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# Bioactivity Potential of *Gracilaria salicornia, Padina boergesenii, Polycladia myrica*: Antibacterial, Antioxidant and Total Phenol Assays

**Zohreh Ramezanpour[1*], FatemeGhanbariPirbasti[2], Somayeh RasouliDogaheh[3]**

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**Abstract**

Antibacterial activities of MeOH and aqueous extracts of *Gracilaria salicornia* (C. Agardh) Dawson, *Padina boergesenii* Allander and Kraft, *Polycladia myrica* (S. G. Gmelin) Draisma, were examined against Gram-positive bacteria *Staphylococcus aureus* Rosenbach 1884, *Pseudomonas aeruginosa* (Schröter 1872) Migula 1900, and *Escherichia coli* (Migula 1895). Indeed, extracts of wet samples showed 5.3±0.58 to 34.3±0.6 mm antibacterial activity. Furthermore, antioxidant activities of algae evaluated using DPPH and ABTS radical scavenging methods. Whereas, in the DPPH method, aqueous extract of *Polycladia myrica* showed the highest antioxidant activity, MeOH and aqueous extracts of *Gracilaria salicornia* exhibited the lowest antioxidant activity. Beside, the antioxidant activity of extracts was higher using the ABTS method. Additionally, aqueous extracts showed the lowest $IC_{50}$ values in comparison to MeOH extracts. Total phenolic content of aqueous extract was 5.07±0.08 to 46.73±0.24 mg gallic acid /100 g higher than the MeOH extract. The MeOH and aqueous extracts of *Padina boergesenii* demonstrated the highest TPC among others.

**Keywords**: Antibacterial; Antioxidant; Total phenol; Bioactive compounds; Seaweed.

## Introduction

Seaweeds are marine macroalgae considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites (Bansemir et al., 2006; Yuan et al., 2005). Up to now almost 6000 species of seaweeds have been identified, and 2400 natural products have been isolated from macroalgae belonging to Rhodophyceae (red), Phaeophyceae (brown) and Chlorophyceae (green). Wijesinghe et al. (2012) reported that various groups of chemical compounds such as macrolides, peptides, proteins polyketides, sesquiterpenes, terpenes and fatty acids of seaweeds are effective in antibacterial activity. Wide variety of biological activities are attributed to marine macroalgae such as antimicrobial (Ibtissam et al., 2009; Rhimou et al., 2010;

1-Department of Ecology, International Sturgeon Research Institute,Agricultural research education and extension organization (AREEO), Rasht, Iran
2-Laboratory of Biotechnology, Clean Nature Explores Company, Rasht, Iran.
3-Faculty of Science, University of South Bohemia,ČeskéBudějovice, Czech Republic.
*E-mail address: zohreh66@gmail.com

Tajbakhsh et al., 2011b), antiviral (Kim and Karadeniz, 2011), antifungal (de Felício et al., 2010), anti-allergic (Na et al., 2005), anticoagulant (Shi et al., 2010), anti-HIV (Thuy et al., 2015), anticancer (Kim et al., 2011), larvicidal (Bianco et al., 2012), antiplasmodial (Ravikumar et al., 2011), and antioxidant activities (Devi et al., 2011), and antithrombotic and cellular proliferation activities (Guerra Dore et al., 2013).

Seaweeds and plants contain diverse phenolic compounds, such as flavonoids, phenolic acids, lignins and tannins, which are recognized as compounds that contribute in antioxidant activity (Grassmann et al., 2004). Some studies improved a correlation between phenolic content and antioxidant activity (Jiménez et al., 1999).

Gracilaria species (Rhodophyceae) are one of the most valuable macroalgae and the main source of high-quality agar (Praiboon et al., 2006). Indeed, Gracilaria is a source of some food in human consumption and in pharmaceutical components (Torres et al., 2019). Fouladvand et al., (2011) reported anti-leishmanial activity of *Gracilaria corticata* (J. Agardh) J. Agardh, collected from the Persian Gulf. Furthermore, different aspects of bioactive compounds of *Gracilaria corticata* such as antimicrobial activity against human pathogens (Govindasamy et al., 2011), antiobesity (Kannan et al., 2014a), antioxidant (Guaratini et al., 2012, Kannan et al., 2014b), anti-inflammatory (Shu et al., 2013), anti-yeast (Sasidharan et al., 2011) and anti-proliferation (Murugan and Iyer, 2012) were studied

Likewise, brown algae are rich sources of bioactive compounds that possess different biological activities. For example, *Padina boergesenii* Allander and Kraft has been shown to have a hepatoprotective effect against CCl4-induced liver damage and also antioxidant activity (Karthikeyan et al., 2012).

The objective of the study was finding out the antibacterial potentials of some common algae growing in coastal waters of the Persian Gulf in Qeshm Island. Bacterial resistance to available antimicrobial drugs has become a challenge (Brooks et al., 2007). Thus, discovering new, economical and effective antibacterial agents and antioxidants with natural bases sounds very interesting to researchers.

## Material and methods

*Material preparation*

Solvents, ascorbic acid (PubChem CID: 54670067) and Gallic acid (PubChem CID: 370) were purchased from Merck. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (PubChem CID: 74358), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (PubChem CID: 35687). Additionally, Folin-Ciocalteu's reagent supplied from Sigma and Aldrich companies. Biochemicals including nutrient agar and nutrient broth purchased from Merck. Finally, the absorption rate measured by spectrophotometer (Unico 2100, China). Indeed, freeze dry (Christ, Alpha 1-2 LD Plus) used for removing solvents.

*Sample collection*

*Gracilaria salicornia* (C. Agardh) Dawson, *Padina boergesenii* Allander and Kraft and *Polycladia myrica* (S.G.Gmelin) Draisma (formerly *Cystoseira myrica*) were collected from the Persian Gulf (55' 57 ºE and 26' 56 ºN) in Qeshm Island, Iran, from late October 2013 to December 2014. In addition, this region supports massive growth and distribution of these algae. *Gracilaria salicornia* and *Padina boergessenii* were identified by Ramezanpour (recorded in NCBI), and *Polycladia myrica* was identified by Dr. Michael Wynne. Then, the algae washed twice with seawater and tap water to remove epiphytes, freeze dried and stored in labeled plastic tubes at -19 °C.

*Preparation of extracts*

10 g of each fresh algal sample used for MeOH extraction (100 ml) by soxhlet extractor for 4 cycles. Then, the mixture was filtered through Whatman filter paper. Next, the extract was freeze dried and stored at −19 °C for further analysis. For aqueous extract, 10 g algal sample refluxed with deionized water (100 ml) for 2 hours. After that, the mixture was filtered through Whatman filter paper. Finally, the extract was freeze dried and stored at −19 °C for future analysis (Magaldi et al., 2004).

*Antibacterial assay*

The gram-positive bacteria *Staphylococcus aureus* Rosenbach 1884 and *Micrococcus luteus* Schroeter 1872 and gram-negative bacteria *Pseudomonas aeruginosa* (Schröter 1872)

Migula 1900 and *Escherichia coli* (Migula, 1895) Castellani and Chalmers 1919 were used as bacterial strains. The bacteria provided from microbiology laboratory of the University of Guilan. The antibacterial activities were evaluated by MeOH and aqueous extracts of *Gracilaria salicornia* (C. Agardh) Dawson, *Padina boergesenii* Allander and Kraft, and *Polycladia myrica* (SG. Gmelin) Draisma against both gram-positive and gram-negative bacterial strains. Additionally, the process performed using well-diffusion method (Balouiri et al., 2016). Then, a colony of each investigated organism was sub-cultured in order to obtain fresh bacteria on the nutrient agar plates at 37 ºC for 18 hours, and fresh suspensions of microorganisms (0.5 McFarland) were prepared. After that, a suspension of 30 µl bacteria added to each nutrient agar plate and was spread through plates by sterile spreader. Extract concentrations of 200, 100 and 50 $mg.ml^{-1}$ were prepared in dimethyl sulfoxide (DMSO). Next, each well received 30 µl of the corresponding concentration of extract. Later, plates incubated at 37 °C for 24 h. Finally, the inhibition zones were measured by coulisse and expressed in millimeters (mm). The experiments were performed in triplicate and the results are reported as mean± standard deviation (mean ± SD) of zone of inhibition. Tetracycline and Penicillin G were used as positive controls. DMSO and deionized water were used as negative controls.

*Antioxidant assays*

*DPPH Radical scavenging activity*

Antioxidant assay by DPPH assay was evaluated according to Jin et al.'s method (2012)

with some modifications. Extracts were prepared at the concentrations of 60, 30, 15, 7.5, 3.75 and 1.875 $mg.ml^{-1}$ in MeOH and in deionized water. 2, 2-diphenyl-2-picryl-hydrazyl (DPPH) was dissolved in MeOH to achieve a concentration of $6.25 \times 10^{-5}$ M. Then, 3.9 ml of DPPH solution added to 0.1 ml of extract solution at different concentrations. Next, the samples shaken vigorously and incubated at 25 ˚C for 30 min. Finally, the decrease of absorbance rate in resulting solution measured at 517 nm. In addition, MeOH used as a blank and 3.9 ml DPPH solution containing 0.1 ml of MeOH or deionized water used as the control. The experiments were conducted in triplicate.

Radical scavenging activity percentage calculated as follows:

DPPH radical scavenging rate (%) = $[(A_{control}-A_{sample})/ A_{control}] \times 100$ (1)

Were, $A_{control}$: the absorbance of all the reagents except the test extracts. Asample: the absorbance of the test extracts and all the reagents. $IC_{50}$ of the samples was calculated by plotting the radical scavenging percentage against the concentration of extracts.

*ABTS Radical Scavenging Activity*

The ABTS assay was performed following the procedure described by Jean et al., (2012). ABTS (7.4 mM) in MeOH and potassium persulfate ($K_2S_2O_8$) (2.6 mM) as an oxidizing agent in MeOH were mixed in equal volumes, then the $ABTS^{\bullet+}$ radical cation was produced by reacting stock solution for 12 hours in the dark at room temperature. In order to give the absorbance of 1.1 ± 0.02 at 734 nm, the stock solution was diluted with MeOH. Then, extracts were prepared at concentrations of 60, 30, 15, 7.5, 3.75 and 1.875 $mg.ml^{-1}$ in MeOH for MeOH and aqueous extracts. Next, 150 µl of sample solution was added to 3.0 ml $ABTS^{\bullet+}$ solution and this mixture was shacked and incubated in the dark for 2 hours. Finally, the absorbance of each solution was recorded at 734 nm. Moreover, MeOH and water were used as blanks. Indeed, Radical scavenging activity was calculated by formula of DPPH radical-scavenging assay and the $IC_{50}$ values of each compound for ABTS assay were calculated by plotting the inhibition percentage against concentration of the extracts.

*Total phenol content*

Evaluation of total phenolic content (TPC) was done by the Folin–Ciocalteu colorimetric method according to Skerget et al. (2005). Next, Folin-Ciocalteau reagent (2.5 ml) was diluted 1:10 with distilled water and added to 0.5 ml of extract (1 $mg.ml^{-1}$ of distilled water). After 2 minutes freshly prepared aqueous solution of $Na_2CO_3$ (2 ml, 20%) added and shacked vigorously, then, the mixture incubated at 25 ˚C for 30 minutes and the absorbance of solution was measured at 760 nm. In addition, blank solution contained 0.5 ml of distilled water instead of the extract. Furthermore, Gallic acid was used in 0.001 to 0.01 $mg.ml^{-1}$ concentrations as a standard. The results reported as equivalent to milligrams of gallic acid per 100 gram of dry weight extract (mg GAE/100 g). The experiments were performed in triplicate.

*Statistical analysis*

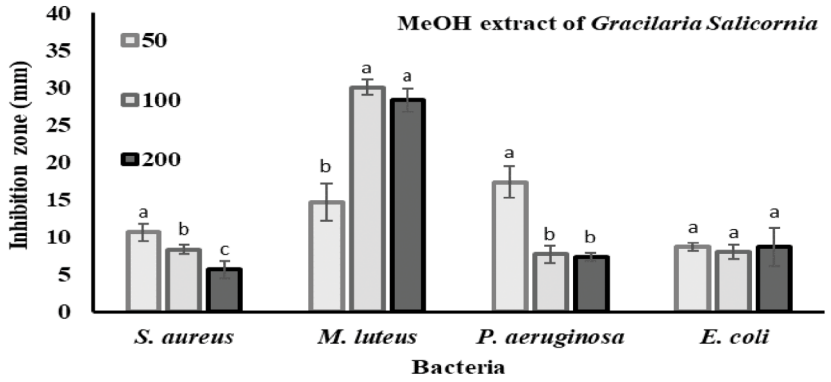
The statistical package SPSS 26 used for

analyses. Two-way ANOVA analysis was performed to compare the means. Values presented as mean ± SD of triplicate experiments.

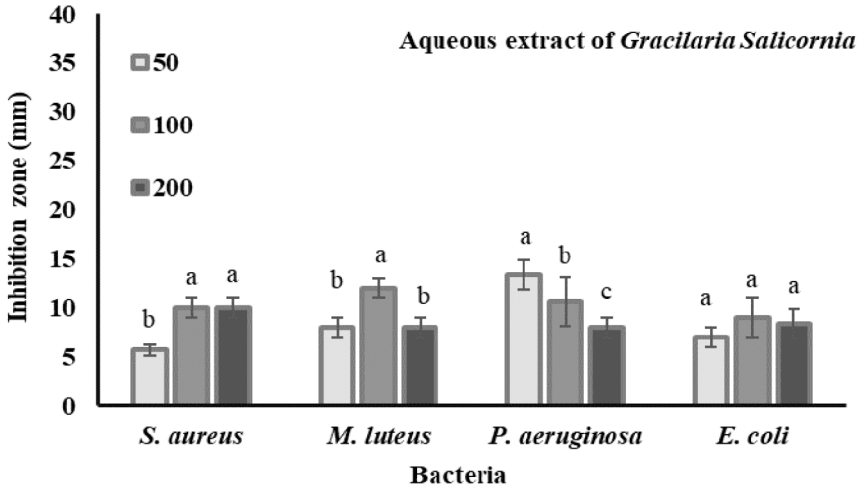
## Results

*Antibacterial assay*

Antibacterial effect of MeOH and aqueous extract of *Gracilaria salicornia* is shown in Figure 1a, b. Indeed, antibacterial activity of MeOH extract of *Gracilaria salicornia* against all the tested bacteria was not significantly different at 100 and 200 mg.ml$^{-1}$ concentrations except *Staphylococcus aureus*. Further, there was a significant difference between antibacterial activity of *Gracilaria salicornia* at 100 and 200 mg.ml$^{-1}$ concentrations against *Micrococcus luteus* and *Pseudomonas aeruginosa*. There was no significant difference between any concentrations of aqueous extract against *Escherichia coli*. Additionally, the highest antibacterial activity of *Gracilaria salicornia* aqueous extract was at 50 mg.ml$^{-1}$ concentration against *Pseudomonas aeruginosa*.



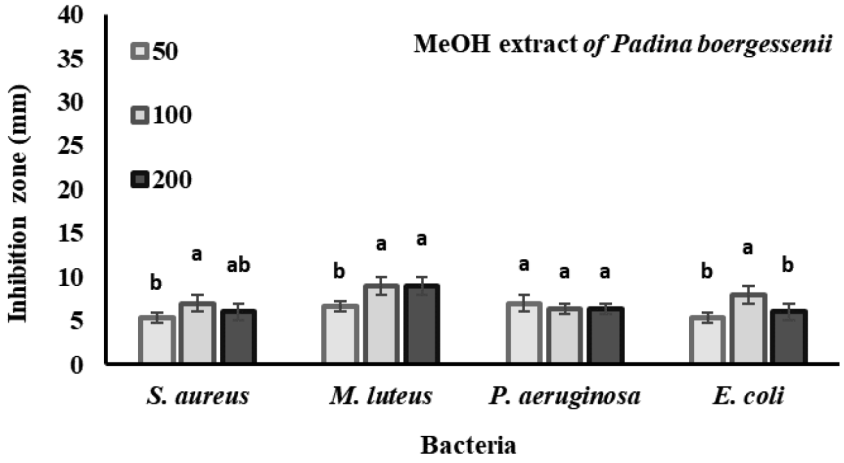

a



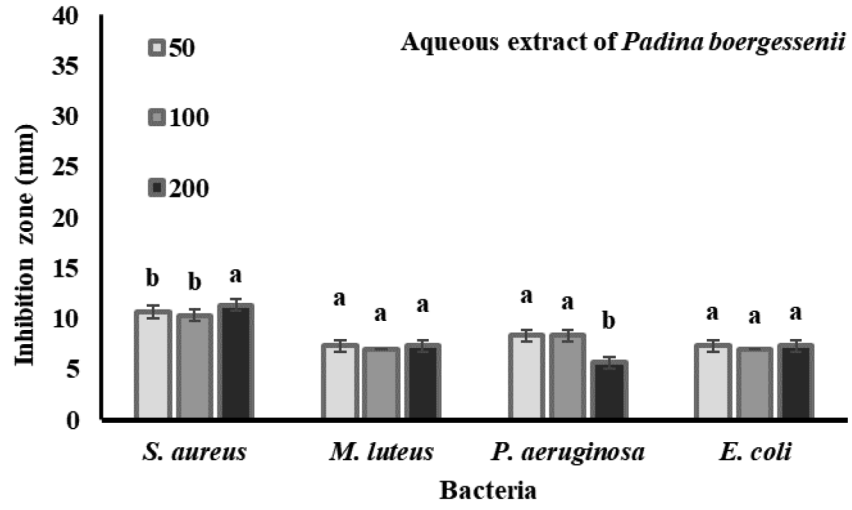

b

**Fig. 1. (a)** Antibacterial activity of MeOH and (b) aqueous extracts of *G. Salicornia*

Antibacterial activity assays of MeOH and aqueous extracts of *Padina boergesenii* against *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia Coli* and *Pseudomonas aeruginosa* are presented in Figure 2a, b. The MeOH extract of *Padina boergesenii* exhibited no significant difference against tested bacteria including *Micrococcus luteus* and *Pseudomonas aeruginosa* at 100 and 200 mg.ml$^{-1}$ concentrations. While Aqueous extract of *Padina boergesenii* showed significant difference only at 200 mg.ml$^{-1}$ concentration against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, exhibited no significant difference against *Micrococcus luteus* and *Escherichia coli* at all concentrations.



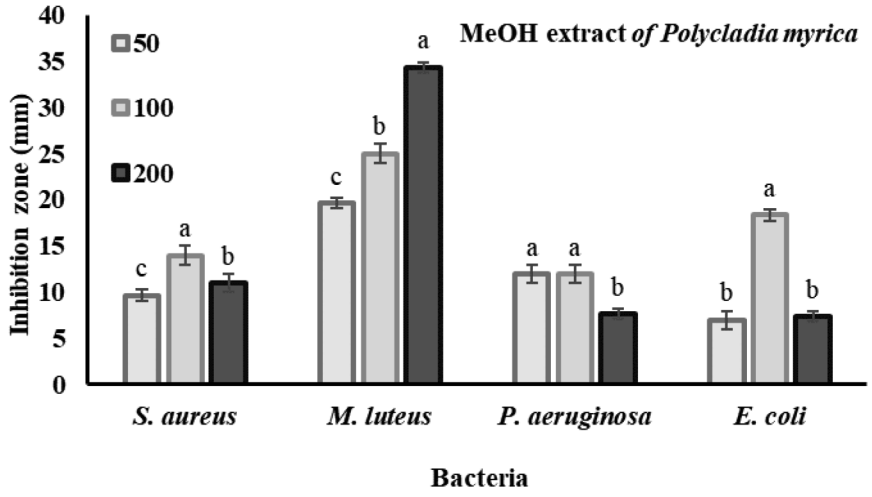

a



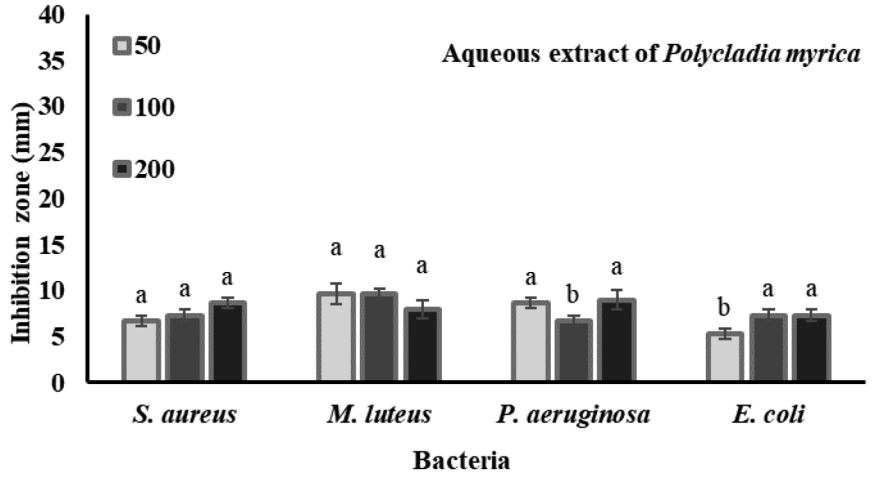

b

**Fig. 2.** (a) Antibacterial activity of MeOH and (b) aqueous extracts of *P. boergesenii*

Inhibition zone of MeOH and aqueous extracts of *Polycladia myrica* is represented in Figure 3. MeOH extract of *Polycladia myrica* inhibited most powerful at the concentration of 200 mg.ml$^{-1}$ against *Micrococcus luteus*, then other concentrations in different tested bacteria. While, there are significant differences at the concentration of 100 and 50 mg.ml$^{-1}$ against *Pseudomonas aeruginosa* and *Escherichia coli*, no considerable differences were observed between antibacterial activity of aqueous extracts of *Polycladia myrica* against *Staphylococcus aureus* and *Micrococcus luteus*.




a




b

**Fig. 2.** (a) Antibacterial activity of MeOH and (b) aqueous extracts of *P. myrica*

The antibacterial activities of extracts were not comparable to penicillin and Cefixime as positive controls.

*Antioxidant activity*

The DPPH radical scavenging activities of MeOH and aqueous extracts of *Gracilaria salicornia*, *Padina boergesenii* and *Polycladia myrica* are depicted in Figure 4. Additionally, both extracts of *Gracilaria salicornia* possessed the lowest antioxidant activity. Furthermore, aqueous extract of *Polycladia myrica* possessed the highest antioxidant activity. In addition, the antioxidant activities of MeOH and aqueous extracts of *Gracilaria salicornia* were variable from 3.09 ± 0.22 to 27.71 ± 0.39 mg.ml$^{-1}$. Indeed, the antioxidant activities of MeOH and aqueous extracts of *Padina boergesenii* were from 21.39 ± 0.4 to 53 ± 0.39 mg.ml$^{-1}$ and 37.14 ± 0.37-55.19 ± 0.39 mg.ml$^{-1}$ for *Polycladia myrica*. Finally, the antioxidant activity of extracts is not comparable to ascorbic acid (100%) at similar concentrations. Although, there was a significant difference between all concentrations of aqueous extract of *Gracilaria salicornia*, there was no significant difference between some concentrations of MeOH extract. While, in the MeOH extract of *Padina boergesenii* there was no significant difference between concentrations of 30, 15, 7.5 mg.ml$^{-1}$, in aqueous extract there were significant differences between all concentrations except 7.5 and 3.75 mg.ml$^{-1}$. There was significant difference between concentrations of MeOH and aqueous extracts of *Polycladia myrica* except 7.5 and 3.75 mg.ml$^{-1}$. Finally, there was no significant difference between similar concentrations of MeOH and aqueous extracts of *Polycladia myrica*.

The results of ABTS and DPPH assays are depicted in Figures 4 and 5. Further, MeOH and aqueous extracts showed higher antioxidant activity using ABTS method in comparison with DPPH method. Furthermore, Minimum and maximum antioxidant activities according ABTS assay was observed in both MeOH and aqueous extraction, 15.24±0.57 mg.ml$^{-1}$ of *Padina boergesenii* and *Gracilaria salicornia* 96.41±0.76 mg.ml$^{-1}$, respectively. Additionally, there was a significant difference between MeOH and aqueous extracts of *Polycladia myrica* in ABTS assays.

The $IC_{50}$ values of MeOH and aqueous extracts for DPPH and ABTS assays are represented in Table 1. While, $IC_{50}$ values of aqueous extracts in *Padina boergesenii* and *Polycladia myrica* are lower than MeOH extracts, in *Gracilaria salicornia* MeOH extract was lower than aqueous extract in ABTS method.

*Total phenol content*

TPC results of aqueous and MeOH extracts *Gracilaria salicornia*, *Padina boergesenii* and *P. myrica* are depicted in Table 2. TPC of MeOH and aqueous extracts of *Padina boergesenii* was higher than *Gracilaria salicornia* and *Polycladia myrica* and there was significant difference between TPC of MeOH and aqueous extracts of each alga.

## Discussion

Bioactive components have already

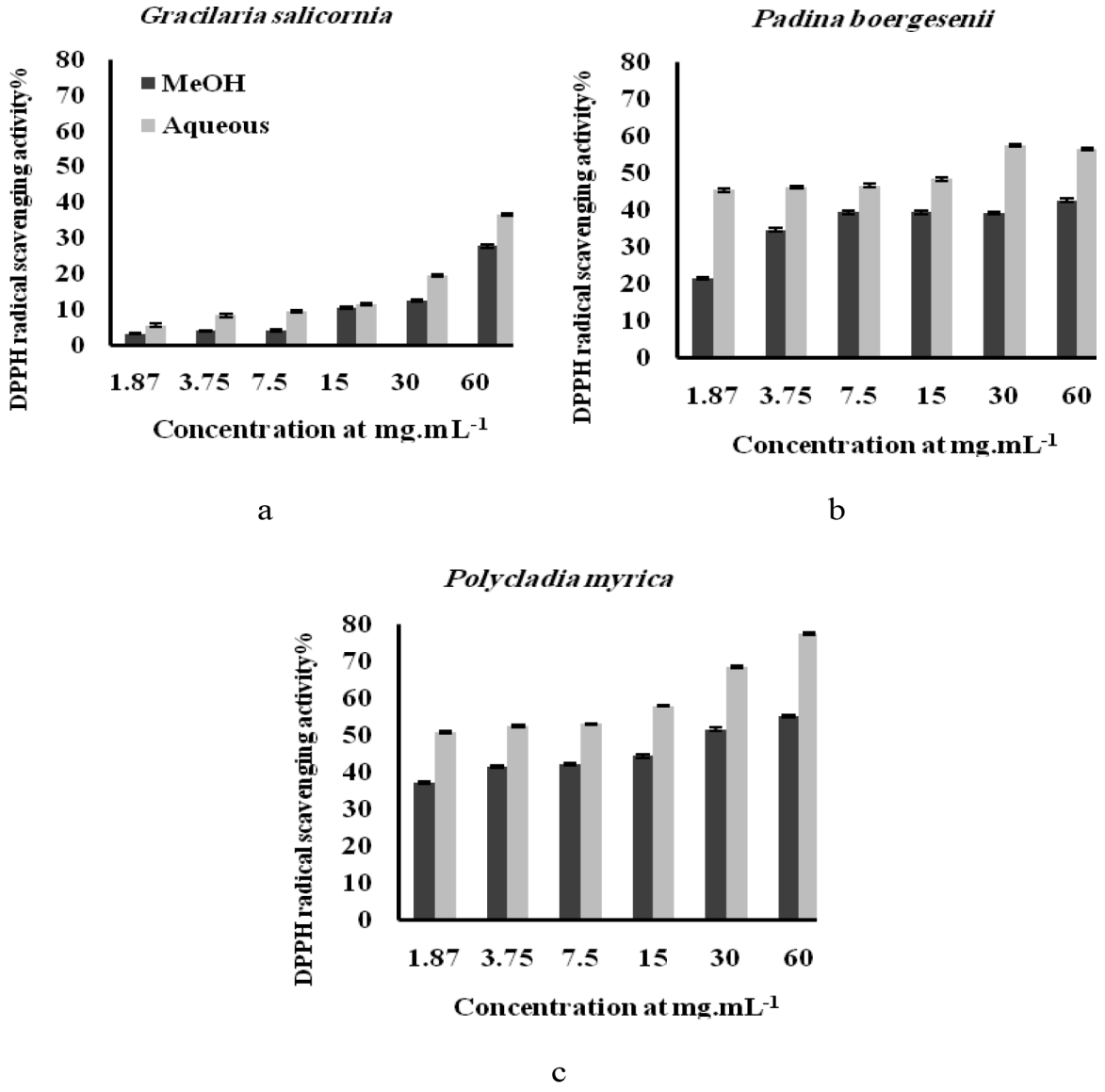



**Fig. 4.** DPPH radical scavenging activity of activities of MeOH and aqueous extracts of (a) *G. salicornia,* (b) *P. Boergesenii,* and (c) *P. myrica*

been known in seaweed such as sterols, terpenoids, phenols, flavonoids, halogenated components, fatty acid compounds (Holdt et al., 2011), and polysaccharides (Barros et al., 2013). Additionally, there are numerous records on antibacterial activities of different extracts of *Gracilaria* spp., *Padina* spp. and *Cystoseria* spp. against various bacterial species.

Sastry and Rao, (1994) reported antibacterial activity of MeOH and $CHCl_3$ *Gracilaria corticata* extracts against *Pseudomonas aeruginosa* and benzene extract of the same species against *Salmonella typhi* and *Escherichia coli*.

MeOH extract of *Gracilaria canaliculata* exhibited the highest antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria. Furthermore, Isoamyl alcohol and hexane extracts of *Graci-*

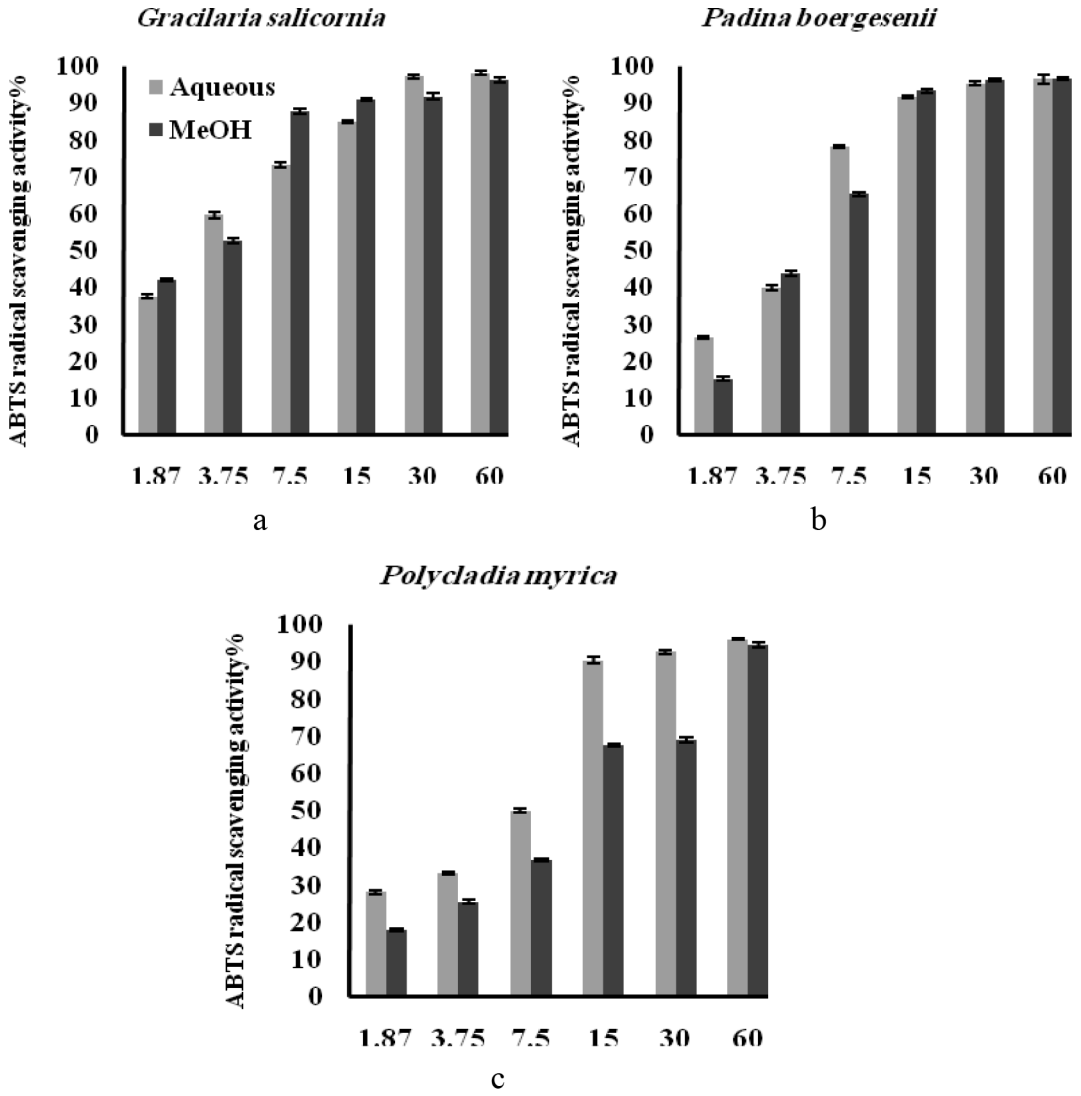



**Fig. 5.** ABTS radical scavenging activity of MeOH and aqueous extracts of (a) *G. salicornia*, (b) P. *boergesenii,* and (c) *P. myrica*

**Table 1**. $IC_{50}$ values of antioxidant activity by DPPH and ABTS methods. Significant differences for each alga are indicated by different letters as determined by T-test ($p<0.05$)

| Algae | Extract | $IC_{50}$ ($mg.ml^{-1}$) by DPPH | $IC_{50}$ ($mg.ml^{-1}$) by ABTS |
|---|---|---|---|
| *Gracilaria salicornia* | MeOH | $119.7\pm0.4^{a}$ | $1.58\pm0.30^{a}$ |
| | Aqueous | $90.1\pm0.7^{b}$ | $2.78\pm0.28^{b}$ |
| *Padina boergesenii* | MeOH | $22.23\pm0.52^{a}$ | $6.53\pm0.22^{a}$ |
| | Aqueous | $17.41\pm0.58^{b}$ | $4.27\pm0.12^{b}$ |
| *Polycladia myrica* | MeOH | $35.07\pm1.34^{a}$ | $21.61\pm0.32^{a}$ |
| | Aqueous | $10.14\pm0.31^{b}$ | $10.31\pm0.10^{b}$ |
| Ascorbic acid | | $0.038\pm0.04$ | $0.001\pm0.02$ |

Table 2. Total phenolic content of MeOH and aqueous extracts of *G. salicornia*, *P. boergesenii* and *P. myrica* (Data are expressed as mean ± standard deviation of triplicate samples).

| Total phenolic content (mg GAE/100g) | Extraction solvent | Alga |
|---|---|---|
| *G. salicornia* | MeOH | 5.07±0.08[a*] |
| | aqueous | 7.34±0.07[b] |
| *P. boergesenii* | MeOH | 20.22±0.11[a] |
| | aqueous | 46.73±0.24[b] |
| *P. myrica* | MeOH | 14.62±0.26[a] |
| | aqueous | 29.22±0.21[b] |

* Significant differences for each alga are indicated by different letters as determined by T- test($p<0.05$)

*lariopsis longissima*, *Gracilaria foliifera*, *Gracilaria corticata*, *Gracilaria canaliculata* and *Gracilaria edulis*, showed the highest and lowest antibacterial activity against human bacterial pathogens, respectively (Prabhahar et al., 2012).

*Gracilaria edulis* showed highest antibacterial activity against *Staphylococcus aureus* (Vallinayagam et al., 2009). EtOH extract of *Gracilaria cortica* resulted more antibacterial activity against *Pseudomonas aeruginosa* (Jeyanthi et al., 2013; De-Campos et al., 1988).

Adaikalaraj et al., (2012) recorded high antibacterial activity potential of *Gracilaria ferugosoni* and *Gracilaria verrucosa*. Additionally, in the present study all concentrations of MeOH and aqueous extracts of *Gracilaria salicornia* showed a low to high gradient antibacterial activity.

This study showed moderate antibacterial efficiency of aqueous and MeOH extracts of *Padina boergesenii* against all tested bacteria. El-Fatimy and Abdel-Moneim (2011) mentioned that while *Padina antillarum* showed no effective activity against the tested bacterial strains *Padina pavonica*, *Padina gymnospora*, and *Padina tetrastromatica* have antibacterial effect especially against *Staphylococcus aureus* (Rizvi, 2010).

Further, in this study MeOH extract of *Polycladia myrica* showed considerably higher antibacterial activity against *Micrococcus lutus* than other tested bacterial strains. *Cystoseira compressa* (Dulger and Dugler, 2014) and *Cystoseira barbata* showed moderate inhibitory activity against the *Staphylococcus aureus* and *Salmonella typhimurium* (Ertürk and Taş, 2011; Ozdemir et al., 2006).

Extraction of *Sirophysalis* (formerly Cystoseira) *trinodis* was respectively effective against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and

*Pseudomonas aeruginosa* (Tajbakhsh et al., 2011a). While, Diethyl ether and EtOH extracts of *Cystoseria mediterranea*, *Cystoseriata mariscifolia*, *Cystoseira sedoides* and *Cystoseira compressa* exhibited antibacterial activity on *Staphylococcus epidermidis aureus, Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Tüney et al., 2006; Ainane et al., 2014;), extractions of *Cystoseira crinita* were not effective against *Escherichia coli* (Berber et al., 2015).

In addition, there are obviously similarities between the results of present study and other authors. Furthermore, the reasons for high or moderate antibacterial activities could be due to innate differences in various algal species or strains. Additionally, Production of substances, geographical zones and habitats, seasonal variations, life phase also could be effective in antibacterial potentials of different extracts.

While, the $IC_{50}$ of MeOH extract was higher. In this study MeOH extract showed lower antioxidant activity in comparison with aqueous extracts. The result of the research is in contrast with Ganesan et al. (2008) who reported higher antioxidant activity for MeOH than aqueous extract. However, the $IC_{50}$ value is not comparable with ascorbic acid. Moreover, Vijayavel et al. (2010) reported higher antioxidant activity for MeOH extract of *Gracilaria salicornia* (Hawaiian marine algae) in comparison to present study, but EtOH and MeOH extracts of *Gracilaria birdiae* and *Gracilaria cornea* showed higher antioxidant activity (Souza et al., 2011). *Polycladia myrica* had higher antioxidant activity than *Gracilaria salicornia* and *Padina boergesenii*.

Widowati et al., (2014) reported low antioxidant activity for MeOH extract of *Gracilariopsis longissima*. According to Elalla and Shalaby, (2009) aqueous extract showed lower antioxidant activity in comparison to EtOAc and petroleum ether extracts. Antioxidant activities of non-polar and polar extracts of *Gracilaria manilaensis* showed that MeOH extract had the lowest antioxidant activity and is not comparable with ascorbic acid and vitamin E (Abdullah et al. 2013). *Stephanocystis* (formerly Cystoseira) *hakodatensis* was recognized as the best source for antioxidants based on its high phenolics and fucoxanthin content (Airanthi, et al. 2011).

Guner et al. (2013) reported *Gracilaria compressa* as a natural source of antioxidant. *Cystoseira tamariscifolia* and *Bifurcaria bifurcata* assayed by the β-carotene–linoleic acid system as high antioxidant activities (Zubia et al., 2009). Hydroalcoholic fraction of *Polycladia myrica* showed reasonable antioxidant activity (Sadati et al., 2011).

The content of phenolic compounds of *Gracilaria salicornia*, *Padina boergesenii*, and *Polycladia myrica* (*Polycladia myrica*) were determined by Folin-Ciocalteu reagent, and their values are expressed as mg gallic acid per 100g dry sample. Additionally, Phenolic compounds are very important constituents of natural compounds because of their scavenging ability due to their hydroxyl groups. Furthermore, some

studies showed a relationship between phenol content and antioxidant activity (Cao et al., 1997; Devi et al., 2008b). While, MeOH extract of *Gracilaria edulis* contained more phenol (Ganesan et al., 2008), *Gracilaria manilaensis* showed moderate phenol content in comparison with EtOAc and acetone extracts (Abdullah et al., 2013).,This results is in disagreement with aqueous extract of *Gracilaria salicornia* which showed more phenol than MeOH extract. Finally, a positive correlation between antioxidant activity and phenol content of *Gracilaria birdiae* and *Gracilaria cornea* has been reported (Souza et al., 2011).

In this research phenol content of MeOH extract of *Gracilaria salicornia* was higher than *Gracilaria edulis* (Murugan and Iyer, 2012). Moreover, aqueous extract of *Gracilaria salicornia* revealed higher phenol content in contrast with *Gracilariopsis longissima* (Elalla and Shalaby, 2009). Moreover, aqueous extract of *Gracilaria salicornia* showed higher antioxidant activity and more phenol content in comparison to the MeOH extract.

Aqueous extract of *Polycladia myrica* showed more phenol content compared with the MeOH extract in present study. Previous works proved high content of total phenol compounds (TPC) in *Stephanocystis hakodatensis* (Airanthi et al., 2011), *Cystoseira abies-marina* (Barreto et al., 2012), *Cystoseira tamariscifolia* (Zubia et al., 2009), *Polycladia myrica* (Sadati et al., 2011; Moein et al., 2015) and *Cystoseira tamariscifolia* possessed higher TPC (Zubia et al., 2009). TPC in *Padina tetrastromatica* (Chia et al. 2015) was measured 5 times more than *Padina Pavonica* (Husni et al., 2014). Moreover, TPC of aqueous extract of *Padina pavonica* was more than MeOH extract which is in consistency with our results. In addition, MeOH extract of *Padina boergesenii* from Gulf of Mannar Biosphere possessed higher TPC than diethyl ether extract (Karthikeyan et al., 2011). In accordance with our results, aqueous extract *Padina tetrastomatica* had higher TPC than MeOH extract (Chandini et al., 2008). Furthermore, *Padina antillarum* showed high raise TPC from 1240 to 2040 mg GAE. 100 g dried sample for different concentration of MeOH extracts. The result indicated that, aqueous extract of *Padina boergesenii* showed higher antioxidant activity and more phenol content in comparison with MeOH extract and other extracts.

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# *Phormidium* sp. Improves Growth, Auxin Content and Nutritional Value of Wild Barley (*Hordeum spantaneum* L.)

**Fatemeh Alzahra Neyshabouri[1], Ali Akbar Ghotbi-Ravandi[1*], Zeinab Aghashariatmadri[1], Seyed Mohammad Farahabadi[1]**

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**Abstract**

Cyanobacteria are oxygenic, photosynthetic prokaryotes with unique potential to enhance plant growth, development, and productivity. These microorganisms have the ability to stimulate plant growth by producing growth-inducing phytohormones and increasing the solubility of soil nutrients. In the present study, the plant growth promoting potential of *Phormidium* sp. on the growth indices of wild barley seedlings was evaluated. In this regard, *Phormidium* sp. is a filamentous and non-heterocystous cyanobacterium. Indeed, filaments are unbranched and usually in fine, smooth, layered microscopic or macroscopic mats. The results showed that dry weight and length of root and shoot, the content of nitrogen (2%), potassium (2%), and auxin (8%) phytohormone of wild barley seedlings treated with *Phormidium* sp. had a significant increase compared to the control group. Accordingly, the use of *Phormidium* sp. as a plant growth-promoting cyanobacteria seems promising alternative to chemical fertilizers.

**Keywords**: Auxin, Biofertilizer, Cyanobacteria, Growth phytohormones, Nitrogen content

## Introduction

The production of healthy plant-based products to meet the nutritional needs of the world's growing population depends on the use of fertilizers to supply plant nutrients. The use of microorganisms as bio-inoculants is believed to be echo-friendly approach to maintain soil fertility (Kour et al., 2020). Some microorganisms especially plant growth-promoting (PGP) bacteriacan enhance plant growth and protect plants from disease and abiotic stresses (Souza et al., 2015). Micro-organisms that are well studied for their beneficial effects are the plant growth-promoting rhizobacteria (PGPR), mycorrhizal fungi and symbiotic rhizobia (Castro-Sowinski et al., 2007; Yang et al., 2009; Wang et al., 2012; Mendes et al., 2013; Willis et al., 2013). Along with the fungal and bacterial species, cyanobacteria are another group of microorganisms that potentially improve soil fertility and crop productivity through contribution in

1-Department of Plant Sciences and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
*E-mail address:a_ghotbi@sbu.ac.ir

biological nitrogen fixation, phosphate solubilization, mineral release, phytohormone secretion and siderophore production (Hu et al., 2002; Singh, 2014). Cyanobacteria distributed in a wide range of harsh habitats including fresh water, hot springs, arctic and antarctic (Singh, 2014). *Phormidium* sp. is a genus of filamentous cyanobacteria and belongs to Oscillatoriales order (cheng et al., 2019). *Phormidium* sp. has the ability to produce cytokinin (Hussain et al., 2010), auxin (Boopathi, 2013), and gibberline (Gupta and Agarwal, 1973) hormones. It is observed the seed germination of tobacco increased up to 40% by adding the extracellular extract (ECE) of this cyanobacterium to tobacco culture medium (Boopathi, 2013). Also, it was found that *Phormidium* was the superior species for biological crust formation (Hu et al., 2002). This cyanobacterium has received less attention as a plant growth-promoting bacteria.

*Hordeum vulgare* L. subsp. *Spontaneum* is considered as progenitor of cultivated barley (Ghahremaninejad et al., 2021) and its hybrid with cultivated barley is fertile. This plant is a source of various stress-resistant genes and used as a model for modification of cultivated barley (Guo et al., 2009). The aim of this study was to investigate the potential of *Phormidium* sp. to elicit the growth of wild barley and possible underlying mechanisms.

## Material and methods

*Plant Material and Growth Condition*

The seeds of wild barley (HS) were obtained from "Seed and Plant Research Improvement Institute". The barley seeds were surface sterilized in 70% ethanol for about 2 min, 6% sodium hypochlorite solution for about 5min, rinsed three times in distilled water, and then placed on wet filter paper in Petri dishes to germinate in 25 °C. Later, germinated seeds were sown in pots containing 30% perlite and 70% peat moss and grown under controlled condition after 30 hours later, (25 °C, 16 h photoperiod with light intensity of 74 µmol photons $m^{-2}s^{-1}$ and 65% relative humidity). After seeds sowing, the Hoagland solution was added to pots (Hoagland and Aron, 1950). Sampling from control and cyanobacterium treated plants were performed on tenth day after cyanobacterial treatment. Leafs and root samples were taken per treatment and immediately stored at -80 °C for further analysis.

*Culture and purification of cyanobacteria*

*Phormidium* sp. was isolated from the surface soil of an estuary in Abadan city with length 48° 18' 19,929", width 30° 27' 04, 148 and height -1.1 above sea level. The microalgae species purified through repeated subculturing in the BG11 solid medium, using artificial illumination with a 16/8 hour light-dark cycle, and 25 ± 2 °C temperature for culturing the sample. Finally, the purified cyanobacterium was identified by binocular optical microscope Olympus, Model BH-2 and based on Desikachary (1959). In this experiment, two concentration of suspensions were prepared. About two weeks before planting, 100 ml of cyanobacterial suspension with a concentration of 4 $g.L^{-1}$

was added to pots. After planting, 50 ml of cyanobacterial suspension with a concentration of 2 $g.L^{-1}$ was added to the plants.

*Measurement of growth indices*

Plants were grown in the greenhouse with the standard condition for ten days. After harvesting (ten day after planting), root and shoot length were measured. After that, plants were dried in oven (24 h at 50 °C) and dry weight was determined (Rezaee et al., 2019).

*Measurement of auxin*

The plant samples were pulverized in liquid nitrogen. In order to extract the auxin, the samples placed overnight at 4 °C in methanol with sodium diethyl dithiocarbamate. After centrifuge at 10000 g, the supernatant was removed and the residue was mixed again for 60 minutes. Next, the supernatant was vacuumed at 40 °C to remove methanol. Then, the mixture was dissolved in 2 ml of 1 M formic acid. Finally, the auxin content of samples were measured by HPLC (RI-DIV-D made in Germany) (Ge et al., 2007).

*Measurement of nutrients content*

In order to measure elements concentration, the roots and shoots of the plant were separated and digested with $HNO_3$-$HCLO_4$ (2: 1v/v). Then, determination of minerals were measured with Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) (5100, Agilent, California, United States) (Guo et al., 2007).

*Statistical analysis*

The experiment used a completely randomized design with three replicates. Mean comparison between control and cyanobacteria treatments was performed using Student's t-test ($P \leq 0.05$).

**Results**

The results of the pot studies showed a significant increase in the vegetative characteristics of the treated plants compared with the controls. Additionally, *Phormidium* sp. treatment led to significant ($P \leq 0.05$) increase in shoot and root growth indicators like root length and dry weight of root and shoot. Root length (20%) and dry weight of root (10%), shoot length (30%), and dry weight of shoot (20%) enhanced by *Phormidium* sp. treatment (Fig. 1a, b).

*Phormidium* sp. treatment resulted in significant ($P \leq 0.05$) increase in nitrogen content in both shoot and root compared to the control group (Table 1). Potassium content in both root and shoot of wild barley was significantly increased ($P \leq 0.05$) in *Phormidium* sp. treated plants compared to the control group (Table 1). Sulfur content in plants treated with cyanobacteria decreased significantly ($P \leq 0.05$) in both shoot and root compared to the control group (Table 1). *Phormidium* sp. treatment led to significant reduction ($P \leq 0.05$) of calcium content in roots and shoots of wild barley (Table 1). The results of hormone analysis revealed a significant increase ($P \leq 0.05$) in auxin phytohormone content in *Phormidium* sp. treated seedlings compared to the control group (Figure 2).

**Discussion**

Cyanobacteria and microalgae are one of

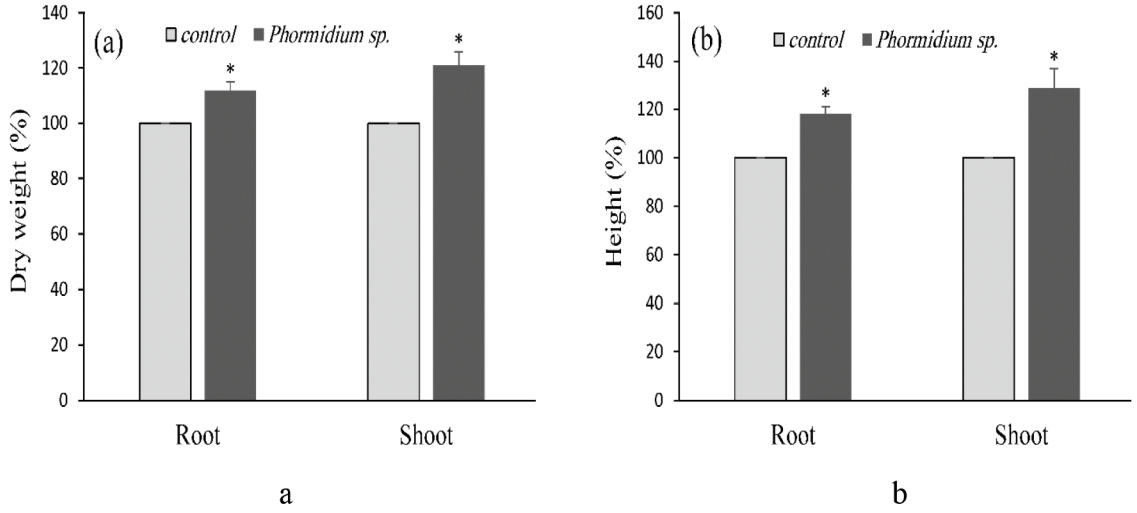


**Fig. 1.** Effects of *Phormidium* sp. treatment: (a) on dry weight (%), and (b) height (%) of root and shoot in wild barley *(Hordeum spantaneum*)

**Table 1.** Effects of *Phormidum* sp. treatment on nutrient content in root and shoot of wild barley (*Hordeum spantaneum*)

| | | Nutrient content (µg $g^{-1}$) | | | |
|---|---|---|---|---|---|
| Organ | Treatment | N | K | S | Ca |
| Root | Control | 3225.9 ± 22.8 | 2481.7 ± 24.2 | 187.6 ± 12.0 | 1076.7 ± 12.5 |
| | *Phormidium* sp. | 3369.6 ± 86.6 * | 2570.2 ± 21.8 * | 172.9 ± 22.8 * | 1038.3 ± 22.7 * |
| Shoot | Control | 8811.2 ± 89.7 | 3474.5 ± 33.9 | 102.2 ± 11.3 | 1040.6 ± 5.1 |
| | *Phormidium* sp. | 8995.8 ± 76.4 * | 3598.3 ± 30.5 * | 81.2 ± 16.8 * | 1021.8 ± 6.5 * |

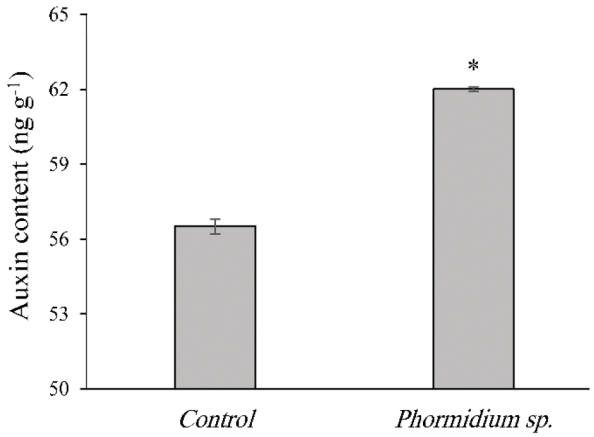


**Fig. 2.** Effects of *Phormidium* sp. treatment on shoot auxin content (ng.$g^{-1}$) of wild barley (*Hordeum spantaneum*)

the primary photosynthetic microorganisms of the soil. Due to the important characteristic of nitrogen fixation, the utility of cyanobacteria in agriculture to enhance production is beyond doubt (Singh, 2014). In the present study, it found that the use of *Phormidium* sp. promoted the growth indices of wild barley. *Phormidium* sp. increased the dry weight and length of roots and shoots of wild barley. Similar to our findings, it reported that *Phormidium* significantly increased dry weight and length of roots and shoots in *Zea mays* (Younesi et al., 2019). This enhancement in growth and dry matter production may be due to the secretion of phytohormones such as auxin (Boopathi et al., 2013), gibberellin (Gupta and Agarwal, 1973), and zeatin (Hussain et al., 2010; Hussain and Hasnain, 2011) by *Phormidium* sp. Indeed, auxin (Indole-3-acetic acid, IAA) content in the shoot of wild barley increased by *Phormidium* sp. Additionally, auxin is a key regulator of plant growth and development, orchestrating cell division, elongating and differentiating, developing embryo, tropismming root and stem, apical dominance, and transiting to flowering (Balzan et al., 2014). Recently IAA phytohormone isolated from *Phormidium* sp. that coexist with mangrove root (Boopathi et al., 2013). Also, IAA was isolated from *Phormidium* sp. in a study by Hossein et al. (2010).

In the present study, nitrogen and potassium content of both shoot and root tissues were significantly enhanced by *Phormidium* sp. Treatment. While, *Phormidium* is a non-heterocystous cyanobacterium, that can stabilize nitrogen in the absence of oxygen or micro-oxyc condition (Bergman et al., 1997). Similar to our findings, it was reported that *Phormidium ambiguum*, as a non-stabilizing nitrogen cyanobacterium, was able to increase soil nitrogen levels (Chamizo et al., 2018). Indeed, cyanobacteria play an important role in the bio-nutrient cycle. Additionally, they have an extraordinary ability to manage agricultural ecosystems. Further, they improve potassium, iron and other soil nutrients and facilitate the use of these nutrients for plants (Singh, 2015). In the present study, potassium content of both root and shoot of wild barley significantly increased in response to *Phormidium* sp. treatment. Similar to our results, an increase in potassium and phosphorus content observed in tomato seedlings treated with *Aphanothece* sp. cyanobacterium (Mutale-joan et al., 2020). In this experiment, cyanobacterial treatment reduced the amount of sulfur in both root and leaf tissues of wild barley. The reduction in plant sulfur content may be due to the consumption of part of soil sulfur by cyanobacteria. In this regard, iron-sulfur clusters (Fe-S) act as a protein cofactor in many important physiological processes including photosynthesis, respiration, and nitrogen fixation of cyanobacteria and other photosynthetic organisms (Balk and Pilon, 2011). In the study performed by Aziz and Hashem (2003), cyanobacterial inoculation slightly increased the available sulfur content of the soil compared to the control. So far, not much research has been done on the effect of cyanobacteria on the solubility of sulfur

and potassium in the soil and its uptake by plants.

Furthermore, the calcium content of both root and shoot was also significantly decreased in response to *Phormidium* sp. treatment. Since *Phormidium* has the ability to calcify, calcium in the soil may be used in the calcification process (Shiraishi et al., 2017). In the chamomile plant treated by *Nostoc carneum* ISB88, *Nostoc punctiforme* ISB90, and *Wollea vaginicolla* ISB89 it was found that the amount of soil calcium has also increased (Zarezadeh et al., 2020). As yet, very little research has been done on the distribution of calcium and sulfur in the soil by cyanobacteria.

In general, according to the results of this experiment, *Phormidium* sp. enhanced the content of nitrogen, potassium elements and auxin phytohormone. As a result, plant growth indices such as dry weight, root and shoot length have increased. Therefore, the use of this cyanobacterium as a plant growth promoting rhizobacteria (PGPR) seems promising ecofriendly method in boosting growth of barley plants.

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# Bioaccumulation and the Effect of Selenate Concentration on Growth and Photosynthetic Pigment Content of *Spirulina platensis*

**Fatemeh Yaghoobizadeh[1*], Hamid Rajabi Memari [2], Mohammad Roayaei Ardakani[3]**

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**Abstract**

Selenium (Se) is a necessary microelement for microalgae growth. Moreover, it might be directly act and cause a decrease in primary production in microalgae. Nowadays, there is a great focus on preparations of Se-enriched products. In the current study, the effects of various selenate concentrations on *Spirulina* evaluated as a primary stage to large-scale production of Se-enriched *S. platensis*. Zarrouk medium supplemented to investigate the stimulatory/inhibitory effects of selenate on the growth at various concentrations. Biomass dry weight and cell mass measured on $OD_{550}$. Thereafter, inhibitory and algicidal concentrations were determined. Furthermore, its effects on morphology and changes of some important pigments in response to the metal challenge investigated, too. While the results showed that at 5 and 10 ppm concentrations growth was supported, the dry weight of microalgae decreased at selenate levels above 50 ppm. Besides, the inhibitory and lethal effects of selenate were at 100 ppm and 300 ppm, respectively. In addition, morphological changes observed at this concentration. Additionally, chlorophyll, carotenoid and phycobiliproteins, showed a stimulatory effect at 5-50 ppm, 10 ppm, and 10 ppm, respectively.

Moreover, *Spirulina* uses for foods production because some chemicals are unique compounds. Simple cultivation method and high quality of its protein, and no toxic effects, cause its feasibility for large-scale production.

**Keywords**: *Spirulina platensis*, Selenate, Photosynthetic Pigment Content, Toxicity

## Introduction

Disorder in aqueous ecosystems as a result of metal pollution will damage biological diversity and enhance the biological accumulation and concentrated pollution in food chains, as well (Mane et al., 2013). Because many chemical and physical characteristics are effective for determining the metals' impact on aqueous organisms, it is

1- PhD student of Microbial Biotechnology, Iran, Khouzestan, Ahvaz, Shahid Chamran University of Ahvaz, Faculty of Science, Department of Biology, Microbilogy Laboratory
2- Assistant Professor of Biotechnology, Iran, Khouzestan, Ahvaz, Shahid Chamran University of Ahvaz, Faculty of Agriculture, Department of Agronomy and Plant Breeding, Biotechnology Laboratory
3- Professor of Microbiology, Iran, Khouzestan, Ahvaz, Shahid Chamran University of Ahvaz, Faculty of Science, Department of Biology, Microbilogy Laboratory
*E-mail address: yaghoobizadeh@gmail.com

difficult to determine the metals' effects on these organisms (Mane et al., 2013).

According to Bertrand's known diagram, there is a certain range of positive effects on humans for each micronutrient, which in lower or higher amount than it becomes harmful for organisms. Therefore, it is obvious that the precious selection of required dosages is the most important work in designing of therapeutical substances (Frontasyeva et al., 2009).

Among these elements, Se is a natural trace element whose essential range can change from natural micronutrients to the toxic compound so that it affects organisms in a dose-dependent manner (Babaei et al., 2017; Gojkovic et al., 2014; Schiavon et al., 2016; Sun et al., 2014; Umysova et al., 2009). This element is essential for many biological functions in human, animals, and microorganisms (Schiavon et al., 2016). Moreover, it plays important role in decrease the harmful effects of free radicals (Frontasyeva et al., 2009). Because of its role in mammalian development, endocrine systems, immune function, slowing down aging, etc., this element has a highly important role in human health (Frontasyeva et al., 2009; Gojkovic et al., 2014; Sun et al., 2014; Umysova et al., 2009). Se function has tight connectivity with vitamin E and beta-carotene (which are present in *Spirulina platensis* biomass); therefore, these elements sometimes utilize together with therapeutical purposes (Frontasyeva et al., 2009).

This opinion generally exists that Se compounds are better and safer than inorganic Se as food supplements, therefore, various biological Se-enriched compounds such as garlic, yeast, lactic acid bacteria (LAB) are commercially available. In this regard, Se supplementation using microorganisms has been attracted attention in the last decades (Chen et al., 2008).

Se toxicity and deficiency are dependent on its availability in the environment. In aqueous environments, Se exists as two main oxidation states (selenite; $Se^{IV}$, $-SeO_3^{2-}$, $Se^{3+}$ and selenate; $Se^{VI}$, $-SeO_4^{2-}$, $Se^{6+}$). Additionally, selenate has high water solubility, therefore, has more bioavailability for aqueous organisms rather than selenite (Gojkovic et al., 2014; Khademi and OraghiArdebili 2017; Schiavon et al., 2012; Schiavon et al., 2016). In aqueous environments, Se exists from natural sources and human activities such as agricultural lands irrigation, mining, and combustion. Common concentration in freshwaters is in the 0.13-2.50 nmol/L range but in polluted areas, higher amounts (up to 5 μmol/L) have been observed (Fournier et al., 2010; Sun et al., 2014).

Phycoremediation is an improvement of environmental pollution decrease/remove using microalgae (Soeprobowati and Hariyati, 2014). In ecosystems, microalgae are the major concern because they accumulate Se from the water column and transform it to organic Se before its transfer to higher organisms (Fournier et al., 2010; Sun et al., 2014).

However, studies showed that both selenate and selenite are toxic for microalgae at higher concentrations, selenate is more lethal

than selenite (Babaei et al., 2017; Khademi and OraghiArdebili, 2017). These types can absorb by t algal cells and affect growth in a dose-dependent manner (Gojkovic et al., 2014; Schiavon et al., 2016). Nevertheless, plant cells have both enzymatic and non-enzymatic strategies against damage effects of oxidative stress (Chen et al., 2008).

Besides Se toxicity effects in microalgae, essential need to Se has been reported in at least 33 species belong to six phyla but its biological importance has been unknown yet (Schiavon et al., 2016). Indeed, Se effects on metabolism and bioaccumulation, studied in various groups of microalgae such as *Spirulina*, *Scenedesmus*, *Chlorella* or *Chlamydomonas* (Babaei et al., 2017; Gojkovic et al., 2014).

Further, at low concentration, Se motivate the growth of some algae (*Thalassiosira pseudonana*) and at high level, it could be toxic for algae and cause growth repression, cell ultrastructure modification, and reactive oxygen species (ROS) transformation which can cause cellular damage, and low storage products (Babaei et al., 2017; Khademi and OraghiArdebili 2017; Schiavon et al., 2012; Schiavon et al., 2016).

However, microalgae can metabolize inorganic Se to its organic forms e.g. selenoproteins, volatile compounds, and seleno-aminoacids especially selenomethionine and seleno-cysteine as a part of their detoxification process probably because of their reductive metabolism; Se can stop growth at certain concentrations (Babaei et al., 2017; Khademi and OraghiArdebili 2017). Indded, over absorption of Se can cause metabolic reactions, and raise the probability of photo-oxidative damages (Babaei et al., 2017). Because rapid growth and high amounts of other inorganic substances such as calcium, sodium, potassium, iron micromineral, and nutrients such as protein (58.5%), ash (12%), carbohydrate (7.5%), lipid (7%), and crude fiber (0.95%) in contrast to other organisms, single-cell protein in cyanobacteria are crucial. On the other hand, in some studies on environmental pollution remediation using *S. maxima* $Cd^{2+}$ accumulated in various layers of its cell wall (Costa et al., 2003). In like manner, *Microcyctis aeruginosa* removed $Cd^{2+}$ (90%), $Hg^{2+}$ (90%) and $Pb^{2+}$ (80%) (Chen et al., 2005). In addition, *Spirulina* is an efficient biosorbent so that process equilibrium has reached during 5-10 minutes (Soeprobowati and Hariyati, 2014). *S. platensis* is a filamentous cyanobacteria (Soeprobowati and Hariyati, 2014) enriched by proteins (60-70%). Additionally, *S. platensis* cantains lipids, pigments, antioxidants, and vitamins used commercially as animal feed or human food supplements (Khademi and OraghiArdebili, 2017). Moreover, *Spirulina* becomes the simplest Se-supplementable algae from aqueous environments by changing the culture conditions (Li et al., 2003).

Several studies showed that this alga is a good carrier for Se accumulation. Moreover, Se accumulation could cause an improvement of *S. platensis* quality by enhancement of biomass production, photosynthetic pigments, and protein concentration (Chen et

al., 2008).

Currently, there is a need to investigate the effects of different Se concentrations on *Spirulina* growth to produce Se-enriched *Spirulina* in large-scale amounts (Mane et al., 2013). In the current study, *Spirulina platensis* (from Nogen Company, Ahvaz, Iran) selected as the subject organism because previous studies investigated Se effect on this species. The study aimed to determine the effect of different selenate concentrations on the growth ability of selected strain, selenate tolerance range in *S. platensis* culture, and its effect on photosynthetic pigments (including chlorophyll, phycocyanins, and beta-carotene). Moreover, this study could present some aspects of Se tolerance and increase our perception of Se interaction with *S. platensis*.

## Material and methods

*Microalgae cultivation*

Cyanobacteria strain (*Spirulina platensis* NCC A300) was prepared from the local culture collection of Nogen Company, Ahvaz, Iran. The primary standard inoculated to Erlenmeyer as follows. It was necessary to obtain mid-exponential cells of microalgae. For this, a Zarrouk medium (pH 11.0) was prepared. Medium composition was as follows (g/L): $NaHCO_3$ 16.8, $K_2HPO_4$ 0.5, $NaNO_3$ 2.5, $MgSO_4.7H_2O$ 0.20, EDTA $Na_2.2H_2O$ 0.08, $CaCl_2.2H_2O$ 0.04, $FeSO_4.2H_2O$ 0.01, trace element mixture A 10 ml/L, trace element mixture B 1.0 ml/L. And autoclaved at 121 ºC, 1 atm for 15 min. Then, inoculation was done as 1% of basal medium and incubated at 28±2 ºC with 16/8 hours (light/dark) photo-period for 30 days (Li et al., 2003; Mane et al., 2013).

*Growth kinetics study*

For the determination of mid-exponential cells, sampling was done at predefined times (every day) during the incubation period and growth rate was determined by Optical Density measurement at 550 nm using a spectrophotometer (analytikjena, spekol, 2000) (Khademi and Oraghi Ardebili, 2017).

It should be noted that growth kinetic studies were done at the presence of 0, 5, 10, 25, 50, 100, 300, 1000, and 2000 ppm selenate concentrations, respectively. Then, the stimulatory or inhibitory effects of selenate on growth profile of *S. platensis* were determind. Moreover, biomass concentration determined by dry weight measurement (Gojkovic et al., 2014). To achieve this goal, 1 ml of cultured media was withdrawn, washed to remove unwanted materials, and centrifuged at 10000 rpm for 10 min. The supernatant discarded and the cell pellet placed at 80 ºC, overnight. All experiments were done in triplicates (Sun et al., 2014). Dry weight expressed as g/L and calculated by the difference between the primary and final weights (Gojkovic et al., 2014; Khademi and OraghiArdebili, 2017). Moreover, pH measured during incubation time to investigate the pH changes during microalgae culture (Sun et al., 2014).

*Determination of minimum algistatic and minimum algicidal concentrations*

The tolerance of microalgae to selenate toxicity investigated using a broth medium

containing different amounts of selenate oxyanions. In this regard, 30 ml of Zarrouk medium in 100 ml Erlenmeyer was prepared and supplemented with different selenate concentrations (5, 10, 25, 50, 100, 300, 1000 and 2000 ppm), separately (Li et al., 2003). It is noteworthy that, this wide concentration range used to determine the connection between Se dosage and physiological and ultra-structural changes (Schiavon et al., 2016). Then, the Erlenmeyers incubated at predefined conditions. Following, sampling done and growth determination was performed as above to study growth kinetics at the presence of heavy metal stress. Ultimately, those cultures, which did not have any signs of growth, considered as minimum algistatic concentration. While the algicidal effect of selenate was investigated in solid Zarrouk medium by culturing 50 µl of no algae growth cultures (Sun et al., 2014).

*Effect of selenate on cell morphology*

An approach to investigate the effect of heavy metals on microalgae is the study of ultrastructure and morphology changes. Accordingly, samples were withdrawn from culture media supplemented with various concentrations of selenate and observed using a light microscope (Olympus) (Belokobylsky et al., 2004; Pelah and Cohen, 2005; Schiavon et al., 2012; Schiavon et al., 2016; Umysova et al., 2009).

*Pigments extraction and analysis*

To determine the effect of selenate toxicity on pigments content, the number of essential pigments (chlorophyll a, carotenoid, phycobilliproteins) was measured using acetone. For this, the culture of *S. platensis* withdrawn on the 30th day of cultivation, then their cells were breakdown using vigorous vortex for 2 min. After centrifugation, supernatant was collected and subjected to further study as follows (Sharma et al., 2014).

*Pigment analysis*

According to Sharma et al. (2014), the amount of photosynthetic pigments was measured in both types of media: supplemented with various concentrations of selenate; without any metal. Using a vortex mixer breaking microalgae cells done and chlorophyll and total carotenoid contents were determined in 80% acetone. After centrifugation, the supernatant collected (because it contains pigments). The absorbance of the supernatants of all extraction steps was measured using spectrophotometer in the most suitable wavelength for each pigment, as it has been mentioned in the following sections (Nanodrop 1000, ThermoScientific, USA) and the concentrations of pigments were calculated (Babaei et al., 2017).

*Chlorophyll estimation*

5 ml of homogenized cyanobacterial suspension centrifuged for 10 min at 4000 rpm and supernatants discarded. 5 ml of 90% acetone was used to extract chlorophyll-a (Chl-a). The tubes wrapped with aluminum foil and placed in the dark for 24 hours. Finally, samples centrifuged for 15 min at 5000 rpm, and the supernatant collected for further analysis. Absorbance measured at 630 nm (A630), 645 nm (A645), and 665 nm (A665) and 90% acetone used as blank. The Chl-a concentration calculated using

the following equation (Mane et al., 2013; Sharma et al., 2014):

$C = 11.6\ A665 - 1.31\ A645 - 0.14\ A630$ (1)

In a given volume of culture, Chl-a concentration determined by the following formula:

$\text{Chl-a}\ ((mg/l)) = c_e / v_c$ (2)

Wherein C, Ve, and Vc are value obtained from the above equation (1), volume of extract (ml), and volume of culture (l), respectively (Khademi and OraghiArdebili, 2017).

Phycobiliproteins estimation

5 ml of cell suspension centrifuged for 10 min at 4000 rpm to obtain the pellet. The cell pellet washed with distilled water. Thereafter, washed pellet used for the total extraction of Phycobiliproteins with 5 ml of sterile phosphate buffer (0.05 M, pH 6.7). Extraction repeated by three times freezing and thawing cycles. The samples centrifuged for 15 min at 10,000 rpm and supernatant collected. The absorbance read at 562 nm (A562), 615 nm (A615), and 652 nm (A652) against phosphate buffer as blank. Using the following formula, concentration of allophycocyanin (APC), phycocyanin (PC), and phycoerythrin (PE) were calculated (Sharma et al., 2014):

$APC = A652 - 0.208\ (A615) / 5.09$ (3)

$PC = A615 - 0.474\ (A652) / 5.34$ (4)

$PE = A562 - 2.41(PC) - 0.849(APC) / 5.09$ (5)

Phycobiliprotein's concentration can be determined by the following formula:

$\text{Phycobiliprotein}\ (mg/ml) = c \times v_e / v_c$ (6)

In these equations: APC, PC, and PE represented Phycocyanin, Allophycocyanin, and Phycoerythrin, respectively. C is the sum of PC, APC, and PE. According to equations 3-5, Ve is the volume of extract (l), and Vc is the volume of culture (l) (Sharma et al., 2014).

*Carotenoid estimation*

5 ml of homogenized cyanobacterial suspension centrifuged as the previous stage at 4000 rpm for 10 min. The pellet washed 2-3 times with distilled water to remove adhering salts and biomass collected. Pellet was broken down with 5 ml of 90% acetone and the samples were centrifuged 15 min at 5000 rpm for carotenoid extraction. The supernatant was collected and read the absorbance rate at 450 nm (A450). Finally, carotenoids (Cart) concentration calculated using the following formula: (Sharma et al., 2014)

$C = A450 \times V \times f \times 10 / 2500$ (7)

Where, C is the total amount of carotenoid (mg/ml), V is the volume of extract (l) and f is the dilution factor.

*Statistical analysis*

All statistical analysis performed by SPSS software (version, 19). The significant differences level between the investigated pigment content in different medium compared by Chi-Square.

## Results

*Change in microalgae's culture in response to various concentrations of selenate*

In these set of experiments, after addition of various selenate concentrations from low and stimulant (5 ppm) to high, and inhibitor concentration (2000 ppm), growth and physiology of *S. platensis* studied and compared with control.

Figure 1 represents growth kinetic of mi-

croalgae is impressed at the different concentrations of selenate. The concentrations of selenate in 5, 10 and 25 ppm result more regular changes in biomass production yields (Fig. 1, Fig. 2). It should be noted that when culture media supplemented by 50, 100, and 300 ppm of Se, dry weight was increased. However, by increasing the incubation time, dry weight reduced especially at 50, and 300 ppm of selenate concentration.

Furthermore, Se toxicity evaluated by the decrease the exponential growth rate (Khademi and OraghiArdebili, 2017). Moreover, the minimum growth rate considered as a key determinant of Se toxicity. So that with impressing of any metabolic reaction, a corresponding amount of this determinant will be decreased (Gojkovic et al., 2015; Khademi and OraghiArdebili, 2017).

*Effect on pH value*

While the pH of culture media before selenate addition and *S. platensis* inoculation was 9.0, the overall pH during incubation period tends to increase (Fig. 3).

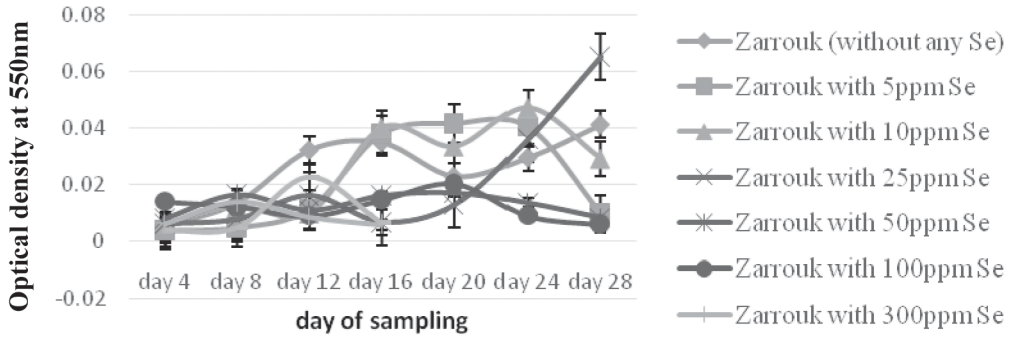



**Fig. 1.** Growth curve of *S. platensis* in Zarrouk medium supplemented with and without various selenate concentrations during 30 days. The error bars of the mean $OD_{550}$ data and dry weight measurement represents the standard error

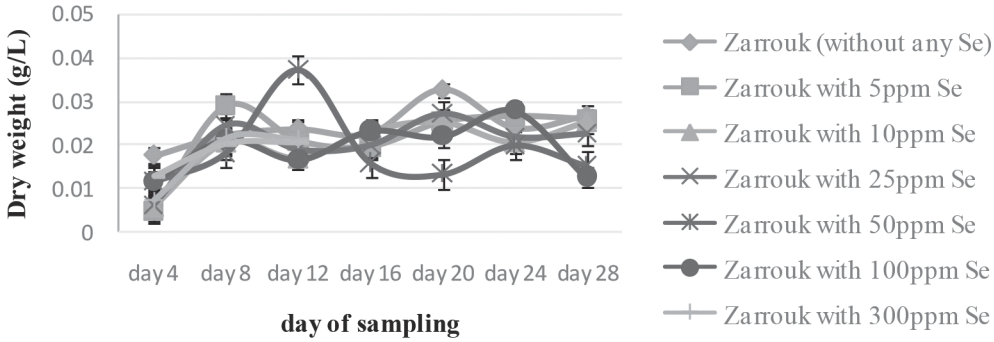



**Fig. 2.** The dry weight of *S. platensis* in Zarrouk medium during 30 days. The error bars of the mean $OD_{550}$ data and dry weight measurement represents the standard error

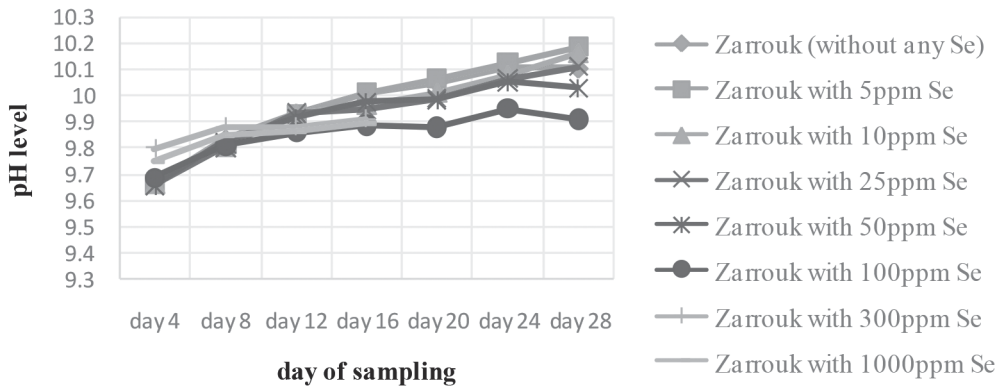



**Fig. 3.** pH changes of *S. platensis* culture in different selenate concentrations during 30 days. The error bars of the mean gathered data from $OD_{550}$ and dry weight measurement represents the standard error

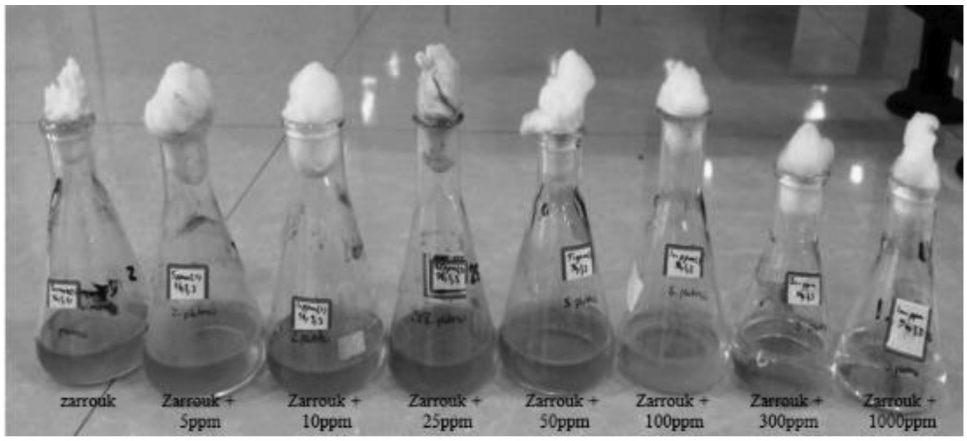



**Fig. 4.** Growth response of *S. platensis* at the MIC determination stage at various selenate concentrations after 7 days

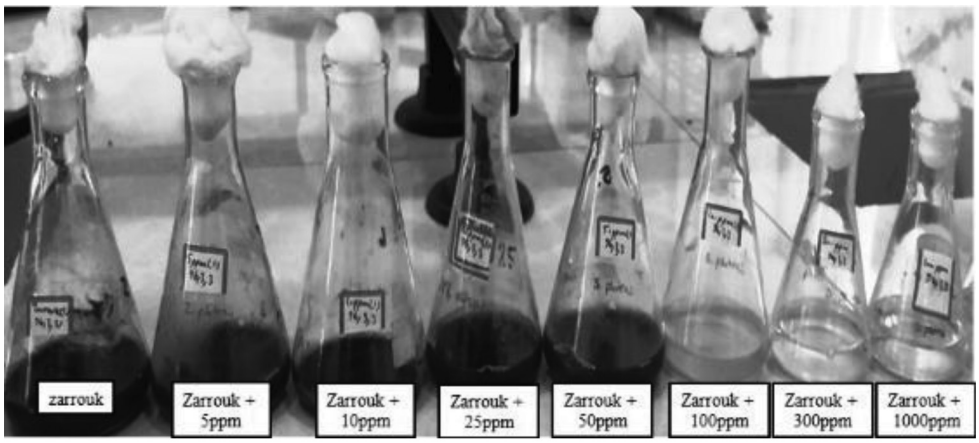



**Fig. 5.** Growth response of *S. platensis* in the MIC determination stage to different selenate concentrations after 30 days

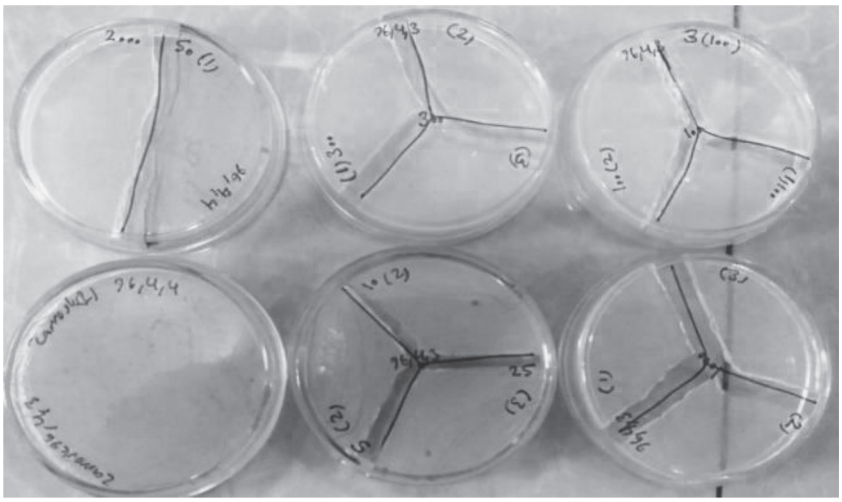
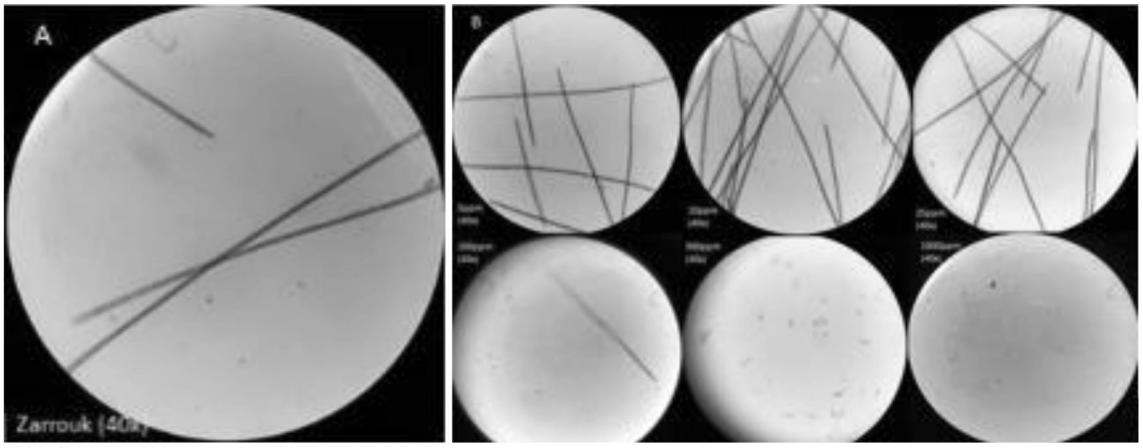


**Fig. 6.** The algicidal effect of *S. platensis* at various selenate concentrations in Zarrouk medium




a b

**Fig. 7.** Microscopic shape of *S. platensis* in (a) Standard Zarrouk medium, and (b) Zarrouk medium supplemented by various selenate concentrations

*Minimum Algistatic and Minimum Algicidal determination*

To investigate inhibitory or lethal effects of selenate on microalgae growth pattern, Zarrouk media which was supplemented with various dosages (5, 10, 25, 50, 100, 300, 1000, and 2000 ppm) of selenate was applied. Growth changes monitored after 7 and 30 days of the incubation (Figs 4 and 5, respectively). At the end of the incubation period, the growth rate recorded visually and the first culture, which did not have any sign of growth, was considered as minimum algicidal concentration (Fig. 4).

Those cultures, which did not have any sign of growth were cultivated to investigate the lethal activity of selenate oxyanion. For this mean, 50 µl inoculum was withdrawn and cultivated on solidified Zarrouk medium without any metal presence. After the incu-

bation, plates investigated and the first plate that did not have any growth considered as minimum algicidal concentration (MIC). Moreover, Selenate in this concentration has a lethal effect on the growth of this microalga (Fig. 6).

Furthermore, selenate has inhibitory and lethal effects on the studied strain at 100 ppm and 300 ppm concentrations, respectively (Fig. 6).

*Effect of selenate on the microscopic shape of S. platensis*

Investigation the heavy metal effect on the ultrastructure of microalgae and cyanobacteria is an issue to show the ability of these microorganisms to cope with heavy metal stresses. In this regard, selenate effect on filament and septa shape of *S. platensis* was investigated (Fig. 7). The results show that filamentous shape would start to change at 100 ppm and disrupted at 300 and 1000 ppm of selenate concentration.

*Effect of selenate on pigment content of microalgae*

The results of the selenate effect on studied pigments of *S. platensis* revealed different chl-a content at various selenate concentrations (Table 1). This order is 5 ppm> 25 ppm> 50 ppm> 10 ppm> zarrouk medium> 1000 ppm> 300 ppm> 100 ppm= 2000 ppm. Furthermore, Se-Toxicity affects several physiological features of *S. Platensis*.

While, carotenoid content changes in response to various selenate concentrations was in this order: 10 ppm> 25 ppm> 50 ppm> 5 ppm> zarrouk medium> 100 ppm> 300 ppm> 1000 ppm> 2000 ppm.

As the results show, this trend is somewhat rational. An increase in the carotenoid amount in response to the high level of Se accumulation, may be present a mechanism for resistance against selenate toxicity because of their protective effects on membrane integrity. Carotenoids may also protect chloroplast membranes from generated damages of produced ROS. However, some studies report different results, which show Se concentrations do not have negative effects on pigment contents.

The results show that the highest amount of each phycobilliprotein is 300 ppm, allophycocyanin in 1000 ppm and phycoerythrin in 300 ppm.

**Discussion**

Several studies reported therapeutical and preventive substances based on blue-green algae (*S. platensis*) (Frontasyeva et al., 2009). On the other hand, some studies investigated the stimulatory/inhibitory effects of selenate on this valuable cyanobacterium. In this regard, our study provides new insight into Se effects on microalgae, especially concerning to its toxicity.

Se is an important microelement because its narrow concentration range placed between its essential and toxic effect on human and animal health (Schiavon et al., 2012; Zheng, 2017). Furthermore, low Se uptake can cause cancer stimulation, immune system dysfunction, cardiovascular diseases, fertility reduction, and hypothyroidism, while high Se level in food regime, affects on cardiometabolic and induce some acute

**Table 1.** Changes in pigment content of *S. platensis* mg/l in response to various selenate concentrations

| | Chl a (mg/l) | Cartenoid | Phycobiliprote in (mg/l) | phycobiliprotein | | | |
|---|---|---|---|---|---|---|---|
| | | | | PC | APC | PE | Phycobiliprotein (mg/l) |
| **Zarrouk** | 0.034180467 | 2.2667E-06 | 3.77783E-07 | 0.00012806 | 9.01768E-05 | 0.000237195 | 7.59053E-05 |
| **5ppm** | 0.102760065 | 1.6667E-06 | 2.778E-07 | 0.000131303 | 0.000207414 | 0.000260651 | 9.98947E-05 |
| **10ppm** | 0.041732198 | 0.000062 | 1.03333E-05 | below the detection limit | 0.000144527 | 0.000329409 | 5.88221E-05 |
| **25ppm** | 0.066564525 | 2.7533E-06 | 4.589E-07 | below the detection limit | below the detection limit | 0.000180256 | below the detection limit |
| **50ppm** | 0.043502142 | 2.3467E-06 | 3.91117E-07 | below the detection limit | 9.28016E-05 | 0.000238547 | 0.000029387 |
| **100ppm** | 0.014163735 | 1.2533E-06 | 2.08883E-07 | below the detection limit | 0.000354884 | 7.17971E-05 | 2.55406E-05 |
| **300ppm** | 0.015770555 | 0.00000088 | 1.4666E-06 | 0.001192539 | below the detection limit | 0.000812214 | 0.000317884 |
| **1000ppm** | 0.016610001 | 8.267E-07 | 1.37783E-07 | below the detection limit | 0.000641206 | 0.00017519 | 9.71508E-05 |
| **2000ppm** | 0.012948333 | 0.0000003 | 0.00000005 | 0.000187266 | below the detection limit | 0.000268543 | 6.91574E-05 |

$P<0.05$ is considered statistically significant (0.95 confidence level). Chl a: chlorophylle A; Cart: cartenoid; PC: phycocyanin; APC: allophycocyanin; PE: Phycoerythrin.

toxicity signs which is naturally occurred and are known as selenosis (Frontasyeva et al., 2009; Schiavon et al., 2016). Indeed, these high concentrations are toxic because producing reactive oxygen species (ROS) which induce DNA oxidation, DNA double strands breakage, and cell death (Schiavon et al., 2016). This assumed that Se substitution with sulfur in S-containing proteins, and its prooxidant ability for catalysis thioloxidation and concomitant production of superoxide, might be its toxicity agent (Chen et al., 2008; Fournier et al., 2010). Additionally, during protein synthesis, this substitution, will change protein structure and function and thereby will cause a teratogenic effect on animals such as fish (Fournier et al., 2010).

Further, Se toxicity impressed by various factors such as oxidation state, Se concentration, microalgae species and characteristic, and environmental condition. So that, it is difficult to realize Se toxicity in aqueous environment (Babaei et al., 2017; Fournier et al., 2010; Schiavon et al., 2016). Therefore, analysis of specific toxicity is required for selected species before Se-enriched biomass production (Babaei et al., 2017). On the other hand, algal sensitivity to elements is strongly depends on species. Umysova et al., (2009) showed that selenate concentrations depend on studied species and may be inhibited 3-fold growth (Umysova et al., 2009). Different organisms react differently to the same metal and may be affect more or less by various metals (Mane et al., 2013).

Li et al. (2003) investigated sodium selenite effect on various parameters of *Spirulina* and showed that sodium selenite causes growth stimulation at concentrations lower than 400 mg/L (especially at 4-50 mg/L). However, they emphasized that the enrichment of *Spirulina* cultures by ranges of 0.5-40 mg/L selenite concentrartion is the most appropriate concentration; this salt has a toxic effect at concentrations above 500 mg/L on algae.

Se toxicity mainly attributed to stronger bioaccumulation of microalgae in comparison to macroalgae, inactivation of Cys-contained enzymes, inhibitory effects on photosynthetic electron transfer chain, which overall result of these mechanisms causes reduction of photosynthetic yield, metabolism disorder, and finally constrained of growth rate (Khademi and OraghiArdebili, 2017).

*S. platensis* growth is supported at 5 and 10 ppm concentrations (Fig. 1). On the other hand, the dry weight of microalgae decreased at selenate levels above 50 ppm. Khademi and OraghiArdebili (2017) reported that an increase of selenate concentration up to 10 mg/L reduced significantly the growth rate of *Spirulina* sp. This trend is observed in the current study at above 50, 100, and 300 ppm concentrations (Fig 1.). Sun et al. (2014) showed that Se is required as a nutrient to biosynthesis of proteins and lipids. They represented that growth stimulatory effect of Se is attributed to its antioxidant activity which increases chlorophyll-a and reduces LPO and ROS.

According to Babaei et al. (2017), 2.5 mg

Se/g DW and 8.5 mg Se/g DW of biomass, caused a slight stimulatory effect on growth rate during the first three days of experiment. Growth and photosynthetic inhibition observed up to 85 mg/g DW Se concentration. Besides, our results are in consistent with this study.

In the current study, the dry weight of microalgae decreased at selenate levels above 50 ppm which is in accordance with Khademi and OraghiArdebili (2017). This is probably due to the selenate effect on the cell division in microalgae (Geoffroy et al., 2007; Khademi and OraghiArdebili, 2017). In accordance with Sun et al. (2014), reduction in biomass at 50, 100, and 300 ppm of selenate concentration, is result of toxic effects of high concentration of selenate. The effect of 0, 5, and 10 mg/L of selenate concentration on the growth and antioxidant activity of *Spirulina platensis* investigated by Khademi and OraghiArdebili (2017) during 7 days cultivation period. The results showed that 5 mg/L of selenate concentration caused growth improvement but it was toxic in 10 mg/L concentration. Moreover, chlorophyll and carotenoid content in selanate-treated microalgae did not have any significant change in comparison to control culture. They stated that Se affects on *Spirulina* physiology at low concentrations.

The results of Chen et al. (2008) determined that using various Se concentrations (0-250 mg/L) caused Se accumulation in a dose-dependent pathway. Results indicated that at concentrations lower than 150 mg/L, Se causes increase in biomass concentration, photosynthetic pigment content including Lutein, beta-carotene and chlorophyll a, and the activities of glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), and Guaiacol-dependent peroxidases (POD). These results confirm that antioxidant enzymes play a crucial role in cell protecting against Se stress. Higher Se concentrations above 175 mg/L cause more accumulation and activities of GPX, SOD, CAT, POD and reduction in biomass concentration and photosynthetic pigments contents. Moreover, continuous reduction of chlorophyll-a content has been reported in control cultures and treated cells with 200 mg/L of Se after 0-11 days of incubation (Chen et al., 2008).

Umysova et al. (2009) investigated the effects of Se compounds at 0-100 mg/L ranges on *Scenedesmus quadricauda* and the level and chemical form of Se are crucial factors in cellular response and revealed that Se toxicity increased by sulfate-deficiency conditions and this is a result of Se interference by sulfur metabolism. It has also been reported that at the above concentration of 50 mg/L, most cells died after 1 or 2 days of incubation. Moreover, Se toxicity cause chloroplasts bleaching, cell malformation, and finally death.

Fournier et al. (2010) also studied the toxic effect of selenate on unicellular green algae *Chlamydomonas reinhardtii* as a function of sulfate ion concentration. It showed that maximum cell density and growth rate decreased by elevation of selenate in culture medium especially in the presence of

low-level sulfate ion. The same results obtained in the current study. According to these results, selenate toxicity correlates with its bioaccumulation in algae and shows that toxicity depends on intracellular effects of selenate rather than surface effects. Mane et al. (2013) reported that Se has stimulatory effects on *Anabaena ambigua* (at 3.0 mg/L), *Anabaena subcylindrica* (at 0.5 mg/L), *Nostoc commune* (at 0.5 mg/L), *Nostoc muscorum* (0.5 mg/L), *Spirogyra* sp. (0.1 mg/L) and *Spirulina* sp. (0.1 mg/L).

Based on Schiavon et al. (2012) after cultivation of *Ulva* sp. at the 6 first days of incubation, the pH of culture media was identical between all treatments but a considerable increase of pH from 8.46 for 0 concentration to 9.71 for 100 µM concentration of selenate observed at the end of the experiment (incubation period was 10 day). Nonetheless, there was no significant difference between control culture and supplemented medium with 2.5, 10, and 50 µM of selenate. Based on the results, the plastids are major aim for Se toxicity, ultrastructure studies in various microalgae, e.g. *Chlamydomonas reinhardtii* showed that both selenate and selenite could damage thylakoid membranes and cause overproduction of starch granules (Schiavon et al., 2016; Umysova et al., 2009).

According to Mane et al. (2013), at lower Se levels, Se has stimulatory effects on total chlorophyll content, protein, carbohydrate, starch, and free amino acids of studied algae. Moreover, all biochemical parameters of algal strains decreased gradually as in a dose-dependent manner. Furthermore, Babaei et al. (2017), indicated that a low Se level (about 19-65 µM) partially stimulates photosynthetic activity during 24 hours after cultivation. Sun et al. (2014) emphasized a similar trend and the inhibitory effect of Se salts on photosynthetic yield depends on ultrastructural changes. Probably, chloroplasts are the first goal of Se toxicity, so that their stroma, thylakoid, and pyrenoids are impressed and extra accumulation of starch and formation of condensed Se granules in their structures is reported using electron microscope. Finally, increasing in Se concentration (190 µM) causes a significant decrease in photosynthetic activity and at the growth rate. This matter is consistent with the data other studies on various algae such as *Spirulina*, *Chlamydomonas* or *Chlorella sorokiniana* (Geoffroy et al., 2007; Morlon et al., 2006; Schiavon et al., 2016).

Schiavon et al. (2016), reported that thallus morphology did not impress by selenite/selenate. They concluded that resistance to high selenate concentrations (200 and 400 mg/L) had been probably due to the exclusion mechanism for Se which may be include down regulation of at least one sulfate transporter. Although there was no effect on total morphology but chloroplast ultrastructure had affected, our results showed that in concentrations above 100 ppm, microscopic morphology of microalgae starts to change and the overall structure disrupted finally. However, in another study conducted by Belokobylsky et al. (2004), an increase of selenate level did not affect the morphol-

ogy of *Arthrospia platensis* Gomont, Gojkovicet al. (2014) considered this result. Likewise, they observed typical changes on chloroplast structure due to challenge with selenate oxyanions using electronic microscopi. Indeed, granulated stroma and reduction of its density and variations in thylakoids observed in contact with 40 and 100 mg/L of selenate (Gojkovic et al., 2014).

Effect on the pigment amount:

Carotenoids play crucial role in cell response to oxidative stress as one non-enzymatic antioxidant, which arises from ROS. Indeed, singlet oxygen in stress situations causes severe metabolic disorders using oxidative damage to cell components (Sun et al., 2014). Further, it found that carotenoids protect the photosynthetic membrane from photo-oxidation using effective removal of singlet oxygen and returning chlorophyll to the primary state. Sun et al. (2014), reported an increase in carotenoid content during the cultivation. However, in our study, the chlorophyll and carotenoid contents decreased at Se concentrations above 50 and 25 ppm, respectively. Subsequently, it can be a result of lipid peroxidation in chloroplast membranes. These effects caused by cellular damage or death from Se toxicity (Sun et al., 2014).

Mane et al. (2013) reported the inhibitory effects of some metals (such as iron, copper, silver, zinc, etc.) on chlorophyll contents in algae, which are in accordance with this study.

As it was demonstrated in the present study, when the selenate concentrations increase to upper levels (100-2000 ppm) chlorophyll amount will increase in 5, 10, 25, and 50 ppm of selenate concentration and were showed reduction of this the pigments is reduced. On this basis, an increase of chlorophyll amount in treated cells with low levels of Se (5-50 mg/L) may be due to efficient removal of ROS using CAT, SOD, and GPX. Also, these results stated by Sun et al. (2014).

Gojkovic et al. (2014), investigated the effect of 40 mg/L (212 μM) selenate on *Chlorella sorokiniana*. The results indicated that chlorophyll and carotenoid amounts did not change but the growth rate decreased up to 25%. Schiavon et al. (2012) reported that chlorophyll amount did not have any significant change in cultured thallus at the presence of selenate in comparison to control culture. However, an increase of these pigments observed after three days of cultivation in the presence of high selenate levels. In contrast, the carotenoid amount significantly rose during 3 to 10 days of experiment especially in a 50-100 mg/L range. On the other hand, at low levels of selenate (2.5-50 mg/L) any effect on the microscopic structure of thallus did not observe and in the higher concentrations (100 mg/L) thallus thickness decreased.

Gojkovic et al. (2015), reported that the total amount of chlorophyll and carotenoid increased during the first 48 hours of cultivation at control culture and nearly constant up to 96 hours of cultivation. Thereafter, the levels of pigments in the control culture de-

creased as the self-shading effect.

For Se-enriched biomass production and development of biotechnological processes, a better perception of total Se effect on microalgae is required (Babaei et al., 2013). Due to the increase in bioavailability, Se is essential both in biomedicine and as a complementary diet for domestics. It seems that further studies need to conduct usage of Se-*Spirulina* as antioxidant for aquaculture feed and human food (Sun et al., 2014). Further, more studies need to find key variables, which cause growth inhibition or even growth failed on large scales at different Se Concentration.

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# Study the Effect of the Terrestrial Cyanobacterium *Nostoc commune* Aqueous Extract on Seed Germination and Seedling Growth of Rice

**Mohammad Hossein Abedi Firoozjaei[1], Seyedeh Batool Hassani[1*], Ehsan Nazifi[2], Somayeh Keypour[3]**

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**Abstract**

Seed priming has a significant effect on seed germination and improves the establishment of crops. In this study, rice seeds (*Oryza sativa* L. cv. *Shiroodi*) were primed by different concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 g/l) of *Nostoc commune* Vauch aqueous extract. Furthermore, seedling growth, seed germination rate and percentage were examined. The results showed that *N. commune* extract had a significant positive effect on increasing the percent and rate of seed germination. Indeed, the length of roots and shoots in seedlings obtained from primed seeds with *N. commune* extract increased comparing to those of the seedlings from primed seeds with water. Additionally, the highest seed germination percentage and seedling length was obtained by 0.025 g/l *N. commune* extract. Based on the results, seed germination and seedling growth was not affected positively by seed priming with *N. commune* extract at concentrations more than 0.1 g/l. Therefore, sowing rice seeds with a low concentration of *N. commune* extract can improve seed germination and seedling establishment.

**Keywords**: Seed Priming, *Oryza sativa*, *Nostoc commune*, Germination rate, Germination percentage

## Introduction

Rice (*Oryza sativa* L.) has global importance, especially in Asian countries. Due to its importance to human life, the year 2004 is designated as the international year of rice by the United Nations (Gnanamanickam, 2009). It was reported that rice seeds affected on the cultures, diets, and economies of thousands of people worldwide (Van Nguyen and Ferrero, 2006).

As previous works demonstrated, increasing seed germination rate and initial seedling establishment are crucial factors affecting rice production and yield (Jongdee et al., 2002). Seed germination is a crucial stage in the development of plants. There is a direct relationship between the seed germination stage and proper plant production and yield

1-Department of Plant Science and Biotechnology, Faculty of Biological Sciences and Technology, Shahid Beheshti University, Tehran, Iran.
2-Department of Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Mazandaran, Iran.
3-Department of Biology, Farhangian University, Tehran, Iran.
*E-mail address: b_hassani@sbu.ac.ir

(Murungu et al., 2003; Sawan et al., 2009). Seed priming with biological compounds is one of the valuable techniques to accelerate seed germination and improve the early establishment of crops (Lee and Kim 1999; Farooq et al., 2006, 2007). Beneficial effects of seed priming with algae was analysed for several crops such as rice, barley, cucumber, tomato, cotton, berseem clover, corn, and lettuce (Seifikalhor et al., 2019; Shariatmadari et al., 2013; Thajuddin and Subramanian, 2005; Younesi et al., 2019; Rezaee et al., 2019; Soltani et al., 2019). Previous researches indicated that priming the seeds with cyanobacteria increases seed germination and seedling growth in crops and medicinal plants (Shariatmadari et al., 2015; Seifikalhor et al., 2019; Chookalaii et al., 2020).

Cyanobacteria are the first photosynthetic organisms that produced oxygen in photosynthetic pigments (Whitton and Potts, 2012; Liberton et al., 2013). Indeed, cyanobacteria have a unique potential to increase plant productivity in various environmental conditions (Chatzissavvidis and Therios, 2014; Umesha et al., 2018). Water-retaining capacity by cyanobacteria jelly structure, nitrogen fixation, plant growth stimulants production such as auxin, gibberellin, and cytokinin, vitamins, and amino acids are the significant features that improve plant growth (Hashtroudi et al., 2013; Saadatnia and Riahi, 2009).

It seems that considering the positive physiological effects of seed priming with cyanobacteria the concentration and type of extract is required to be optimazed for each species. The present study investigates the efficacy of different concentrations of *Nostoc commune* water extract on seed germination and seedling growth of rice.

## Material and methods

*Plant material*

Seeds of *Oryza sativa* cv. *Shiroodi* obtained from International Rice Research Institute in Amol (IRRI). It is noteworthy to mention that *Shiroodi* cultivar has the highest level of cultivation in the northern regions of Iran.

*Preparation of N. commune extract*

Naturally, growing colonies of *N. commune* collected from the soil in Babolsar, Mazandaran, Iran (36°42'47"N, 52°41'25"E) during September 2020. Then the colonies were washed under tap water to remove the soil and air-dried at room temperature in the shade. Then, the sample was grounded using an electrical blender. Next, the *N. commune* powder (0.5 gr) was suspended in autoclaved distilled water (100 ml) and placed on a shaker at room temperature overnight. Also, the mixture was sonicated for 30 minutes for achieving better extraction. After that, the sonicated mixture was centrifuged at 4000 rpm for 5 minutes at room temperature. Finally, the supernatant was filtered through Whatman filter paper No. 1 and a clear extract obtained.

*Seed sterilization*

The seeds incubated in distilled water containing 2 drops of detergent per liter for sterilizing in 10 minutes. Then, the seeds were

soaked in 1% sodium hypochlorite for 10 minutes. Finally, the seeds were washed twice with autoclaved distilled water for 2 minutes.

*Seed priming, treatments, and cultivation conditions*

*N. commune* extract was diluted to eight concentrations; 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 g/l for treating the rice seeds. Ten seeds treated with 5 ml of each concentration in separate Petri dishes, each with three replications. All the Petri dishes incubated under sterile laboratory conditions at 25 ± 2 °C. Indeed, cultures were placed at laboratory conditions for 3 days in the dark. Finally, cultures kept in a photoperiod of 16 h light with 5500 lux /8h darkness for seven days to study the growth rate of seedlings.

*Measurement of rate and percentage of seed germination*

Germinated seeds (seeds with root emerged) were counted daily and seed germination rate was calculated by the following equation (Agrawal, 1991)

growth rate$\sum$ = The number of germinated seeds at (n) day/ (n) day (No. 1)

Where n is the number of the day which germinated seeds counted.

The percentage of seed germination was calculated using the following equation.

percentage of seed germination = (number of germinated seeds at third day / total number of seeds) × 100 (No. 2)

*Measurement of seed growth parameters*

Seedlings transferred to 16 h light/8 h dark conditions within three days of germination in the dark. After seven days the length of seedling, shoot, and root measured by ImageJ software (version 1.44P; US National Institutes of Health, Bethesda, Maryland, USA) (Collins, 2007).

*Statistical analysis*

The experiments conducted with completely randomized designs arrangedat least three replications. Statistical analysis performed using SPSS software (version 18). Microsoft Excel 2016 software used to create charts. The data presented as the mean ± standard error. Significance of differences between the data obtained from One-Way ANOVA analysis followed by Duncan test ($p<0.05$).

**Results**

*The effect of N. commune extract on rice seed germination*

Although the results indicated that different concentrations of *N. commune* extract had a significant effect on the germination percentage of rice seeds, no significant effect on seed germination was reported (Table 1).

Furthermore, comparison of germination percentages showed that only 0.025 g/l of *N. commune* extract increase significantly seed germination percentage (Figs 1 and 2). While, the highest values of germination (93%) occurred when the extraction applied in 0.025 g/l only 70% of untreated seeds germinated. Although an increase and decrease in seed germination percentage observed at 0.05, 0.1 g/l and 0.2, 0.4 g/l of *N. commune* extract, respectively that it was not significant (Figs 1 and 2).

However, the highest germination rate of rice seeds (approximately 10 germinated

seeds per day) was obtained in primed seeds with 0.025 g/l *N. commune* extract. Indeed, negative effect of higher concentrations of *N. commune* extraction (0.3 and 0.5 g/l) on seed germination rate is shown in Figure 3.

*The effect of N. commune extract on rice seedling growth*

The results of rice seedling growth indicated the significant effect of *N. commune* extract concentration on the length of seedling, root, and shoot of rice seedling (Table 2).

Further, the highest seedling length was

**Table 1.** One-way analysis of variance (ANOVA); effect of different concentrations of *N. commune* water extract on percentage and germination rate of rice seed ($p < 0.05$)

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Percentage of seed germination | Between Groups | 2429.167 | 7 | 347.024 | 4.899 | .004 |
| | Within Groups | 1133.333 | 16 | 70.833 | | |
| | Total | 3562.500 | 23 | | | |
| rate of seed germination | Between Groups | 25.685 | 7 | 3.669 | 1.655 | .191 |
| | Within Groups | 35.481 | 16 | 2.218 | | |
| | Total | 61.167 | 23 | | | |

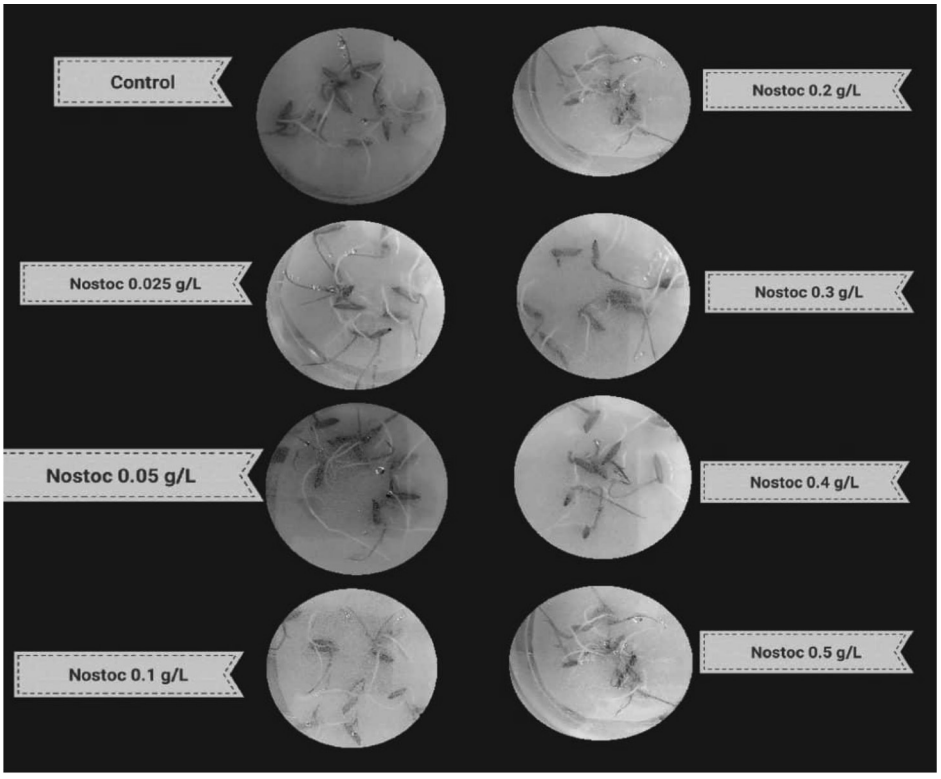


**Fig. 1.** Effect of different concentrations of *N. commune* water extracts on rice seed germination

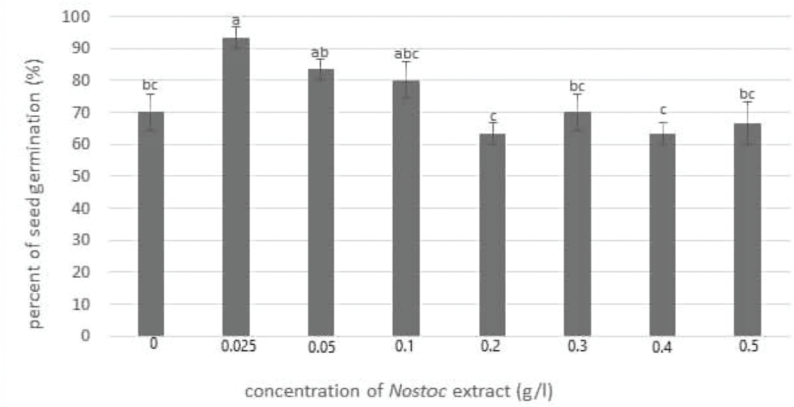


**Fig. 2**. The effect of different concentrations of *N. commune* extract on the germination percentage of rice seed. The bars are the means of three repetitions ± standard error. Letters indicate a significant difference between the means at the probability level of P <0.05

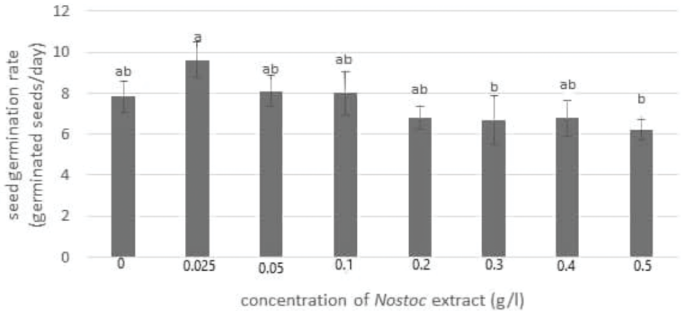


**Fig. 3**. The effect of different concentrations of *N. commune* water extract on rice seed germination rate. The bars are the means of three repetitions ± standard error. Letters indicate a significant difference between the means at the probability level of P <0.05.

observed in seeds treated with 0.025 g/l *N. commune* extract (Figure 4). However, seedling height under treatments of 0.05 and 0.1 g/l of *N. commune* extract was significantly increased; higher concentration of *N. commune* extraction (0.5 g/l) had no affect on seedling height (Figure 4).

There was a significant increase in root length at concentrations of 0.025-0.2 g/l *N. commune* extraction. The results showed that concentrations higher than 0.2 g/l *N. commune* water extract had no positive effect on root length (Figure 5).

Although the highest shoot length was obtained in seedlings treated with 0.025 g/l of extract, the result showed no significant increase comparing to the control, or a negative effect at high concentration of *N. com-*

**Table 2**. One-way analysis of variance (ANOVA) of *N. commune* water extract concentrations on rice seedling growth parameters at p <0.05

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Seedling height | Between Groups | 11.132 | 7 | 1.590 | 6.414 | .001 |
| | Within Groups | 3.967 | 16 | .248 | | |
| | Total | 15.099 | 23 | | | |
| Shoot height | Between Groups | 4.642 | 7 | .663 | 5.698 | .002 |
| | Within Groups | 1.862 | 16 | .116 | | |
| | Total | 6.505 | 23 | | | |
| Root height | Between Groups | 2.262 | 7 | .323 | 4.478 | .006 |
| | Within Groups | 1.154 | 16 | .072 | | |
| | Total | 3.416 | 23 | | | |

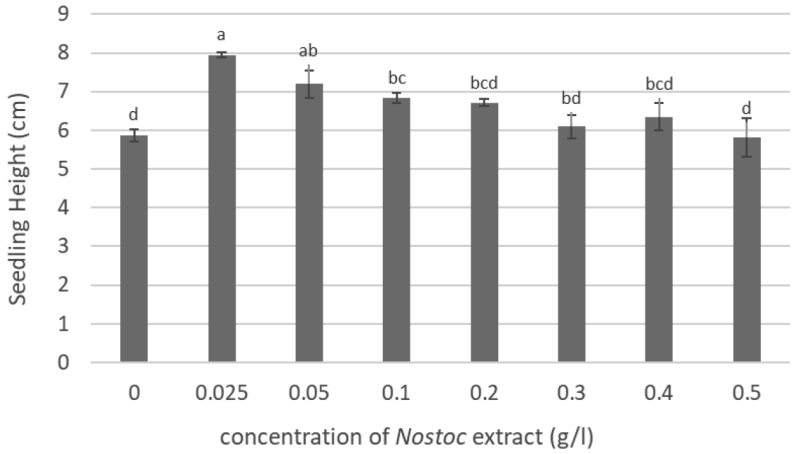


**Fig. 4**. Effect of different concentrations of *N. commune* extract on rice seedling height. The bars are the means of three repetitions ± standard error. Letters indicate a significant difference between the means at the probability level of P <0.05

*mune* extract on shoot growth. The shoot height was significantly reduced at 0.5 g/l *N. commune* extract (Figure 6).

## Discussion

The percentage and rate of seed germination improvement in crops leads to seedling establishment, plant growth, and a strong root system (Murungu et al., 2003). In addition, increasing the seed germination and production of healthier seedlings provide a better chance for the plant to withstand the environmental stresses. Besides, these factors allow the farmers to introduce more resistant cultivars for agriculture (Conrath et al., 2001; Kalefetoğlu et al., 2009; Borges et

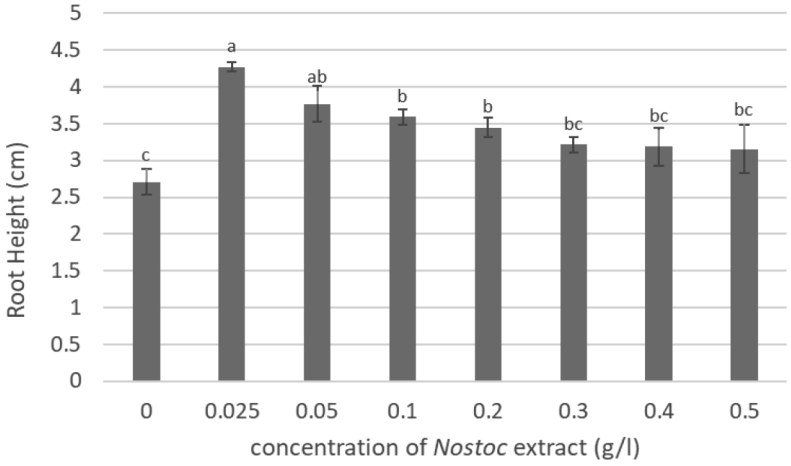


**Fig. 5**. Effect of different concentrations of *N. commune* water extract on rice root length. The bars are the means of three repetitions ± standard error. Letters indicate a significant difference between the means at the probability level of P <0.05

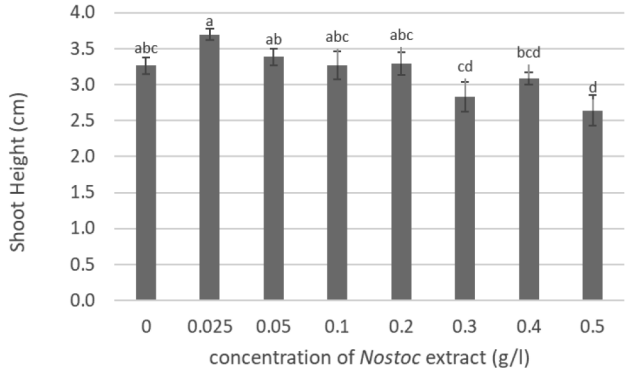


**Fig. 6.** Effect of different concentrations of *N. commune* extract on rice shoot height. The bars are the means of three repetitions ± standard error. Letters indicate a significant difference between the means at the probability level of P <0.05

al., 2014; Ahmadpour et al., 2016). Several studies focused on increasing the seed germination percentage and improving the initial establishment of seedlings in crops (Seifikalhor et al., 2019; Thajuddin and Subramanian, 2005) and medicinal plants (Chookalaii, et al., 2020) by priming and treating the seeds with biological compounds.

In this study, priming *Shiroodi* rice seeds with *N. commune* extract increased the germination percentage and rate. Previous researches have shown that priming the rice

and alfalfa seeds increased the rate and percentage of seed germination (Lou et al., 2004; Basra et al., 2006). Indeed, primed seed performs better metabolic processes of germination, especially the hydrating step and the hydrolytic activity. Further, seed priming increases catalase and superoxide dismutase activity (Yan, 2016; Falahhosseini et al., 2017). In addition, seed priming appears to increase germination by reducing damage to proteins, RNA, and DNA. Following the priming of seeds, the activity of phosphatase, synthesis of RNA, DNA, and cell division increases (Eskandari, 2013).

In the last decade, the study of biotechnology of microalgae and active compounds derived from cyanobacteria is progressed (Dahms et al., 2006). *N. commune* is known to be rich in growth-promoting substances such as vitamins and amino acids (Abed et al., 2009). Furthermore, *Nostoc* sp. can poduce phytohormones, including auxin, gibberellin, and cytokinin (Hashtroudi et al., 2013; Esch, 2014). Cytokinins increase the cell division in seeds and activate the alpha-amylase enzyme, that increas germination rate and percentage (Craigie, 2011). Moreover, an algal extract contains gibberellin and auxin that cause the breaking of seed dormancy, stimulate seedling growth, and proper establishment of seedling in the soil (Yamaguchi and Kamiya, 2001; Gayathri et al., 2017; Tan et al., 2021). However, low or high concentration of auxin reduces the cell division in root, intermediate concentration of auxin have a positive effect (Campanoni and Nick, 2005).

In this study, the positive effect of *N. commune* extract observe at 0.025 g/l concentration (0.025 g/l). It seems that at low concentration of *N. commune* extract the level of auxin is lesser to improve seed germination. Therefore, the concentration of algal extract is crucial to achieve better germination and establishment.

In addition, treated rice seedling growth was improved using low concentration of *N. commune* extract. Similarly, several studies confirmed that cyanobacteria increase seed germination and shoot length in rice (Sinha et al. 1999; Saadatnia and Riahi, 2009). Julia et al. (2020) reported that *Macrocystis pyrifera* extract had a positive effect on seed germination and seedling establishment of lettuce. Also, priming of *Medicago sativa* L. seeds with the extracts of *Oscillatoria* sp. and *Spirogyra* sp. increased seed germination and seedling length (Brahmbhatt et al., 2015). Generally, the higher rate of seed germination and growth rate of rice seedlings provide farmers a greater opportunity for replanting and reduce the germination time. This research performed on *Shiroodi* cultivar, which has a high cultivation area in northern Iran. It seems that it is necessary to conduct similar research on other rice cultivars and other crops to analysis the effect of *N. commune* and other algal extract concentration on seed germination and growth.

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

**Pardis Irankhahi[1], Hossein Riahi[1*], Zeinab Shariatmadari[1], Neda Soleimani[2]**

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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 μg $ml^{-1}$ 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-

1-Department of Plant Sciences and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
2-Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
*E-mail: h-riahi@sbu.ac.ir

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 μmol photons $m^{-2}s^{-1}$) 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, genomics 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 (TCTACGCATTTCAC 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 µmol photons $m^{-2}s^{-1}$) 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× 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 × g) for 3 min. Finally, the supernatant separated and sterilized by passing them through a 0.22 µm 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 × 106 cells $ml^{-1}$ in RPMI medium supplemented with 11 mM sodium bicarbonate, 2 mM l-glutamine, 100U $ml^{-1}$ penicillin, 100 µg $ml^{-1}$ streptomycin, and 5% fetal bovine serum. The cell line was plated (200 µl $Well^{-1}$) into 96-well microplates (Nunc, Wiesbaden, Germany); $3\times10^5$ 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 µl 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 µl 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) cyotoplasmic extract at 50 µg $ml^{-1}$ 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 level 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 µg $ml^{-1}$) 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-

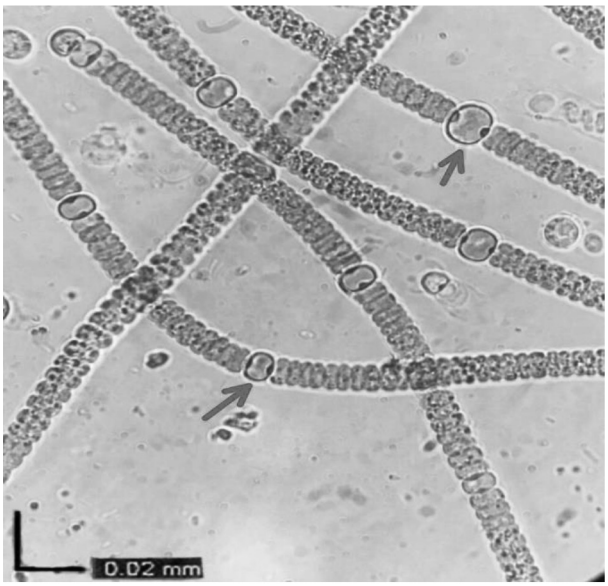


**Fig. 1**. *Nodularia harveyana* ISB112, the heterocysts are marked with arrows

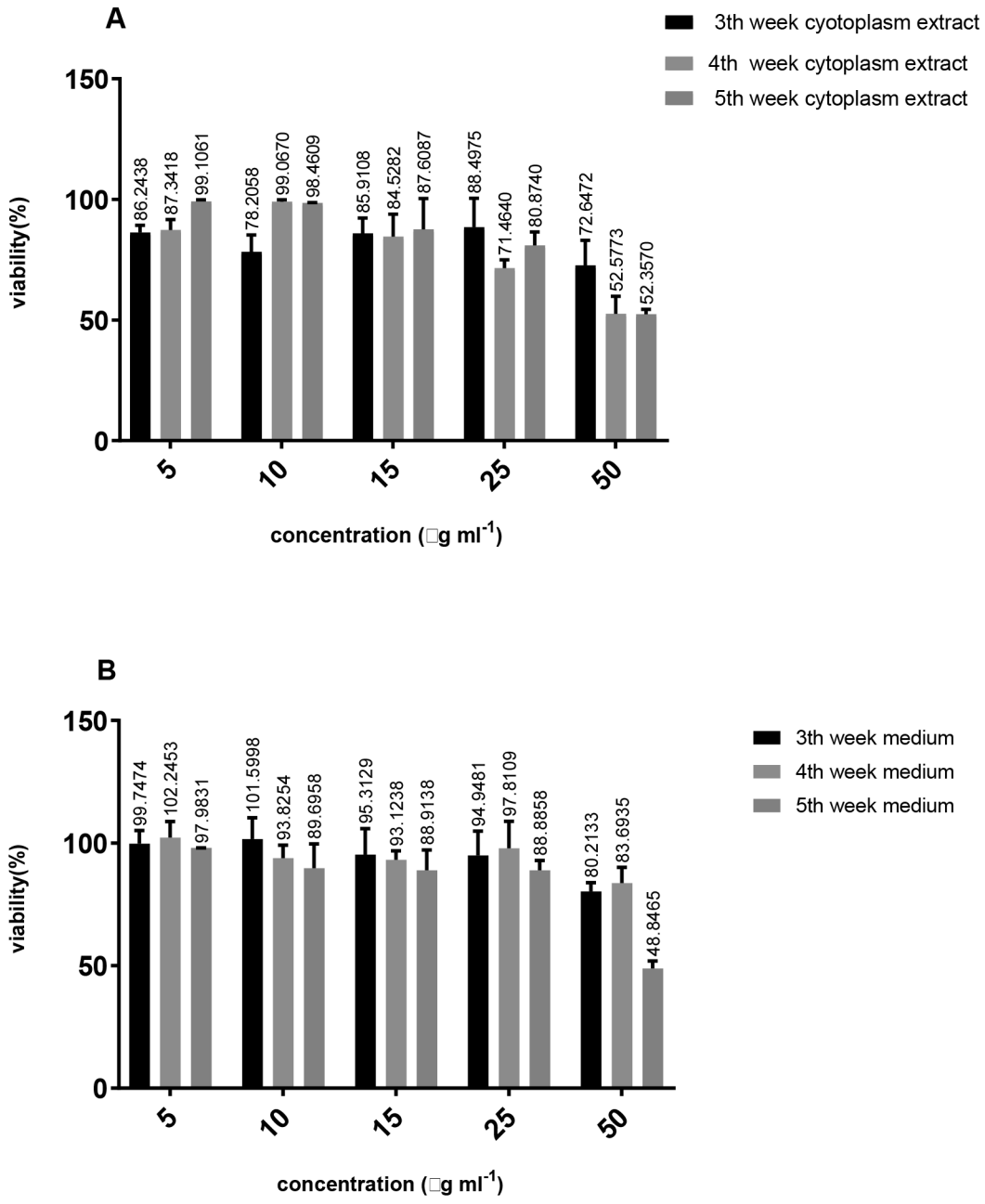


**Fig. 2.** Cytotoxicity of *Nodularia harveyana* ISB112 during a period of three, four, and five weeks of cyanobacteria growth to human melanoma cell line (B16) by MTT assay.

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

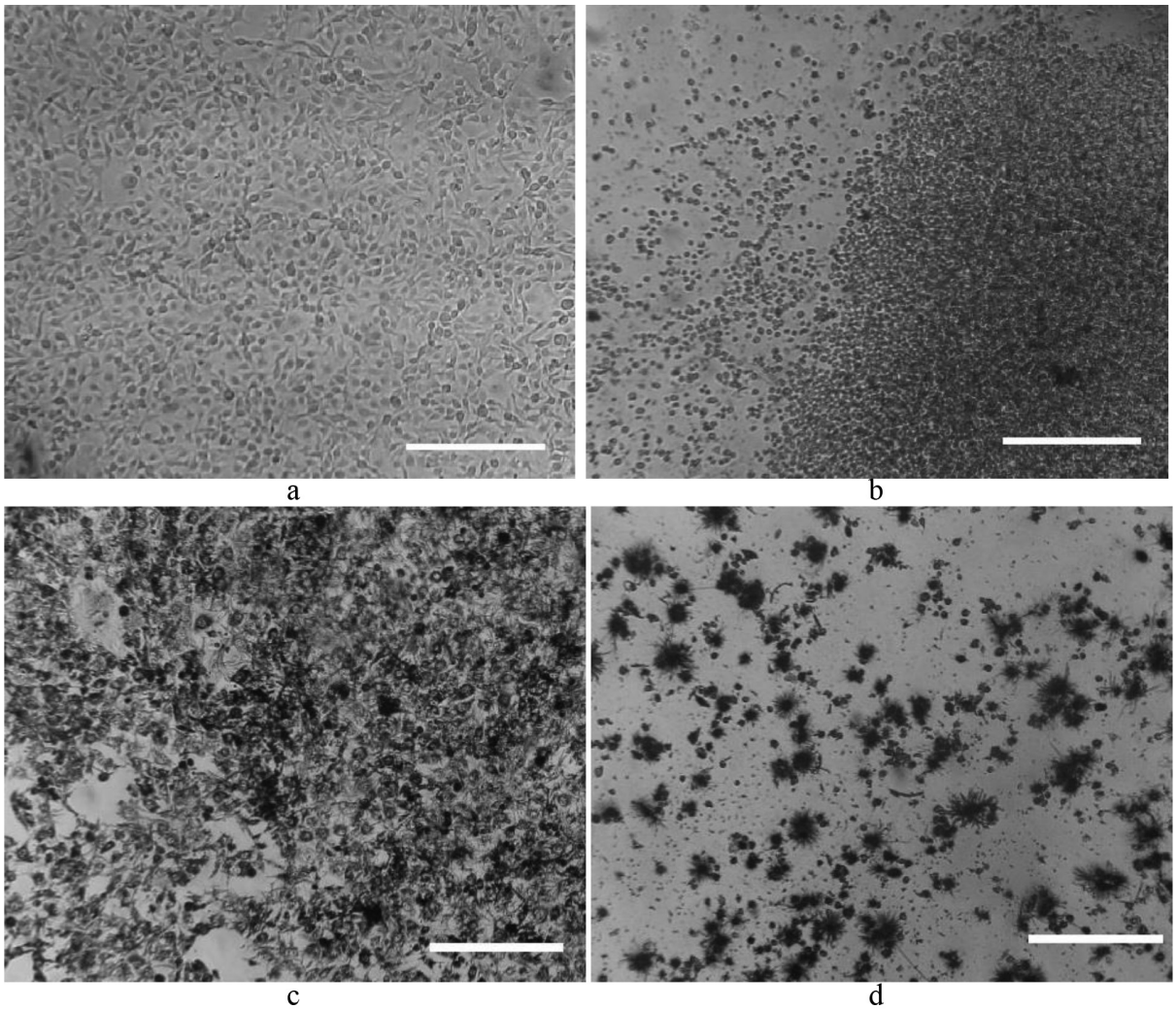


**Fig. 3**. Cells observation under an inverted microscope; (a) melanoma cells (B16) before MTT test, (b) Melanoma cells after treatment with the extract, (c) melanoma cells showing formazan crystals- control group, (d) melanoma cells showing formazan crystals- test group 48 h after treatment. Scale 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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# The Impact of Combined Alkalinity and Time Pretreatments on Light Harvesting System in Tresterial Cyanobacterium *Fischerella* sp. FS 18 (Oscillatorials, Cyanophyta)

**Fatemeh Yadollahi[1], Shadman Shokravi[1*], Mehrali Mahmoudjanlo[1], Mahdi Ebadi[2]**

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**Abstract**

Possibility of change in the phycobilisome status, photosynthetic pigments, photosynthetic ratios, and photosynthetic parameters of soil cyanobacteria *Fischerella* sp. FS 18 investigated. Neutral and extreme alkaline pH (7, 9), and short time incubation including 20, 40, and 60 minutes treatments. After purification, cyanobacteria were subjected to extreme alkaline treatment for one hour at 20, 40, and 60 minutes intervals. Colorimetric assays of phycocyanin, allophycocyanin, phycoerythrin, chlorophyll) and a comparison of the combined effect of time and alkalinity on photosynthetic ratio performed. Indeed, the photosynthesis-light curves compared with direct measurements. The results showed that the combined treatment of time and alkalinity after 20 minutes of inoculation significantly increased the performance of the photosystem and stability of the phycobilins. While, under the 40 min and both neutral and alkaline treatments, the yield of photosystem II, increased the production of thephotosystem I, and significantly the linear fraction of the photosynthesis-light curve. Although, the needed energy to achieve maximum photosynthesis reduced. Further, the maximum photosynthesis was completely different at 40 min pretreatmentand without pretreatment. Furthermore, the results show no specific regularity and trend at20 and 60 minutes times treatment. Thus, the production of light collecting-antennas is influenced by both time and alkalinity treatments. In consequent, 60 minutes or less treatment times, cause a significant change in the structure and performance of the photosynthetic apparatus. While, alkaline treatments at a short time significantly save energy and enhance photosynthesis.

**Keywords**: Pretreatment, Time, Cyanobacteria, Alkalinity, *Fischerella* sp. FS 18

## Introduction

In this paper, the effect of alkaline pretreatment for 1 hour on *Fischerella* sp. FS 18 studied. Unlike most cyanobacteria, *Fischerella* sp. FS 18 grows better in neutral media (Soltani et al., 2007). Abbasi et

1-Department of Biology, Gorgan Branch, Islamic Azad University, Gorgan, Iran
2-Department of Chemistry, Gorgan Branch, Islamic Azad University, Gorgan, Iran
*E-mail: shadmanshokravi@yahoo.com

al. (2019, 2020) studied *Calothrix* sp. FS 56 and *Fischerella* sp. FS 18 and revealed the effect of short-term (24 h) treatments on efficiency and biomass production. Based on the research, cyanobacteria tend to live and reach their optimum growth rate and photosynthesis capacity in alkaline pH conditions. It is noteworthy that the effect of the shorter 24-hour time treatment compared to longer time (96 hours) is more visible (Abbasi et al., 2019). In such conditions, the performance of the phycobilisome and rate of photosynthesis increases. Abbasi et al. (2019), showed that the activity of photosystems, particulary photosystem 1, increase significantly within 24 h under alkaline treatment (Abbasi et al. 2019). This major achievement can apply to both pure and biotechnological mass cultivation. It assumed that there is a possibility of better result in less than 24 hours. Amirlatifi et al. (2018) represented that time treatments less than 24 h (ten, six, four and two hours after inoculation) in different salinity environments (17, 80, and 160 mM) on *Fischerella* sp. FS 18 provide higher growth rate and photosynthesis capacity.

Tang and Vincent (1999) bagan the investigation of the pretreatments in the cyanobacteria. Their findings on Oscillatorials showed that applying temperature treatments at intervals of 5 °C could completely change the behavior of cyanobacteria. Rosen and Mares, (2016) studied the effect of pretreatments and initial cyanobacterial habituation. They showed that the use of pretreatments can completely change the expected behavior. The application of time is among the most crucial factors in this fundamental change (Downing, 2014). When quite different results observed in a simple pretreatment, by developing molecular techniques we can use molecular biology analysis in this prokaryotic cyanobacteria.

This study aimed to use alkaline shocks to evaluate the impact on optimization. It is assumed that if the time below one hour can somehow affect the photosynthetic apparatus to enhance its performance, there will be a significant economic savings in the future mass cultivation process of this cyanobacterium. The interval between 24 hours and one hour is considerable, and time reducing will be associated with economic efficiency. In addition, the derived results are useful for a better understanding of environmental cyanobacterial fluidity.

Cyanobacteria are basophil organisms (Witton and Potts, 2000; Soltani et al., 2006). Alkaline conditions are a major evolutionary barrier that separates cyanobacteria from other photosynthetic prokaryotic organisms in the response to the environmental factors (Shokravi et al., 2007). The movement of the environment and the transfer of cyanobacteria from alkaline to neutral or acidic conditions have altered the behavior of these organisms and even enabled them to live in acidic conditions (Shokravi et al., 2010). Apparenty, grow in acidic conditions require the ability of the photosynthetic apparatus trapping $CO_2$ and light energy. Therefore, photosynthetic apparatus fluidity is possible even under the most abnormal conditions in

cyanobacteria.

A few studies conducted on the effect of short time treatment (below 60 min) on soil cyanobacteria in Golestan province, Iran (www.Irandoc.ac.ir). Soltani et al., (2011) investigated the effect of salinity on growth and photosynthesis of *Fischerella* sp FS 18. Shokravi et al. (2014) studied the combined effects of pH and extreme light on growth and pigment status of *Hapalosiphon* sp. FS 56. Rodríguez-Sanchez et al. (2012) and Karseno et al. (2018) revealed the effect of pH on survival, growth, and pigment status of *Mirocheate* sp. FS 101 and *Anabaena* sp. FS 76. Although, some studies do not directly and completely investigate the effect of pH on the physiology and ecophysiology of cyanobacteria (Iranshahi et al., 2013; Amirlatifi et al., 2013). Amirlatifi et al. (2018) studied the combined effects of salinity and carbon dioxide limitation on optical physiology and biochemistry on *Calothrix* sp. FS 56.

## Material and methods

The pure culture of *Fischerella* sp FS 18 obtained from the algae bank of Shahid Beheshti University. Indeed, collection and cultivation technique data provided by Soltani et al. (2009). Soil samples cultured according to the method of soil cyanobacteria (Kaushik, 1987). Then, colonization, isolation, and subsequent cultures of *Fischerella* sp. FS 18 was prepared (Kaushik, 1987). Identification was performed using Desikachary (1959), Prescott (1962), Anagnostidis and Komarek (1990), John et al. (2003). The Primary culture carried out in BG0-11 solid and liquid medium at 60 µmol/$m^2$, at 28 °C, and pH of 7.8 (Soltani et al., 2009). After the initial growth of the isolated samples, in the pretreatment condition of pH of 7.9 for 20, 40, and 60 minutes and then transferred to the normal culture medium at pH 9. Additionally, the sample selected as a control without pretreatment. The growth curve plotted based on turbidimetry and dry weight (Léganes et al., 1987). Biochemical analysis including chlorophyll, phycocyanin, phycoerythrin and allophycocyanin were performed in the form of microbes using absorbance spectra (Abbasi et al., 2019). Photosystem ratios and photosynthesis-light parameters evaluated using the Clark model oxygraph according to Soltani et al. (2007). The number and rod part to phycobilisome base calculated according to Amirlatifi et al. (2013) and Shokravi et al. (2014). Indeed, initial evaluation of the pretreatment effect performed by comparing absorbance spectra (Amirlatifi et al., 2018). Then, the statistical analysis performed using SPSS (Version 11). Additionally, data were standard based on Poza-Carrion et al. (2001) using RSP (Version 10) (Ghobadian et al., 2015).

## Results

Generally, the effect of short-term treatments (less than one hour), in the photosystem II and phycobilisome, is evident from the comparison of absorbance spectra (Fig. 1). Indeed, there is a uniform order in the two phycobilisome and photosynthetic organelles. This means that the effects of time

and alkalinity treatments were significantly different in treated and untreated phycoerythrin and phycocyanin. In other words, 20 minutes of alkaline treatment changes the phycobilisome contents. In comparison with phycobilisome, the effect of 20 minutes more pronounced in the carotenoids, the situation is different with the phycobilisome and photosystem II. Although, the use of the time of 20 minutes in alkaline conditions has led to a significant increase in light-collecting pigments, however, application of 40 and 60 minutes reduced the production of these pigments. While, 40 and 60-min treatments enhance the performance of the phycobilisome and photosystem II, production and performance of light-collecting antennas around optical systems diminished. In addition, there are unknown peaks in these areas due to the production of light collector pigments, which affected by the time treatment and appear to decrease in concentration and performance after inoculation at 40 and 60 minutes (Fig. 1). Consequently, treatment for 20 minutes increases more than 30-40% production of carotenoid compounds, which is quite acceptable to justify the efficacy of this method for this cyanobacterium.

If the treatment condition changes to, extreme alkaline (pH 9) the results will be changed (Fig. 2). However, the 40 min time treatment under neutral pH increases the concentration and performs the phycobilisome and photosystem II. Whereas, 20 and 60 min times treatment at alkaline condition had a decreasing effect (Fig. 2). Additionally, at extreme alkaline and 20 to 60 min treatment, the performance of photosystem, phycobilisome and light-collecting pigment regions reduced in compared with neutral condition. Unlike neutral treatment, the behavior of the light-collecting part follows a pattern that is true for phycobilisome and photosystem II. Whereas, application of 40 minutes treatment in extreme alkaline condition enhances the performance of photo-

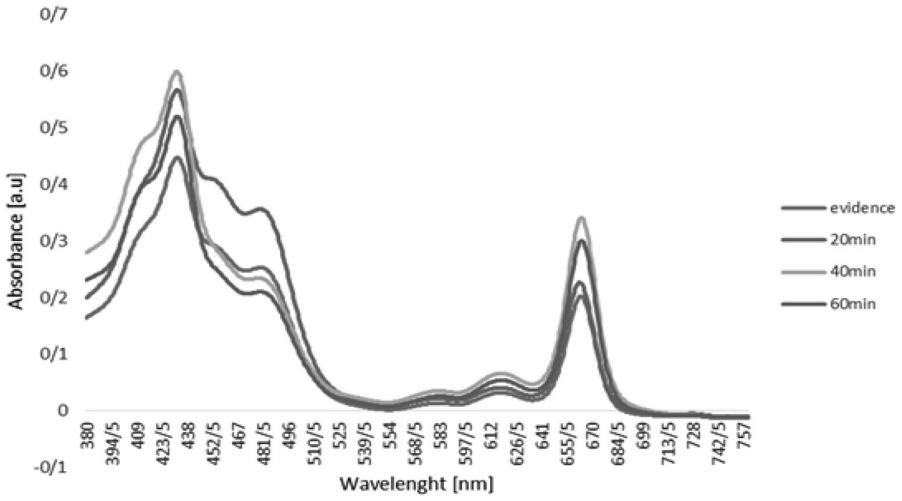


**Fig. 1.** Comparative study of the absorbance spectrum of *Fischerella* sp. FS 18 at pH 9 after 60, 40, and 20 minutes

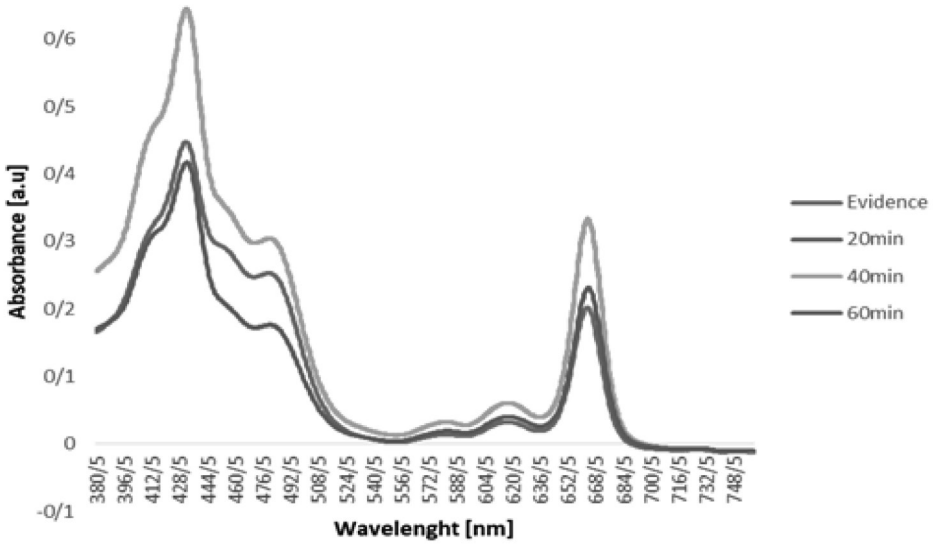


**Fig. 2.** Absorbance spectrum of different time treatments on *Fischerella* sp. FS 18 at pH 9 after 60, 40, and 20 minutes

system II, phycobilisome, and light-collecting antennas. By increasing or decreasing time for 20 min (20 and 60 min treatments), the cyanobacterial photosynthetic apparatus exhibits contradictory behaviors. Indeed, extreme alkaline and treatment for 40 min significantly increases the efficiency of the photosynthetic ratio resulting in the growth and biomass production. However, this increase is far more than the neutral pH and the sensitivity to time in alkaline conditions will increase dramatically.

The photosynthetic ratio compared under optimal conditions based on the absorption spectra without treatment (Table 1). The result from Table 1 is in consistency with the results of the absorbance spectra in Figures 1 and 2. As mentioned before, treatment for 40 min in alkaline in neutral conditions enhances significantly (more than 20%) the photosystem I to II ratio, which confirms the results of the absorbance spectrum. It seems that 40 min treatment in alkaline and neutral conditions (especially alkaline conditions) increases the production of photosystem I ratio and the efficiency of energy transfer from photosystem II to I and reduces the energy loss. Naturally, this reduction in energy loss attributed to increase the performance of the set of photosynthetic ratio, which is the best way to measure directly photosynthesis ratio (Table 2). While, prolonged treatment appears to enhance the performance of the photosynthetic apparatus, this difference is not enough to minimize the role of short time effects. Further, it is still not possible to say whether there is a critical time to enhace photosynthetic ratios under 1, 24, and 96 hours. At present, considering the results for ranges below one hour and above 24 hours treatment in neutral and alkaline conditions perform the highest the photosynthetic yield.

**Table 1.** Photosystem ratios of *Fischerella* sp. FS 18 after 40 minutes and without treatment. The numbers in the parenthesis reported by Abbasi et al., (2019) at 24 and 96 hours treatment

| pH | Time (min) | PSI/PSII |
|---|---|---|
| 7 | 40 | 1.23 (1.48) |
| | 0 | 1.04 ( 1.62) |
| 9 | 40 | 1.56 (2.07) |
| | 0 | 1.13(1.87) |

**Table 2.** Photosynthesis-Irradiance curves parameters of *Fischerella* sp. FS 18 after 40 minutes and without treatment. The numbers in the parenthesis reported by Abbasi et al. (2019) at 24 and 96 hours treatment

| pH | Time (min) | Pmax μmol $O_2$ mg $chl^{-1}h^{-1}$ | A | $I_k$ $\mu E.m^{-2}.s^{-1}$ |
|---|---|---|---|---|
| 7 | 40 | 224.9±12.4 (265) | 1.3±0.1 (1.4) | 157.9 (190) |
| | 0 | 125.3±12.8 (375) | 1.1±0.2 (1.7) | 229.3 (280) |
| 9 | 40 | 322.5±15.2 (525) | 3.9±0.9 (4.9) | 44 (55) |
| | 0 | 155.1±18.3 (409) | 1.9±0.3 (2.4) | 171.2 (161) |

From this point of view, there is sensitivity to time. It seems that sometimes are crucial, higher, and lower than that will reduce photosynthetic ratios and energy transfer efficiency between photosystems.

Table 2 presents the result of direct measurements of photosynthesis-light curves. Furthermore, it is expected that the highest amount of released oxygen is related to extreme alkaline conditions at 40 min. It is noteworthy that there is a two-fold difference (two hundred percent) between the oxygen released under alkaline conditions and no treatment. In addition, a 40 min treatment increases significantly the oxygen production rate from 150 to 320 micromoles per milliliter of chlorophyll per hour. Indeed, the 200% increase in the shadow or linear portion of the photosynthesis-light curve, under the 40-min treatment, is in consistent with the oxygen release rate. While, the 40 min treatment appears to increase the ability to live in limited light conditions, significantly increase the photosynthetic oxygen. Although, the problem of self-shading and the existence in underlying and low-light conditions in mass cultures discussed, this ability survives cyanobacteria. Indeed, increasing the time up to 24 hours increases both capabilities. Furthermore, treatment for

40 minutes enhances both the pigmentation efficiency of the photosystem II reaction center and phycobilisomes especially in alkaline conditions. Consequently, by increasing the production rate of photosystem I, energy loss reduces in the transmission path of photosystem II to. While, this capability increases the oxygen production efficiency and the linear fraction of the photosynthetic-light curve, reduces the energy required to reach the maximum photosynthesis. It can be concluded that the combination of time and alkalinity affects the photosynthetic behaviors in *Fischerella* sp. FS. Although, it is not possible to understand which time treatment, significantly increases the efficiency of the photosynthetic apparatus, time in cyanobacterial intelligency and adaptability, interpret specifically. Indeed, it may be possible to adapt the photosynthetic apparatus to tolerate 24 hours light by altering the circadian rhythms (Yen et al., 2004). Generally, Stigonematales such as *Fischerella* are found in the habitats that do not tolerate 24-hour light conditions, and secondly it is not possible to reduce the impact of subhour time and its inconsistency. Abbasi et al. (2019) observed the behaviors of *Calothrix* sp. FS 56 after time and salinity treatment that required separate analysis in pigment growth and production. Science-based attitudes here are paradox and researchers inevitably take approaches such as agriculture and biotechnology manipulation that have problems. Particularly, many of these mechanisms are unknown to humans (Abbasi et al., 2019; Amirlatifi et al., 2018).

It stands to know that, the phycobilisomes relatively maintain their structure at less than an hour treatments and the absorption spectra of the rod section presented. Indeed, the effect of time under one hour is less pronounces in the photosystems, and the difference between neutral and alkaline conditions is reduced. Whereas, 40 min treatment in both neutral and alkaline conditions enhances the phycobilisome system, the 20 and 60 min treatments have a similar feature. Another point is that the absorption peaks of the phycocyanin and phycoerythrin change at different times, and it may be associated with the restructuring of the phycobilisome. Generally, 20, 40, and 60 min treatments influence the phycobilisome structure.

Indeed, photosystem ratios in cyanobacteria have been the subject of much attention in recent years. In this regard, if we assume that unlike flowering plants, cyanobacteria do not produce a single photosystem I per two photosystems and at the variable ratio. Naturally, we are facing new processes in terms of energy transfer increasing the amount of photosystem I to II results more efficient transmission of energy from the photosystem II to I (Lambers et al., 2008). If this ratio is one the efficiency of photosystem I will reduce due to mutations and prevente loss of transferred energy into five terms, including higher ratios of photosystem I (Paeizi et al., 2012). Additionally, in *Calothrix* sp. FS 56 at different concentration of salinity and alkaline treatment the ratio of photosystems and energy transfer efficiency increased or decreased. Certainly, other key components,

especially phycobilisome are essential and the benefits of increasing or decreasing the photosystem ratio weakened by the low performance of the phycobilisome. Although, if other parts of the photosystem are healthy, using extreme alkaline and 40 min time treatment can significantly increase the ratio of photosystems I and II. Furthermore, it affects the entry of the electron current into the energy-generating reactions and the redaction by reducing the fluorescence and heat processes.

Concerning the same studies, Soltani et al. (2007), Shokravi et al. (2012, 2014), and Safaie et al. (2015) studied the photosynthetic ratio of 2.5. In this regard, the photosystem ratios in investigated cyanobacteria were below 2. Additionally, this was a factor that justified the possibility of interference with other parts of the photosynthetic system. Further, 40 min treatment, especially in alkaline conditions, increase all respects of the photosystems and release more oxygen in low light and low energy conditions. In addition, indicator of the linear part of the photosynthesis-light curve can be due to the increase in the length of photosynthetic antennae or pigment production. Indeed, by increasing the shading coefficient the range of growth and survival will be increase in the depth of the water (Harati et al., 2009) which is a major advantage in the biotechnology of algae. Further, by reducing the amount of energy needed to achieve maximum photosynthesis, the competition potential of the cyanobacteria will increase compared to other algae in similar habitats. Furthermore, The specimens are able to prevent the influx of dimming or shading and remove them. Although, competing cyanobacteria with the same strategies in turn, will stabilize it in the bulk, either pure or in combination with other algae, can expand the range of growth in the natural environment. However, in the studies conducted by Boshruye et al. (2007) and Sasani et al. (2009) on Oscillatorials, a disruption reported after using temperature, light, and salinity treatments. Vakili et al. (2007) studied the effect of 10 min periods of illumination on cyanobacteria *Fischerella* sp.

Shokravi et al. (2011) showed the effectiveness of time treatment on chlorophyll and sugar content. As mentioned above, a few studies presented the effect of the combination of alkalinity and short time, below 60 minutes on the soil cyanobacteria. So far, further studies needed based on combination of different time and alkaline treatments to study the impact of treatments on photosynthetic and biomass production in cyanobacteria.

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