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Enhancement of Total Flavonoid and Phenolic Contents in *Plantago major* L. with Plant Growth Promoting Cyanobacteria

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ABSTRACT

The flavonoid and phenolic compounds are among the main pharmaceutical components of medicinal plants. These compounds are considered as effective anti-oxidant sources. Five cyanobacterial extracts were used to stimulate the plant growth and increase production of specific secondary metabolites in *Plantago major* as a medicinal plant. These cyanobacteria were isolated from the growth bed of the plant in its natural habitats. Nitrate-free BG11 medium was used for preparing axenic monoalgal cultures. Pot experiments were performed by spraying cyanobacterial extracts on the soil of treated plants every 20 days from the time of planting. Growth of plants was evaluated by measuring growth parameters such as plant height, root length, dry and fresh weight of plant, leaf number, leaf area, as well as inflorescence characteristics 60 days after planting. In addition to growth factors, the total amount of phenol and flavonoid of plants was also assessed. Statistical analysis showed that there was a significant difference in the vegetative and reproductive characteristics compared to the control plants. Also, the methanolic extraction of treated and control plants displayed the highest total phenolic and flavonoid content 77.23±3.21 µg of GA mg^{-1} and 389.67±34.43 µg of RU mg^{-1} in plants treated with *Cylindrospermum michailovskoence*. Based on the obtained results, cyanobacterial fertilizers are suggested as the biological elicitors to improve the quantity and quality of medicinal plants products. As a result of this study, chemical content of cyanobacterial extracts and the production of plant growth stimulating substances such as phytohormones can be proposed as factors affecting plant growth parameters and metabolites production.

Keywords: Bioelicitor, Cyanobacterial fertilizers, Medicinal plant, Phytohormones.

INTRODUCTION

Plantago major L. is a perennial medicinal plant that belongs to the family of Plantaginaceae (Zubair *et al*., 2011). Although Eurasia is considered the main original habitat of this plant, its range of distribution is not confined to any specific place, and it can be found as native to many regions (Samuelsen, 2000). This medicinal plant grows in areas up to 3500 meters above sea level, and humid areas such as river banks and coastal regions are the most suitable habitat for its growth. Due to the presence of active metabolites, *Plantago major* L. is used in cosmetic and pharmaceutical industries. Treating dermal diseases, anti-diarrhea, anti-cancer and anti-bacterial properties have been reported in different sources as some of the health benefits of this plant (Samuelsen, 2000; Stanisavljević *et al*., 2008; Mello *et al*., 2015).

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The main pharmaceutical components identified in the *Plantago* species include lipid compounds, caffeic acid, flavonoid and phenolic acid. Phenolic and flavonoid compounds, as the main active metabolites of this plant, are considered as effective anti-oxidant sources in curing cancer (Kuhn and Winston, 2001; Pourmorad *et al*., 2006; Beara *et al*., 2009). It is also reported that p-comaric acid, from phenolic acid family, impedes production of cancer cells and cellular apoptosis by disturbing G2-M division phase (Chang and Shen, 2014; Janicke *et al*., 2011). Some of the researches also show that, due to its strong anti-oxidant properties, *Plantago major* L. reduces the acetaminophen toxicity in the lung (Hussan *et al*., 2015).

Earlier studies indicate that environmental factors, such as biological agents in the soil, can increase the growth of plants, the biosynthesis of their active metabolites, and their resistance to pathogens (Sabbagh *et al*., 2017; Kumar *et al*., 2014). Among them, cyanobacteria play an important role in the stimulation of plant growth and enhancement of bioactive compounds (Saker *et al*., 2000; Shanab, 2001). Production of hormonal and non-hormonal growth stimulating compounds such as auxins, amino acids, sugars and vitamins by these microorganisms is one of the most important factors influencing plant growth (Shariatmadari *et al*., 2015). Moreover, cyanobacteria improve plant growth by improving soil structure through the secretion of mucilage compounds and exclusive polysaccharides, and control fungal and bacterial diseases by means of producing active metabolites (Karthikeyan *et al*., 2007).

In spite of the numerous studies conducted on the growth stimulating effect of cyanobacteria on crop plants and their role in increasing the production of treated plants, there are few reports on the study of medicinal plants. The main purpose of this study was to evaluate the monospecific cyanobacterial extract effects on growth of *Plantago major* L. and assess the amount of phenolic acid and flavonoid compounds. Analysis of the plant growth-stimulating substances, with emphasis on phytohormones and mineral compounds present in the most effective cyanobacterial suspensions, was another purpose of the present study.

MATERIALS AND METHODS

Study Sites and Soil Sampling

In order to isolate the cyanobacteria used in this study, soil samples were collected from the growth bed of seven populations of *Plantago major* L. The plant populations were chosen from Iran's Northern Province, Mazandaran. The soil samples were collected in spring of 2012, based on Rangaswamy method (1966). It should be noted that the specific climatic condition of this province provides a suitable condition for the distribution of *Plantago major* L., in a way that Northern provinces, such as Mazandaran, are considered as the main centers of distribution of this plant in Iran.

Purification and Identification of Cyanobacterial Species

Cyanobacteria were cultured in nitrate-free BG11 medium. The purification of the samples was done through repeatedly subculturing the colonies on the BG11 solid medium. Artificial illumination (74 µmol photons m^{-2} s^{-1}), with a 12/12 hour light-dark cycle, and 25±2ºC temperature was used for culturing the samples. Average pH of the medium was set at 7 (Rinaudo *et al*., 1971). Finally, the purified cyanobacterial strains were identified by binocular optical microscope Olympus, Model BH-2. The identification of the samples was done based on sources such as Desikachary (1959), Wehr *et al*. (2002), and John *et al*. (2002).

Preparation of Cyanobacterial Extract

Cyanobacterial suspensions were prepared through homogenizing 1.0 gr of cyanobacterial biomass after four weeks of

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culturing in the mentioned environmental condition, in 100 mL of sterilized distilled water by means of blender. The cyanobacterial suspension with 1% density was used in this study.

Seedling Growth Test

To select efficient strains, a seedling growth test was performed. For this purpose, the seeds of *Plantago major* L. were collected from native plant populations in Mazandaran Province. After planting *Plantago major* L. in greenhouse condition, the seeds which were used for this study were collected from a single plant in order to minimize the influence of genetic diversity on studied plants and their characteristics.

Air-dried seeds of *Plantago major* L. were soaked in algal extracts (1.0 g fresh algal material in 100 mL of distilled water; experimental) for 24 h. In case of the controls, seeds were soaked in distilled water for 24 hours. Then, the seeds were placed on filter papers wetted with distilled water (control samples) or with 5 mL of algal suspension (treatment samples). After preparation, the plates were placed in a culture chamber at 25ºC temperature, a 12/12 hour light-dark cycle, and light intensity of 74 µmol photons m^{-2} s^{-1}. Each experiment was repeated three times. Efficiency of seedling growth was evaluated by calculating the percentage of seed germination and measuring the length of the seedlings after 10 days of incubation.

Pot Culture

Based on the results obtained from the seedling growth test, three strains of heterocystous cyanobacteria, isolated from the growth bed of *Plantago major* L., and two strains of cyanobacteria, taken from the Algal herbarium of Shahid Beheshti University, were used for the continuation of this study and pot experiments.

Pot culturing of the plant was conducted using sterile soil, which consisted of 60% peat, 25% sand, and 15% natural soil. Plants were grown in the greenhouse with the standard condition for 60 days. In order to minimize the effect of environmental factors, the pots were arranged in a completely randomized design with three replications for each experiment. Inoculation of plants with cyanobacterial suspensions was done every 20 days, through spraying suspensions to the bed soil of the treatment plants. The control plants were irrigated with distilled water during the growth period. The growth parameters including leaf number, leaf area, wet and dry weight of root and leaf, inflorescence length and number were evaluated.

Extraction and Quantification of Total Phenol and Total Flavonoid

Total phenol and total flavonoid of the leaves in the treatment and control plants were evaluated. The plant extracts were prepared by extraction of the dried leaves with methanol for 24 hours. Total phenolic and flavonoid contents of the extracts were quantified by Folin-Ciocalteu assay and Aluminium chloride colorimetric method, respectively (Singleton *et al*., 1999; Bag *et al*., 2015). For the analysis of the total phenolic content, a 1.0 mg mL^{-1} methanolic solution of the extract was prepared. Then, 25 µL of this solution, 125 µL of 10% Folin-Ciocalteu's reagent dissolved in distilled water, and 100 µL of 7.5% $NaHCO_3$ were mixed in a 96 well plate (four replicates). The same procedure was followed for the blank and different concentrations of Gallic acid solutions for constructing the calibration curve. The plate was kept under light protection. The absorbance of the solutions was measured after 2 hours with ELISA reader (Biotek mQuant) at 765 nm. The concentration of total phenol was read (mg mL^{-1}) from the calibration curve and the content of total phenol in extracts was expressed in terms of Gallic Acid equivalent (mg of GA g^{-1} of extract). In order to measure total flavonoid, a methanolic solution of the extract, with the concentration of 1.0 mg mL^{-1}, 4% sodium

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hydroxide, 5% sodium nitrite, and 10% aluminum chloride solutions in water, were used.

For each sample, 25 µL of methanolic solution, 100 µL distilled water, and 7.5 µL 5% sodium nitrite (four replicates) were poured into a 96 well plate. After 6 minutes, 7.5 µL of 10% aluminum chloride, 100 µL of 4% sodium hydroxide, and 10 µL distilled water were added to each well. The plate was kept in the dark, the absorbance of the solutions was read after 15 minutes with ELISA reader at 510 nm, and the mean value of the absorbance was recorded. The same procedure was carried out for the standard solution of rutin to construct the calibration curve. The flavonoid content of extracts was reported in terms of Rutin equivalent (mg of RU g^{-1} of extract (Kamtekar *et al*., 2014).

Extraction and HPLC Analysis of Auxins

Extraction, identification and quantification of the endogenous auxins in the most efficient cyanobacteria, *Anabaena vaginicola* ISB42 and *Cylindrospermum michailovskoense* ISB45, was performed according to the procedure described by Seyed Hashtroudi *et al*. (2013).

Evaluation of Chemical Contents of Cyanobacterial Extracts

Chemical content of the cyanobacterial extracts, such as total nitrogen and inorganic nitrogen, phosphate, sulfate, carbonate and cations were determined by Arian Fan Azma Institute, Tehran, Iran. Laboratory methods of measurements are summarized in Table 1.

Statistical Analysis

One-way ANOVA statistical analysis was performed employing SPSS software, version 16 (Package for the Social Sciences, SPSS Inc., Chicago IL). Means were separated using the Tukey HSD test at $P<0.05$.

RESULTS

Cyanobacterial Extracts as Inoculants for *Plantago major*

According to the data obtained from the seedling growth test, seedlings treated with *Anabaeana vaginicola* ISB42، *Nostoc spongiaeforme* var. *tenue* (ISB63, 64 and 65) and *Cylindrospermum michailovskoense* ISB45 showed a significant difference as compared with the controls (Table 2, Figure 1). Among the cyanobacterial extracts used in the test, *Anabaeana vaginicola* ISB42 had the highest growth promoting effect.

Moreover, the results of the pot studies showed a significant increase in the vegetative characteristics of most of the treated plants compared with the controls. For example, the plants treated with *Anabaeana vaginicola* ISB42 showed a significant difference from the control plants in all of the vegetative and reproductive characteristics (Table 3, Figure 2). Also, the plants treated with *Nostoc spongiaeforme* var. *tenue* ISB65 showed the highest significant increase in leaf area compared with the control plants. Moreover, the plants treated with this strain showed significant increase in most of the measured characteristics. It should be noted that in all of the treatments, increase in the evaluated characteristics was not the same. For example, the plants treated with *Nostoc spongiaeforme* var. *tenue* ISB63 did not show an increase in some of the vegetative characteristics such as leaf area, fresh and wet weight of the leaves as well as fresh weight of root.

Inflorescence characteristics such as the number and length of the inflorescence were the other traits considered in the present study. The results showed that the *Anabaeana vaginicola* ISB42 had the greatest positive effect on the measured inflorescence properties.

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Table 1. Chemical content of algal extracts (1% water extract; fresh algal biomasses homogenates in distilled water).[a]

	Analytical Method	Anabaena (ISB42)	Cylindrospermum (ISB45)	Nostoc (ISB63)	Nostoc (ISB64)	Nostoc (ISB65)
pH	Electrometric	7.43	7.35	6.02	7.03	6.19
EC ($\mu S\ cm^{-1}$)	Platinum Electrode	23.00	25.00	15.00	7.00	10.00
Total Nitrogen (mgL^{-1})	Macro kjeldahl	42.00	34.00	35.00	27.00	34.00
NO_2^- (mgL^{-1})	Colorimetric	0.90	0.80	0.60	0.36	0.16
NO_3^- (mgL^{-1})	Ultraviolet Spectrophotometric	19.80	10.20	9.40	9.60	9.50
NH_4^+ (mgL^{-1})	Nesslerization	27.60	21.10	5.00	1.00	1.10
Phosphate (mgL^{-1})	Vanadomolybdophosphoric acid colorimetric	11.00	11.00	9.00	11.00	14.00
CO_3^{2-} (mgL^{-1})	Titrimetric	0.00	0.00	0.00	0.00	0.00
HCO_3^- (mgL^{-1}	Titrimetric	40.00	40.00	30.00	25.00	25.00
Ca^{2+} (mgL^{-1})	EDTA Titrimetric	3.00	3.00	7.00	5.00	5.00
Mg^{2+} (mgL^{-1})	EDTA Titrimetric	2.00	2.00	5.00	2.00	2.00
Na^+ (mgL^{-1})	Flame Emission Photometric	3.00	3.00	1.50	0.40	11.00
K^+ (mgL^{-1})	Flame Emission Photometric	4.00	8.00	0.11	6.00	9.00

[a] NO_2^-: Nitrite; NO_3^-: Nitrate; NH_4^+: Ammonium; CO_3^{2-}: Carbonate; HCO_3^-: Bicarbonate; Ca_2^+: Calcium, Mg^{2+}: Magnesium; Na^+: Sodium; K^+: Potassium; EDTA: EthyleneDiamineTetraAcetic acid.

Table 3. Effect of 1 % algal culture on growth of *Plantago major* L. 60 days after planting (Mean±SE).

Characters	Treatment					
	Anabaena (ISB42)	Nostoc (ISB65)	Nostoc (ISB64)	Nostoc (ISB63)	Cylindrospermum (ISB45)	Control
Leaf number	8.25± 0.25*	8.00±1.08*	7.75±0 .47*	6.00± 0.81*	7.00±0.70*	4.75±0.75
Leaf area (cm^2)	53.12±4.85*	53.12±4.90*	39.00 ±2.80*	33.43±3.80	28.65±5.26	25.50±2.62
Root length (cm)	33.37±2.08*	22.12±4.21*	27.12±2.63*	26.25±2.68*	20.37±3.06*	16.12±3.70
Fresh weight of leaf (g)	13.51±3.10*	13.67±4.25*	12.05±2.19*	7.27±1.56	9.35±1.04	5.20±0.99
Dry weight of leaf (g)	4.25±1.03*	1.25±0.47*	3.00±1.08*	0.75±0.47	3.5±1.04*	0.75±0.47
Fresh weight of root (g)	10.81±7.81*	5.6±3.03	8.4±8.00*	6.27±5.45	9.32±7.24*	5.82±3.29
Dry weight of root (g)	0.21±.02*	0.32±.018*	0.24±0.01*	0.32±0.01*	0.31±0.02*	0.13±0.01
Number of inflorescence	4.25±1.37*	2.50±1.04*	3.00 ±1.08**	1.25±0.47*	0.75±0.47	0.75±0.47
Length of inflorescence	8.37±1.34**	6.97±0.71*	8.12±0.82**	6.05±1.15**	6.50±0.61**	2.97±0.18

* Significant at the 0.05 level.

Table 2. The effect of cyanobacterial extracts on germination percentage and seedling growth parameters of *Plantago major* L. 10 days after treatment (Mean±SE).

Traetments	Root length (cm)	Leaves length (cm)	Germination percentage
Control	1.30 ± 0.10	0.24±0.00	85
Anabaena vaginicola ISB42	2.23±0.14*	0.40±0.00*	90
Nostoc spongiaeforme var. *tenue* ISB64	1.60±0.05*	0.36±0.03*	87
Cylindrospermum michailovskoense ISB45	1.73±0.08*	0.30±0.03*	87
Nostoc spongiaeforme var. *tenue* ISB63	1.89±0.06*	0.26±0.03*	86
Nostoc spongiaeforme var. *tenue* ISB65	1.83±0.08*	0.36±0.03*	89
Cylindrospermum muscicola ISB88	1.06±0.14	0.24±0.03*	86
Nostoc edaphicum ISB89	1.00±0.23	0.23±0.00	85

* Significant at the 0.05 level.

Figure 1. Comparison between the control and treated seedlings in growth parameters. Treatment conditions: 1% algal extracts (1.0 g fresh algal material in 100 mL of distilled water), (0) Control seedling; (1) Seedling treated by *Nostoc edaphicum* ISB89; (2) Seedling treated by *Cylindrospermum muscicola* ISB88; (3) Seedling treated by *Cylindrospermum michailovskoense* ISB45; (4) Seedling treated by *Nostoc spongiaeforme* var. *tenue* ISB65; (5) Seedling treated by *Nostoc spongiaeforme* var. *tenue* ISB64; (6) Seedling treated by *Nostoc spongiaeforme* var. *tenue* ISB63, (7) Seedling treated by *Anabaena vaginicola* ISB42 (Bar= 1 cm).

Figure 2. Comparison between the control and treated plants in growth parameters. Treatment conditions: 1% algal extracts (5.0 g fresh algal material in 500 mL of distilled water) were sprayed on soil of treated pots; (0) Plant treated by N*ostoc spongiaeforme* var.*tenue* ISB63; (1) Plant treated by *Nostoc spongiaeforme* var. *tenue* ISB64; (2) Plant treated by *Anabaena vaginicola* ISB42; (3) Plant treated by *Nostoc spongiaeforme* var.*tenue* ISB65; (4) Plant treated by *Cylindrospermum michailovskoense* ISB45, and (5) Plant irrigated by distilled water (Control plant) (Bar= 10 cm).

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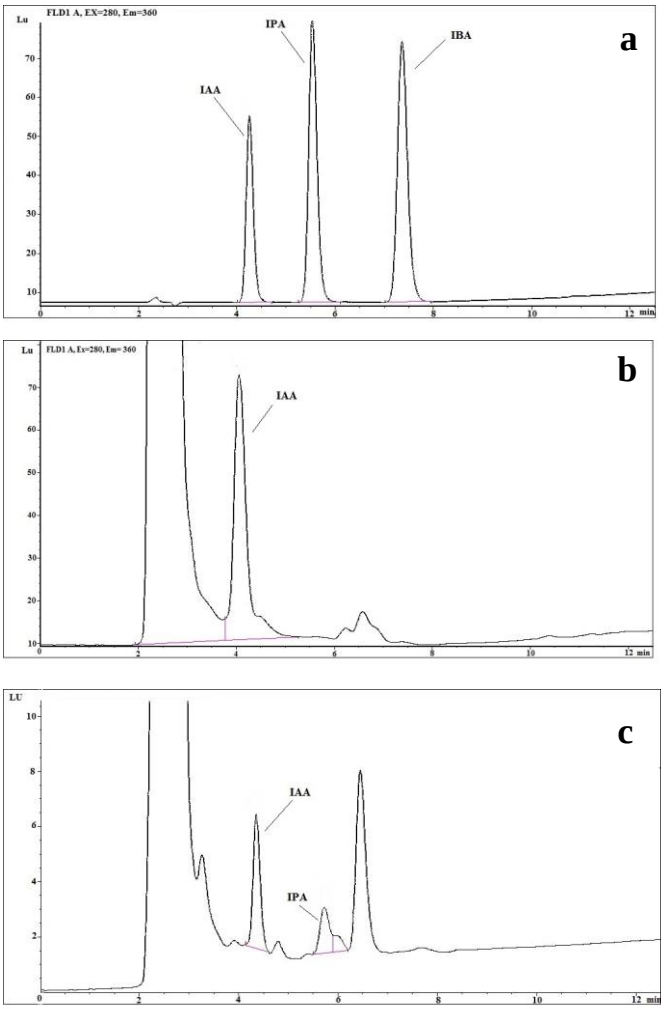

Figure 3. HPLC chromatograms of the ultrasonicated samples for 30 minutes with fluorescence detector: (a) HPLC chromatogram of a 500 ng mL^{-1} standard of three auxins with fluorescence detector. HPLC conditions: UV detection wavelength at 225 nm, fluorescence excitation and emission wavelengths at 280 and 360 nm, respectively, column temperature of 25°C; (b) HPLC chromatograms of the ultrasonicated sample of *Cylindrospermum michailovskoense* ISB45 for 30 minutes, and (c) HPLC chromatograms of the ultrasonicated sample of *Anabaena vaginicola* ISB42 for 30 minutes.

Due to the medicinal importance of the phenol and flavonoid compounds in this medicinal plant, the total phenol and flavonoid of the treated and the control plants were measured and compared (Table 4). Based on the results obtained from the total phenol and flavonoid test, plants treated with *Cylindrospermum michailovskoense* ISB45 and *Anabaeana vaginicola* ISB42 showed the highest content of phenol and flavonoid, respectively, while the control plants had the lowest amount of these compounds.

Determination of Plant Growth Stimulating Factors in Cyanobacterial Extracts

In the chemical analysis of the total content of the cyanobacterial extracts, total nitrogen, ammonium, nitrite and nitrate had the highest amount in *Anabaeana vaginicola* ISB42 compared with the others (Table 1).

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Table 4. Total phenol and flavonoid measurements per microgram per mg of dry extract.

	Total phenolic content (µg of GA mg^{-1} of dry extract)	Total flavonoid content (µg of RU mg^{-1} of dry extract)
Nostoc spongiaeforme var. *tenue* ISB63	63.33 ± 1.33	321.67 ± 11.40
Cylindrospermum michailovskoense ISB45	77.23 ± 3.21	389.67 ± 34.43
Anabaena vaginicola ISB42	70.9 ± 1.75	370.67 ± 21.58
Nostoc spongiaeforme var. *tenue* ISB64	61.53 ± 2.30	285.67 ± 31.13
Nostoc spongiaeforme var. *tenue* ISB65	60.77 ± 1.33	299.56 ± 13.40
Control	55.64 ± 1.60	254.56 ± 26.56

Also, two endogenous auxins, i.e. Indole 3-Acetic Acid (IAA) and Indole 3-Propionic Acid (IPA), were present in the efficient cyanobactrial biomasses. The concentration of IAA in the *Cylindrospermum michailovskoense* ISB44 was higher than the *Anabaena*, whereas IPA concentration was the highest in *Anabaena* species (Figure 3, Table 5).

DISCUSSION

In this study, we investigated the potential of some cyanobacteria strains as a biological elicitor in *Plantago major* L. or broadleaf plantain. It is necessary to mention that because of the presence of the high amount of phenol and flavonoid contents in *Plantago major* L., it is considered an antioxidant herbal medicine (Zubair *et al*., 2011; Beara *et al*., 2009; Gálvez *et al*., 2003). The results of the present study showed that cyanobacteria can significantly increase several growth parameters of the treated plants, from the root to the stem, leaf, and inflorescence growth parameters. This increase was observed both in the seedling growth test and in pot studies (Tables 2 and 3). However, the results show that all of the cyanobacteria do not have this ability to the same degree. Also, cyanobacterial suspensions do not show a similar and positive function in all the studied characteristics.

In previous studies, different reactions of several parts of plants to bioelicitors have been reported. In particular, the significant effects of cyanobacterial extract on the root system of plants such as *Cucurbita maxima* Duchesne, *Cucumis sativus* L. and *Solanum lycopersicum* L. have been proven (Shariatmadari *et al*., 2013). In the mentioned study, the significant increase of root length and weight was observed as a result of the plants' irrigation with cyanobacterial extracts. In our study, the increase of root growth parameters was quiet significant as well (Table 3, Figure 2).

The increase in vegetative growth parameters of treated plants in the present study and previous investigations can have different reasons. In 2007, Karthikeyan *et al*. studied the effect of three strains of heterocystous cyanobacteria on wheat plant and observed an increase in the plant growth and yield. These researchers pointed out that the aggregation of cyanobacteria in the plant

Table 5. Estimated concentrations of three auxins in the most effective cyanobacteria.[a]

Cyanobacteria	Estimated concentration (ng g^{-1}) in DW		
	IAA	IPA	IBA
Cylindrospermum michailovskoense	16751.59	20.54	Nd
Anabaena vaginicola	1935.32	503.10	Nd

[a] DW: Dry Weight; IAA: Indole 3-Acetic Acid; IBA: Indole 3-Butyric Acid; IPA: Indole 3-Propionic Acid, Nd: Not detected.

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rhizosphere and nitrogen fixation by them leads to plant growth promotion. Also, a number of studies have reported the positive effect of these microorganisms on the growth of medicinal plants. Shariatmadari *et al*. (2015) studied the effect of heterocystous cyanobacteria on *Mentha piperita* L. as an economic medicinal plant. The results of the mentioned study showed that cyanobacterial extracts increase growth parameters as well as the amount of essential oil in treated plants. Another study in relation to the effect of cyanobacteria on the growth of medicinal plants was conducted by Riahi *et al*. (2013). In this study, the function of cyanobacteria in relation to medicinal plants, *Matricaria chamomilla* L. (chamomile), *Satureja hortensis* L. (garden savory), and *Mentha aquatica* L. (water mint) were evaluated, and increase in the growth of treated plants was reported. The increase of elements such as total nitrogen, nitrite, nitrate, and ammonium in rhizosphere of treated plants, presence of phytohormones in cyanobacterial extracts, and nitrogen fixation ability of cyanobacteria were proposed as factors influencing the growth and yield of the treated plants.

As mentioned, the ability of nitrogen fixation of heterocystous cyanobacteria is considered as one of the most important factors affecting plant growth and production. In the present study, analysis of the data shows a direct relationship between the consumption of cyanobacterial inoculums and the nitrogen content of the soil. For example, among the three algal strains that had a positive effect on the growth of *Plantago major* L., *Anabaeana vaginicola* extract had the highest amount of total nitrogen and ammonium, as can be seen in Table 1. The plants treated with this strain also had a lengthier root, with more wet weight, in comparison to the other treated plants and the controls. This can be related to the higher nitrogen level of the soil. In a previous study, positive effect of cyanobacteria on plant growth and nutrient uptake was reported by Obana *et al*. (2007). They showed that these microorganisms increased the organic carbon and nitrogen content of the surface soil and enhanced plant growth and nutrient uptake.

The information obtained from previous studies also shows that there is a significant correlation between plant growth parameters and the ionic content of the soil (Mondyagu *et al*., 2012; Shariatmadari *et al*., 2015; Kumar and Nikhil, 2016). The cationic composition of the rhizosphere can affect plant growth due to their significant role in cellular growth, metabolism regulations, and the molecular activity of the plant cells (Shariatmadari *et al*., 2015). The result of cyanobacterial suspension analysis in the present study indicates the ability of algal suspensions to increase soil minerals such as potassium (K^+), Magnesium (Mg^{++}), and Calcium (Ca^{++}). Among several minerals, K^+ is an essential plant nutrient that is required in large amounts for proper growth and plants reproduction. The importance of this cation in plant growth is so great that some researchers believe that potassium after nitrogen is the most important plant nutrient (Prajapati and Modi, 2012). Mg^{++} is one of the most important nutrients involved in many enzyme activities and the structural stabilization of cells and tissues and regulation of cation-anion balance in plant cells (Guo *et al*., 2016). Ca^{++} is another crucial cation that can be considered as a regulator of plant growth and development (Hepler, 2005). In general, it can be said that, through enhancing cations that regulate molecular and metabolic activities in plants, cyanobacteria stimulate the vegetative growth of plants as well as the production of some metabolites in treated plants. In our study, a remarkable increase in growth of the plants treated with *Nostoc spongiaeforme* var. *tenue* ISB65 was also observed, which could be the result of high amounts of cations, especially K^+, in cyanobacterial extract.

In our study, the presence of high amounts of phytohormones such as Indole-3-acetic acid and Indole-3-propionic acid in cyanobacterial extracts was reported. According to the previous reports, it is also

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expected that the cyanobacterial extract have a significant effect on plant growth through producing high amounts of phytohormones such as axins (Shariatmadari *et al*., 2015).

Polyphenols and flavonoids are among the important compounds found in the metabolite complex of this plant and have anti-oxidant properties (Machu *et al*., 2015; Rice-Evans *et al*., 1996). In the present study, the leaf extract of plants treated with cyanobacterial suspensions shows an increase in the phenol and flavonoid contents in comparison to the control plants (Table 4).

Several studies have shown that phenols and flavonoids increase as a result of plant interactions with the stimulating agents such as plant pathogens (Pusztahelyi *et al*., 2015). These metabolites or their precursors are accumulated in high concentrations in plants' leaves and are involved in important defense processes such as disease resistance (Thakur and Sohal, 2013). The results of our study showed that cyanobacteria as a bioelicitor can increase phenol and flavonoid contents in studied plant leaves. In similar studies, the impact of several forms of biofertilizers designed with the help of mycorrhiza, green algae, bacteria, or cyanobacteria on the growth and quality of different medicinal plants' secondary metabolites has been evaluated. Evaluation of the general function of the medicinal plant *Ocimum basilicum* L., treated with mycorrhizal- and algae-based fertilizers as well as a mixture of these fertilizers, is one of the mentioned studies. In this study, a quantitative evaluation of phenolic and flavonoid compounds was also performed (Hristozkova *et al*., 2017; Hristozkova *et al*., 2018). The result of this study showed a significant increase for phenol and flavonoid compounds of the treated plants, as well as in the antioxidant property under the treatment condition. This increase was especially obvious in plants treated with mycorrhiza-algae compound fertilizers. Hristozkova *et al*. (2017) believe that this increase can be the result of the activation of exclusive antioxidant enzymes under treatment condition.

In another study, the effect of plant growth stimulating bacteria *Entrobacter cloacae* on the phenol compounds of *Calendula officinalis* L. was investigated and a meaningful increase of phenol in plants treated with this bacterium was reported (Hormozinejad *et al*., 2018). The study focused on the impact of environmental factors, such as the chemical compound of the soil, on the amount of the secondary metabolites of plants, such as phenol and flavonoid compounds. Based on the report issued by Hormozinejad *et al*. (2018), the increase of the phenol compounds in plants treated with biological fertilizers can be the result of the improvement of the plant's natural functioning under treatment with biological elicitors. Also, Khalil *et al*. (2007) considered the improvement of the nutritional condition of the plants treated with growth stimulating bacteria as the main reason for the increase of phenol and flavonoid compounds in these plants. Dewick (2009) believes that the primary and secondary metabolic pathways interact with each other, in a way that the carbohydrate product of the plant's primary metabolism can function as the pre-material necessary for the development of the exclusive metabolism pathway of phenol compound synthesis. As a result, every factor that can increase the general functioning of a plant and its photosynthetic efficiency could function as the stimulating agent to increase the secondary metabolites such as phenol compounds.

Based on the overall studies in this area, it could be concluded that different microorganisms, such as cyanobacteria, can be used as efficient natural factors for increasing the quality and quantity of medicinal plants' valuable metabolites, and can be used purposefully for increasing the efficiency of medicinal plants production. In conclusion, the studied cyanobacteria strains are able to promote *Plantago major* L. growth and production of phenol and flavonoids in this plant. Altogether, our

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findings suggest that the selected cyanobacteria, isolated domestically, can serve as potential biofertilizer candidates to promote robust production of *Plantago major* L. as a medicinal plant.

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افزایش محتوای فنل و فلاونوئید کل دربارهنگ *Plantago major* L. تحت تأثیر سیانوباکتری‌های تحریک کننده رشد گیاه

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چکیده

ترکیبات فنلی و فلاونوئیدی از جمله مهمترین ترکیبات موثر موجود در گیاهان دارویی هستند که دارای قابلیت آنتی‌اکسیدانی اثبات شده‌اند. در این مطالعه، اثر عصاره پنج نمونه سیانوباکتریایی، به عنوان عوامل تحریک‌کننده رشد و افزاینده متابولیت‌های ثانویه گیاه، بر گیاه بارهنگ بزرگ بررسی شد. سیانوباکتری‌های مورد نظر از بستر رویشی این گیاه واقع در زیستگاه‌های طبیعی آن جداسازی شدند. محیط کشت BG11 جهت آماده‌سازی جدا کشت‌های سیانوباکتریایی مورد استفاده قرار گرفت. مطالعات گلدانی از طریق پاشش عصاره سیانوباکتریایی بر بستر رویشی گیاه در فواصل ۲۰ روزه و به مدت ۶۰ روز صورت گرفت. در انتهای محدوده زمانی مورد نظر، ارزیابی فاکتورهای رویشی و زایشی گیاهان تیمار و شاهد، نظیر طول ریشه و ساقه، وزن تر و خشک گیاه، سطح برگی و خصوصیات گل‌آذین صورت گرفت. همچنین محتوای فنل و فلاونوئید گیاهان پس از طی دوره تیمار ارزیابی گردید. آنالیزهای آماری نشاندهنده اختلاف معنی‌دار شاخص‌های رشد و محتوای فنل و فلاونوئیدی

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گیاهان تیمار در مقایسه با گیاهان کنترل بود. بطوریکه مقایسه عصاره متانولی گیاهان نشانگر بیشینه حضور ترکیبات فنلی و فلاونوئیدی با مقادیر $\mu g\ GA.mg^{-1}$ ۷۷/۲۳±۳/۲۱ و $\mu g\ RU.mg^{-1}$ ۳۸۹/۶۷±۳۴/۴۳ در گیاهان تیمار شده با سیانوباکتری *Cylindrospermum michailovskoence* بود. بر اساس نتایج به دست آمده، سیانوباکتری‌ها به‌عنوان الیسیتورهای زیستی مناسب جهت بهبود کیفیت و کمّیت محصول گیاهان دارویی پیشنهاد می‌شوند. به نظر می‌رسد ترکیبات تحریک کننده رشد نظیر فیتوهورمون‌ها از عوامل مهم تاثیرگذار بر فاکتورهای رشد گیاهان تیمار است.

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