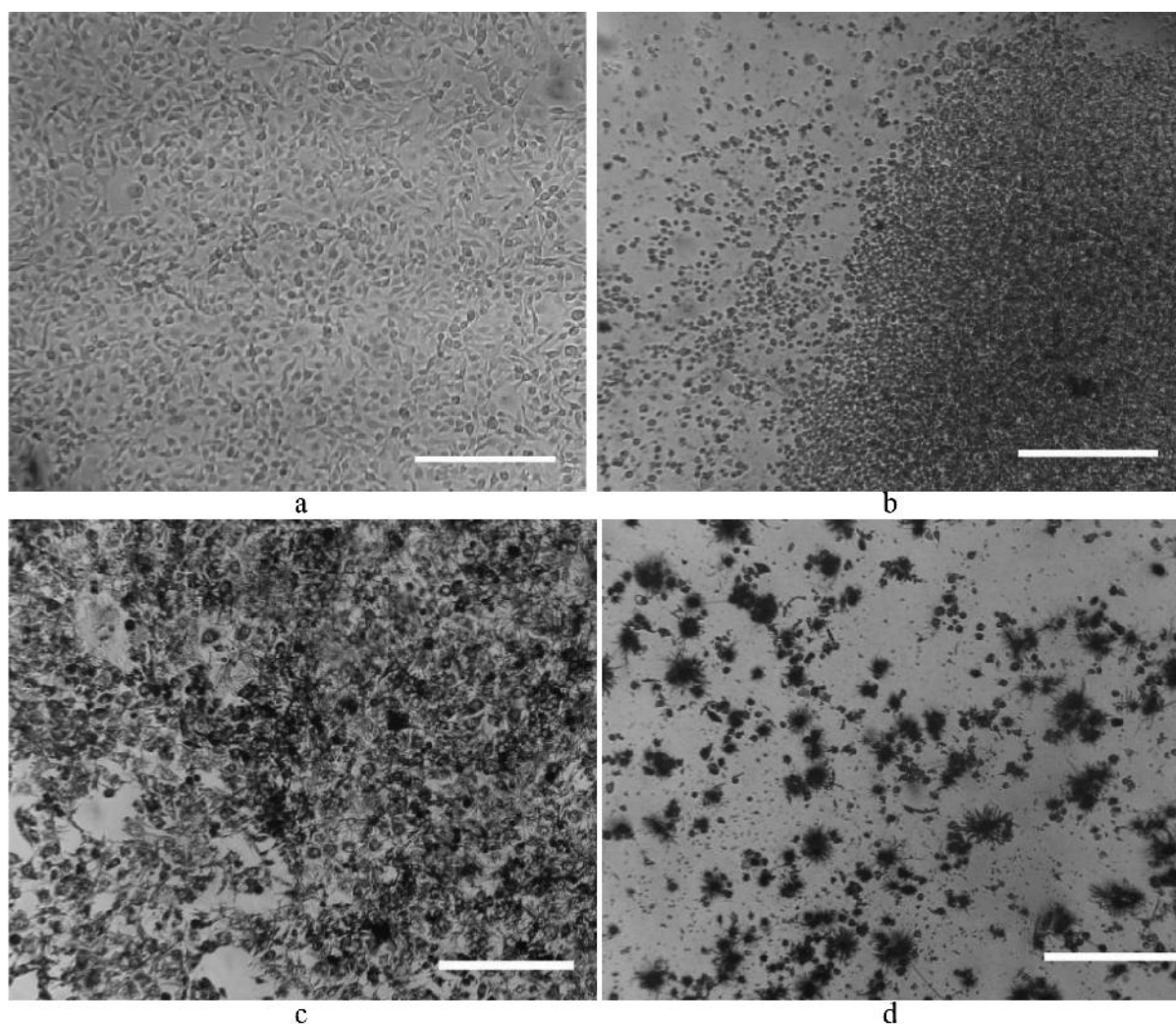


Fig. 3. Cells observation under an inverted microscope; (a) melanoma cells (B16) before MTT test, (b) Melanoma cells after treatment with the extract, (c) melanoma cells showing formazan crystals- control group, (d) melanoma cells showing formazan crystals- test group 48 h after treatment. Scale bar: 20 = μm

cyanobacterial cytoplasmic extract, the final concentration of the extract was 50 µg ml⁻¹ (Figure 2 a). (B) The B16 cells were incubated for 48 hours with cyanobacterial medium, the final concentration of the medium was 5 0µg ml⁻¹. Values are means of three replicates with standard error (Figure 2 b).

Discussion

Cyanobacteria are a great source of biological products. Among a wide variety of biologically active compounds that produced by cyanobacteria, cyanotoxins are of particular interest. Furthermore, cyanobacterial metabolites, which are cytotoxic, considered for the future development of new drugs in a variety of diseases (Volk, 2005; Patterson et al., 1994).

Indeed, most of the biologically active compounds produced by cyanobacteria accumulated in the algal biomass and then release during growth process into the environment (Jaki et al., 2001).

Therefore, in this study for cytotoxic investigation, cytoplasmic extract of algal biomass and culture media of *Nodularia harveyana* ISB112 against melanoma cell line (B16) were tested. The cytoplasmic extract and culture media show cytotoxicity, but their activity was different based on the age of the algae in the culture medium. Likewise, this difference may be due to cell death in the old culture and release the metabolites produce in cell's cytoplasm into the culture medium. We found the metabolite production in the fourth- and fifth-week-old *Nodularia harveyana* ISB112 cultivation is higher than

in the third-week-old. Indeed, cytotoxicity observed at the fourth- and fifth-week-old cyanobacterial extraction, was much more effective and cytotoxicity carried out in a dose-dependent manner.

MTT assay widely use for the study of cytotoxicity and cell viability. Although the cell line cytotoxicity does not indicate in all animals, toxicity studies show that MTT assay correlated to human lethal doses (Surakka et al., 2005; Ekwall, 1999; Evans et al., 2001). However, the genus *Nodularia* is the producer of nodularin, a toxin known as a hepatotoxin and liver tumor initiator, but not all species of this genus produce nodularin (Ohta et al., 1994).

Rehakova et al. (2014) investigated 17 terrestrial and benthic *Nodularia* strains to produce nodularin but only *N. sphaerocarpa* PCC7804/SAG 50.79 produced nodularin. Several previous studies reported that *Nodularia sphaerocarpa* and *Nodularia spumigena* produce nodularin. Furthermore, researches on the benthic *N. harveyana* indicated that the strains were nontoxic, except for *N. harveyana* PCC7804, which produce an isoform of nodularin (Laamanen et al., 2001; Beattie et al., 2000; Moffitt et al., 2001; Moffitt and Neilan, 2004; Saito et al., 2001).

Surakka et al. (2005) indicate that benthic Baltic cyanobacteria contain potentially harmful cytotoxic compounds, even though they do not produce microcystin or nodularin. In their study, one *Nostoc* strain, five *Anabaena* strains, and two *Nodularia* strains were highly cytotoxic to human leukemia

cells. While, *N. harveyana* showed cytotoxic activity, *N. sphaerocarpha* was not toxic. Hrouzek et al. (2005) investigations on cytotoxic effects of methanol extracts obtained from soil cyanobacteria (*Anabaena*, *Calothrix*, *Nodularia*, *Cylindrospermum*, *Tolypothrix* and *Trichormus*) on mammal cell lines (YAC-1, WEHI) showed the cytotoxic effect in 6 of 10 tested cyanobacterial extracts. However, *Anabaena torulosa* and *Cylindrospermum* sp. extracts were significantly cytotoxic and destruct cancer cells.

Furthermore, Acetone extracts of fresh biomass of *Nodularia harveyana* exhibited allelopathic activity against other cyanobacteria and green algae, antifungal activity against plant pathogens, and antibacterial activity against bacteria (Pushparaj et al., 1999).

In our study, *Nodularia harveyana* ISB112 was cytotoxic to the human B16 cell line. The cytotoxicity varied in different weeks of cyanobacterial growth. While, we find that the cytoplasmic extract of *N. harveyana* ISB112 in the fourth- and fifth- week-old of growth was higher than the third-week-old, the medium culture cytotoxicity in fifth-week-old showed the best result. Furthermore, it can happen due to the cyanobacteria cell's death in old cultures and the release of cytotoxic compounds into the environment (culture medium). However, the mechanisms of cytotoxicity and compounds characters need to further studies.

Subsequently, the present and previous studies on soil and benthic strains of *Nodularia harveyana* confirmed cytotoxic and inhibitory effects against cancer cell lines.

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