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An innovative use of microalgal extracts as an alternative to expensive growth regulators *via* biotechnological techniques

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Microalgae are photosynthetic organisms that are described as an abundant source of numerous bio compounds of commercial value, and using this green treasure as a vital alternative to expensive synthetic hormones *via* biotechnological technique is the main objective of research. Murashige and Skoog medium supplemented with crude extract of *Microcystis aeruginosa* and *Haematococcus pluvialis*, separately was used to study its effect on *Cynanchum acutum L*., which is an important wild medicinal plant. Results revealed that all growth parameters have been affected where, the greatest shoot length (5.90 ± 0.45) has been reached with 4 mg/L of *Microcystis aeruginosa* extract, while the highest shoot number (7.50 ± 0.30) and leaf number (16.0 ± 0.59) were with 12 mg/L of *Microcystis aeruginosa* extract. Conversely, the maximum shoot length (12.5 ± 0.44) was reached with 2 mg/L of *Haematococcus pluvialis* extract, while the highest shoot number (6.00 ± 0.36) and leaf number per shootlet (22.0 ± 0.92) were with 5 mg/L of *Haematococcus pluvialis* extract. Biochemical analysis for algae and plant extracts conducted using HPLC proved the presence of phytohormones (zeatin, gibberellin, and kinetin) in algal extracts and nineteen important phenolic and flavonoid substances in plant extracts. The novelty of this study is to use algae as a natural source of growth regulators to achieve an economic benefit in addition to propagating *Cynanchum acutum L.* and increasing its important pharmaceutical products.

Keywords: Wild Medicinal Plant, *Microcystis aeruginosa, Haematococcus pluvialis, Cynanchum acutum L.,* Biostimulants, Biotechnological Technique

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INTRODUCTION

Over the past 26 years, the scientific community and agrochemical industries have given plant biostimulants more attention (Maini 2006; Yakhin et al., 2017). The use of microorganisms as biostimulants and biofertilizers, which are environmentally friendly, may replace chemical fertilization, and provide farmers with economic and ecological benefits, that has recently attracted a lot of attention in the field of crop production. By using biofertilization, they can decrease the usage of chemical pesticides and fertilizers, which are hazardous to the environment and raise health risks for people (Sahu et al., 2012).

Algae could be utilized as an alternative to several pricey chemicals that are added to the medium for *in vitro* production, like vitamins, antibiotics, and synthetic plant growth regulators (Zaccaro et al., 2006). Algal research in the last decades, especially microalgal genomic studies, has reported that phytohormones are produced by a wide variety of microalgae which could produce exogenous and endogenous phytohormones (Prieto et al., 2011; Stirk et al., 2011; Stirk et al., 2014). The most popular phytohormones (i.e., auxins, cytokinin, abscisic acid, ethylene, and gibberellins) have already been identified in a variety of algal lineages, such as green algae, cyanobacteria, and diatoms (Han et al., 2018; El-Sadek & Ahmed 2022).

The present study focused on both *Microcystis aeruginosa* and *Haematococcus pluvialis* as a source of algal extract and evaluated the extracts on all growth parameters and multiplication in terms of increasing the active ingredients of *Cynanchum acutum L*. *M. aeruginosa*, which is the most abundant species of freshwater blue-green algae in South Africa, it grows readily in nutrient-rich, slowly moving water. It is one of the most frequently studied cyanobacteria due to its worldwide distribution. It can form harmful algal blooms of economic and ecological importance. *M. aeruginosa* prefers warm temperatures, as it has the highest laboratory growth rates at 32 °C and is used in the natural production of butylated hydroxytoluene (BHT); an antioxidant and food additive (Tooming-Klunderud, 2007; Babu & Wu 2008; Paerl & Huisman 2008). While *Haematococcus pluvialis* is a green, unicellular freshwater microalgae comprising more than 7000 species distributed in several habitats worldwide. It is considered the best natural source of astaxanthin and the main producing organism of this commercial product (Lorenz 1999; Rao et al., 2010), usually present in temperate zones around the world. The blood-red color of dried-out rock pools is frequently caused by this color due to astaxanthin which is believed to protect the resting cysts from the detrimental effect of UV radiation when exposed to direct sunlight (Dore & Cysewski. 2003).

The huge family of flowering plants known as *Apocynaceae*, or the dogbane family, is made up of vines, perennial herbs, shrubs, forest trees, and succulent herbs. Many species of *Apocynaceae* are poisonous, and many of them have milky latex. Dogbane gets its name from the fact that several *Apocynaceae* herbs were originally used as dog and arrow poisons (Endress & Bruyns 2000). It comprises numerous medicinal plants with a variety of therapeutic activities, and most members have tufted silky-haired seeds, milky juice, and pod-like follicles (Alikhan & Khanum, 2005; Kamel et al., 2014). In Egypt, medicinal plants represent a new promising resource as there is a relatively high representation of medicinal species in the native flora (Batanouny et al., 1999). *Cynanchum acutum L.* has been selected to be the subject of the present study. It is a wild perennial herb known as olliq, modeid, or libbein that is widely dispersed in the Nile Delta (Tackholm, 1974). The phytochemical studies on *C. acutum* have indicated the presence of several natural compounds such as lupeol, lupyl acetate, flavonoid, and two simple coumarins isolated from the aerial parts including scopoletin and scoparone (Halim et al., 1990; Staerk et al., 2002; Heneidak et al., 2006; El-Demerdash et al. 2009). Flavonoids from *C. acutum* showed considerable antioxidant and anti-diabetic effects, and coumarins from the same species exhibited anti-inflammatory, analgesic, and anti-pyritic activity (Awaad, 2000; Fawzy et al., 2008). *C. acutum* alcoholic extract has been shown to have anti-diabetic, insecticidal, antioxidant, anti-cancer, anti-inflammatory, and antipyretic impacts (AbouZeid et al., 2001; Abdelhameed et al., 2021).

This study aimed to research the existence of alternatives (growth regulators in algae extract) to expensive artificial plant growth regulators and replace them with available, natural, and effective alternatives to propagate plants *in vitro* and enhance increasing the active pharmaceutical, natural substances.

MATERIALS AND METHODS

Algae Experiments

Algae Strains and Growth Media Used for Algal Cultivation: Algae species used in this investigation; *Microcystis aeruginosa* (blue-green algae) and *Haematococcus pluvialis* (green algae), were acquired from the Algal Biotechnology Unit, National Research Centre, Egypt. Among the different used algae species, the proper growth medium was varied due to different growth habitats. Two different growth mediums were used. BG-11 (Rippka et al., 1979) was used for cultivation of *M. aeruginosa* and Bold's basal medium used for cultivation of *H. pluvialis* (Bischoff, 1963). Cultivation was carried out in sterilized conical flasks containing 600 ml of the media under continuous illumination. The cultivation time differed from one strain to another depending on the optimum growth rate till reaching the stationary phase which always ranged between (15-20) days. After the determination of optimum growth, a subculture for a suitable volume (1:8) of algae to media was added to 10 liters of sterilized media for propagation. At the end of the cultivation period, the dense cultures were harvested by continuous centrifugation, the supernatants were discarded, and the remaining pallets were used for growth and analysis. The harvested algal cells were dried at 30°C to fine powder and extracted with 70% methanol based on the procedure outlined in (Elakbawy et al., 2021). Then, the methanolic fraction filtrated and evaporated at room temperature. Part of the dry residue was analyzed to study the content of phytohormones in the algae under study and the remaining part was again dissolved in 100 ml sterilized distilled water to create stock algal extract for usage in tissue culture media at different concentrations as an alternative source of exogenous hormones.

Extraction and Determination of Growth Regulators in Algae: The acidic ethyl acetate-soluble fraction was utilized to determine acidic hormones like gibberellin (GA) and indole acetic acid (IAA), while the basic fraction was used to identify cytokinins such as benzyl adenine (BA) and Kinetin (Kin). Combined extracts were evaporated to the aqueous phase in a rotary flash evaporator. The aqueous phase (10 to 30 ml) was adjusted to pH 8.6 with 1% NaOH and partitioned three times with equal volumes of ethyl acetate. The method in detail can be found in (Shindy & Smith, 1975) and (Chen, 1990). The combined ethyl acetate fraction was evaporated to dryness and held for further purification. The aqueous phase was adjusted to pH 2.8 with 1% HCl and partitioned three times with equal volumes of ethyl acetate. The remaining aqueous phase was discarded. The combined acidic ethyl acetate phase was reduced in volume (Fraction I) to be used for the determination of acidic hormones such as IAA, ABA, and GAs. The dried basic ethyl acetate fraction was dissolved in 80% methanol. The methanol was evaporated under vacuum, leaving an aqueous phase which was adjusted to pH 2.8 with 1% HCl and partitioned three times with 25 to 50 ml of ethyl acetate. The ethyl acetate phases were combined (Fraction II), reduced to 5-ml volume, and

stored at -20 C until analysis for neutral auxins. The remaining aqueous phase was to pH 5.5 with 1 % NaOH and partitioned three times with 50 to 100 ml of water-saturated l-butanol. All butanol phases were combined (Fraction III), reduced to 5-ml volume, and stored at -20°C until analysis for cytokinins. Phytohormones analysis by HPLC was performed according to (Kelly et al., 1995; Wasternack & Parthier, 1997; Baydar & Ulger, 1998), and the steps of preparing the samples were modified as (El-Sadek & Ahmed, 2022) at the Desert Research Center, Egypt.

Plant Experiments

Plant Material: Ripe fruits of *Cynanchum acutum L.* wild plants were collected from Al Sadat Axis, New Cairo (30.052, 31.457), Egypt. Fruits were collected during October 2020, dried in a cool dry place, and stored in paper bags in a cool dry place (22-25°C). Seeds were used as a starting explant (Figure 1).

***In Vitro* seed sterilization and germination:** Seeds were collected from dried fruits and rinsed with running tap water for three to four hours followed by distilled water for 15 min. In a laminar flow hood, seeds were fully submerged in varying concentrations of commercial Clorox solution (5.25% sodium hypochlorite) for different exposure times (10, 20, and 30 minutes). After that, seeds were washed three times with sterile distilled water and then cultured on solidified half-strength MS and full-strength MS basal media supplemented with 3% Sucrose and 5 g/L phytagel (Murashige & Skoog, 1962). The pH of all media used was adjusted to 5.8 (Hemphill, 1998; Morre et al., 1998) by 0.1 N HCl or KOH before autoclaving. The media was distributed into 250 ml glass jars, each jar containing 50 ml, and sterilized by autoclaving for 20 minutes at 121ºC. After culturing jars with twenty replicates for each treatment, seeds were incubated 16/8 hrs (light/dark) at 25±2ºC. After 3 weeks of culturing, data was estimated as the percentage of survival (Su), death (D), and contaminated seeds (Cont.).

Impact of Different Concentrations of Plant Growth Regulators on Plant Parameters: The purpose of this experiment was to examine the impact of Murashige and Skoog. (MS) medium supplemented with 3% sucrose, 5 g/L phytagel, and benzyl adenine (BA) alone or in combination with α-Naphthalene acetic acid (NAA) at different concentrations (Table 1) on nodal segment explant obtained from the germinated seeds from the above step. After three weeks, the *in vitro* seedling's growth parameters (shoot number, shoot length, root number, root length, leaves number, fresh weight, and dry weight) were recorded.

Figure 1. *Cynanchum acutum* (a). Complete plant with leaves and flowers, (b). Horny fruits, (c). Dried fruit, (d). Seeds.

Table 1. Different treatments at various concentrations of BA and NAA either separately or together.

Treatments	BA (mg/l)	NAA (mg/l)
T_0	0.0	0.0
T_1	0.5	0.05
T_2	1	0.05
T_3	0.5	0.0
T_4	0.0	0.05

Impact of Varying Concentrations of Algal Extract on Plant Growth Parameters: Three weeks old nodal segments were transferred to a 250 ml jar containing 50 ml of full-strength MS medium supplemented with, 5 g/L agar and 3% (w/v) of sucrose with a combination of twenty different concentrations of algal extract (AE) (M0, M1, M2, M3 to M20 containing 0, 1, 2, 3,….20 mg/L AE respectively), twenty replicates for Each treatment. The incubation conditions were as described previously, after 3 weeks data was recorded as shoot number, shoot length, root number, root length, leaves number, fresh and dry weight. Samples of the control treatment (M0) and plantlet which gave the best result of both shoot length and shoot number, growing on media with algal extract, were collected and dried at room temperature for biochemical analysis.

Identification of Flavonoid and Phenolic Compounds: Phenolic and flavonoid standards: coumaric acid, quercetin, chlorogenic acid, catechin, gallic acid, ferulic acid, caffeic acid, methyl gallate, syringic acid, pyro catechol, ellagic acid, vanillin, naringenin, daidzein, cinnamic acid, apigenin, kaempferol, hesperetin, and rutin were acquired from Sigma Co. (St. Louis, MO, USA). The standards' purity was 98%. An Agilent 1260 series was used for the HPLC analysis. The Eclipse C18 column was utilized for the separation process. Water (a) and 0.05% trifluoroacetic acid in acetonitrile (b) were the constituents of the mobile phase, which had a flow rate of 0.9 ml/min. At 280 nm, the multi-wavelength detector was observed. For every sample solution, there was one injection volume of five microliters. At 40 °C, the column temperature was kept constant. Phenolic-flavonoid content was performed at Chromatography Lab, Central Laboratories Network, National Research Centre, Egypt.

Statistical Analysis

The parameters of each experiment were statically analyzed, and results are presented as mean ± standard error (SE). One-way analysis of variance (ANOVA) and general linear model technique in the Minitab 18 system were used to assess the statistical significance of data from *in vitro* experiments. For mean comparisons, the least significant difference method was applied (Lesik, 2018).

RESULTS AND DISCUSSION

Phytohormone HPLC Study of Methanol Extract of *Microcystis aeruginosa* and *Haematococcus pluvialis*

Data in Table 2 and Figure 2 indicate that zeatin and gibberellins are present in the algal extract used, *M. aeruginosa* and *H. pluvialis* (36.7 and 492 ppm) and (103 and 44.1 ppm), respectively, in addition to gibberellin and zeatin, kinetin has also been found, only in *H. pluvialis* (2.94 ppm). Our findings agree with (Tarakhovskaya et al., 2007; Wang et al., 2022) who reported that indole acetic acid, cytokinin and gibberellin, and other phytohormones were extracted from an aqueous extract of microalgae. Furthermore, (Stirk et al., 2002) showed that Chlorophyta and Cyanophyta produce comparable amounts of cytokinin-like activity. However new research indicates that phytohormones in microalgae have regulatory functions akin to those of higher plants (Lu and Xu, 2015). According to (El-Naggar et al., 2005) the phytohormones (such as auxin and gibberellins) found in *Chlorella kessleriwhen* extract when applied to *Vicia faba*, increased leaf area, seedling growth parameters, germination, pigment content. Our findings also support the findings of (Hussain and Hasnain, 2011), who examined the effectiveness of cyanobacterial strains that secrete hormones (cytokinin and auxin) in promoting plant growth.

***In Vitro* Seed Sterilization and Germination:** Seed sterilization is an essential step in plant tissue culture to determine the optimal concentration and exposure time of the sterilizing agent which results in the lowest contamination and the maximum survival rate. *Cynanchum acutum L.* seeds were exposed to 10, 20, and 30% Clorox with three exposure times (10, 20 and 30 min). Data in Table 3 showed that the low concentration of Clorox (10%) resulted in 50 - 60%, 40 - 50%, and 30-40 % contamination at 10, 20, and 30 min of exposure time, respectively. The same concentration resulted in 40-50%, 50- 60%, and 60 - 70% survival individuals at 10, 20 and 30 min of exposure time, respectively, and no dead individuals were detected. On the other hand, the concentration of Clorox (20%) at 10 min showed 38 - 40% contamination, 10 - 25% at 20 min, and 8 – 10% at 30 min of exposure time. Moreover, at the same concentration (20%) the highly disinfected individuals were 60% at 10 min, 70 - 85% at 20 min, and 80% at

Table 2. HPLC Analysis of *Microcystis aeruginosa* and *Haematococcus pluvialis* methanol extract showing the detected phytohormones by ppm.

Phytohormones	*M. aeruginosa*			*H. pluvialis*		
	Retention Time (min)	Area mAU* (min)	Amount (ppm)	Retention Time (min)	Area mAU* (min)	Amount (ppm)
Zeatine	3.16	0.96	36.7	3.56	2.68	103
Gibberellic Acid	2.55	5.96	492	2.54	0.53	44.1
kinetin	-		-	2.73	2.53	2.94

Table 3. Effect of different Clorox concentrations and three different exposure times on seeds sterilization of *Cynanchum acutum*, where (Su) survival, (D) death, and (cont.) contamination %

10 % Clorox									
Time	10 min			20 min			30 min		
	Su %	D %	Cont.%	Su %	D %	Cont.%	Su %	D %	Cont. %
MS	40	0	60	50	0	50	70	0	30
1\2 MS	50	0	50	60	0	40	60	0	40
20% Clorox									
MS	60	2	38	85	5	10	80	10	10
1\2 MS	60	0	40	70	5	25	80	12	8
30 % Clorox									
MS	30	70	0	40	60	0	20	80	0
1\2 MS	30	70	0	35	65	0	25	75	0

30 min of exposure time. Also, there were 0-2%, 5%, and 10-12% dead individuals at 10, 20, and 30 min of exposure time, respectively. However, at 30% Clorox There was no contamination at 10, 20, and 30 min of exposure time and showed 30%, 35-40%, and 20-25% of the disinfected individuals at 10,20 and 30 min of exposure time, respectively. Also, 70%, 60-65%, and 75-80% of dead individuals at the 10, 20, and 30 min of exposure time respectively. So, a concentration of 20% Clorox at 20 min of exposure time was the most suitable one, giving high percentages of survived individuals with a low percentage of dead and contaminated. The presented treatments of surface sterilization agreed with (El-Bakry et al., 2011) who used 20% Clorox for 20 min after rinsing seeds of *C. acutum* with tap water and then with distilled water for 15 min.

Impact of Varying Concentrations of Plant Growth Regulators on Plant Parameters: Plant growth regulators (PGR) play a significant role in tissue culture technique, and their effect differs from one plant to another and differs according to the part of the plant used and the concentration of them. After 3 weeks data was recorded (Figure 3).

Analysis of variance for shoot number showed significant differences at all treatments (Figures 3 and 4), comparison of means showed that 0.5 mg/l BA (T3) gave the highest mean of shoot number (12.0 ± 0.25), while 0.00 mg/l BA with 0.00 mg/l NAA (T0) was the lowest (1.50 ± 0.17), on the other hand, shoot length treated with 0.05 mg/l NAA alone (T4) gave the highest mean (9.50 ± 0.40), while (T0) again was the lowest (2.90 ± 0.31). For roots, a comparison of means showed that all concentrations had a negative effect on the roots except on (T4) the roots reported and showed significant difference from the control treatment (T0), as the root number was (5.00 ± 0.21) and root length was (3.00 ± 0.10) compared with control root number (1.00 ± 0.16) and root length (0.38 ± 0.13). From the results, due to the objective of our study being direct propagation of the plant, 0.5 mg/l BA (T3) is the best concentration for propagation, so it was used as a control treatment for the rest of the study.

Impact of Different Concentrations of Algal Extract on *Cynanchum acutum L.* Growth Parameters: Nodal segments (2–3 cm) obtained from plantlet multiplicated on T3 were utilized as an explant for multiplication on MS media supplemented with algal extract. There were noticeable effects of using different concentrations of algal extract of both *Microcystis aeruginosa (M)* (Figures 5-7) and *Haematococcus pluvialis (H)* (Figures 8-10) on all multiplication parameters of *Cynanchum acutum*. The treatments varied in their impact as the optimal shoot number and leaf number per shootlet with *M. aeruginosa* extract was (7.50 ± 0.30) and (16.0 ± 0.59), respectively on M12 treatment (MS medium containing 12 mg/l *M. aeruginosa* extract), while with *H. pluvialis* extract, the highest shoot number and leaf number per shootlet were (6.00 ± 0.36) and (22.0 ± 0.92), respectively on H5 treatment (MS medium

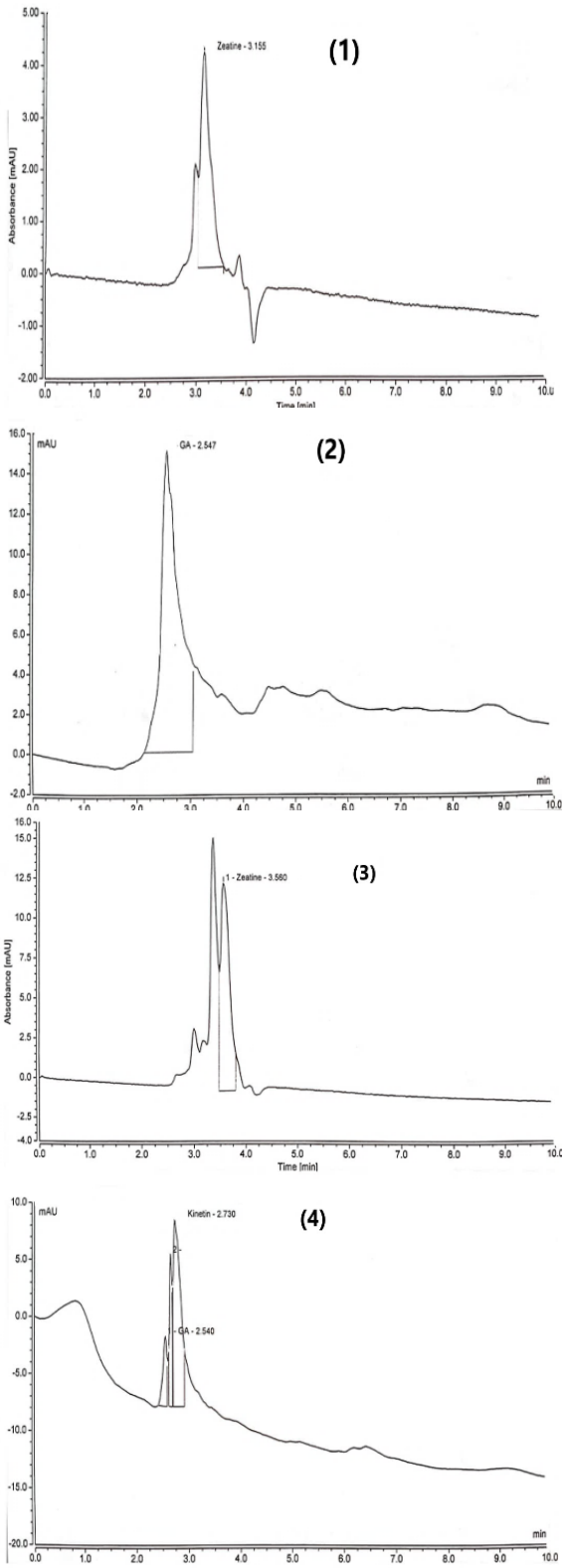

Figure 2. HPLC Analysis for phytohormones of methanol extract, (1,2) *Microcystis aeruginosa* and (3,4) *Haematococcus pluvialis.*

containing 5 mg/l *H. pluvialis* extract), which notably differed from control treatment (2.00 ± 0.17). Conversely, the longest shoot length of M. *aeruginosa*. 5.90 ± 0.45 was on M4 (MS medium with 4 mg/l of *M. aeruginosa* extract), while with *H. pluvialis* extract the greatest shoot length was 12.5 ± 0.44 on H2 treatment (MS medium supplemented with 2 mg/l of *H. pluvialis* extract). Where the highest root number and root length were reported as (6.20 ± 0.55) and (2.60 ± 0.07), respectively with *M. aeruginosa* extract formed on M12 treatment, while with *H. pluvialis* extract the best root number and root length (5.00 ± 0.44) and (5.20 ± 0.26) formed on H5. In terms of highest fresh and dry weight, with *M. aeruginosa* extract were 1.30 ± 0.07 and 0.110 ± 0.008, respectively formed on M12 treatment while with *H. pluvialis* extract was 1.28 ± 0.06 and 0.119 ± 0.009, respectively on H5.

From the above result, it can be clear that the significant function of green and blue-green algae as an excellent substitute source of growth regulators to encourage plant growth was effective compared with the control treatment. Our findings concur with the outcomes produced by (Nakajima et al., 2023) who said that GA plays a significant role in stem or internode elongation in broccoli without lowering yield. Also, GA treatment shortened the growing period without reducing yields. Additionally, gibberellin was also found to have a significant impact on prolonging the flowering period and producing more fruit in Tomatoes (*Lycopersicum esculentum Mill*.) as described in (Novita, 2022). Hudeček et al., 2023 claimed that cytokinin shows beneficial effects in delaying senescence or preserving a healthy level of photosynthetic activity in plants. Furthermore, (Pérez-Rojas et al., 2023) findings indicate that cytokinins have a major influence on the growth of strawberry flowers and receptacles.

The stimulatory responses of *Cynanchum acutum* cultivated on media supplemented with extract of cyanobacteria and green algae may be related to that these algae are an excellent source of vitamins and phytohormone supplements. This result was highly supported by (Chittora et al., 2020) who stated that *via* secreting phytohormones including auxins, cytokinin, and gibberellins, cyanobacteria play a significant role in seed germination. Cultivation of *Knautia sarajevensis* in liquid media containing 2.0 mg/L zeatin resulted in the highest multiplication rate (100%) and an average of 4.67 shoots per explant after three weeks of culture according to (Karalija et al., 2017).

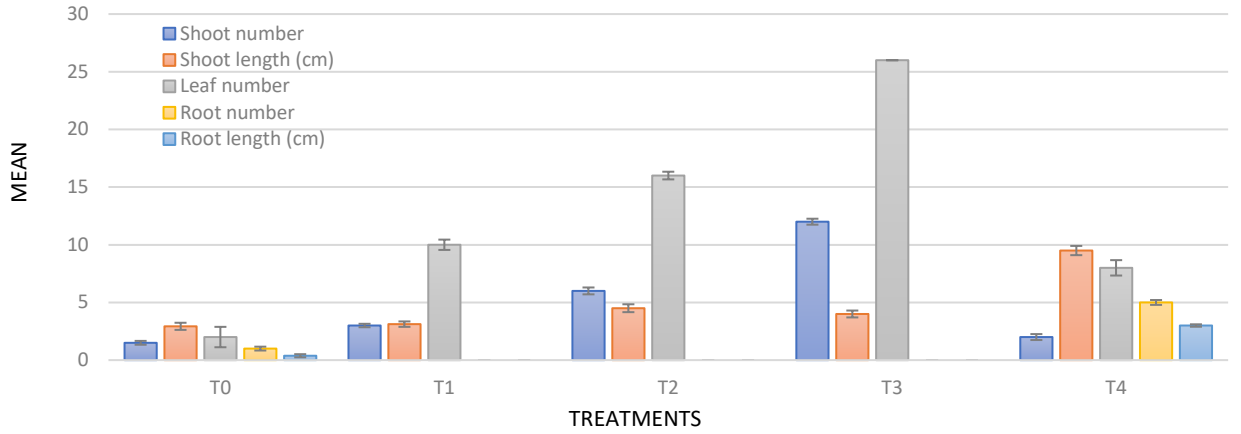

Figure 3. Effects of different treatments with various concentrations of BA and NAA on plant parameters after 21 days from nodal segment culture.

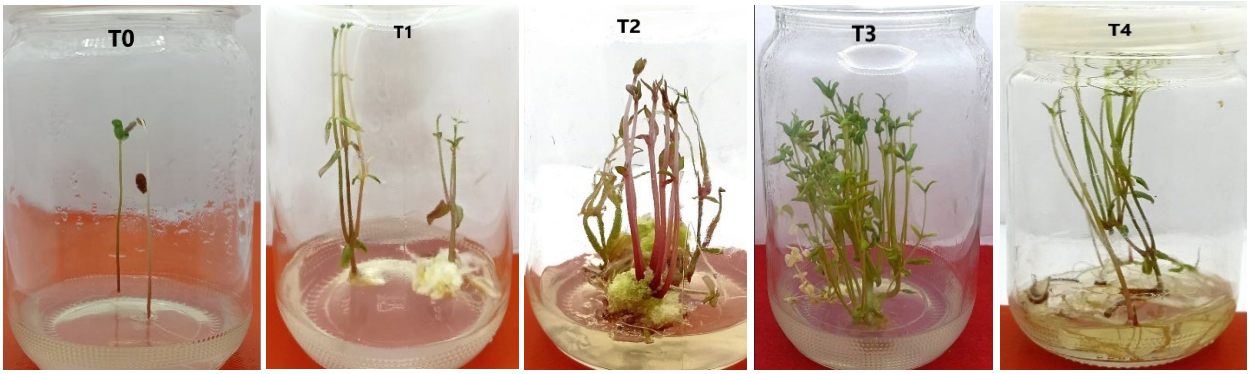

Figure 4. Impact of concentrations of MS + BA and NAA by mg/L after 21 days on growth parameters of *cynanchum acutum. In vitro.* T_0(Contol): MS + 0.00 BA+ 0.00 NAA, T_1:MS+ 0.5 BA + 0.05 NAA, T_2: MS+ 1 BA + 0.05 NAA, T_3: MS+ 0.5 BA + 0.00 NAA, T_4: MS+ 0.00 BA + 0.05 NAA.

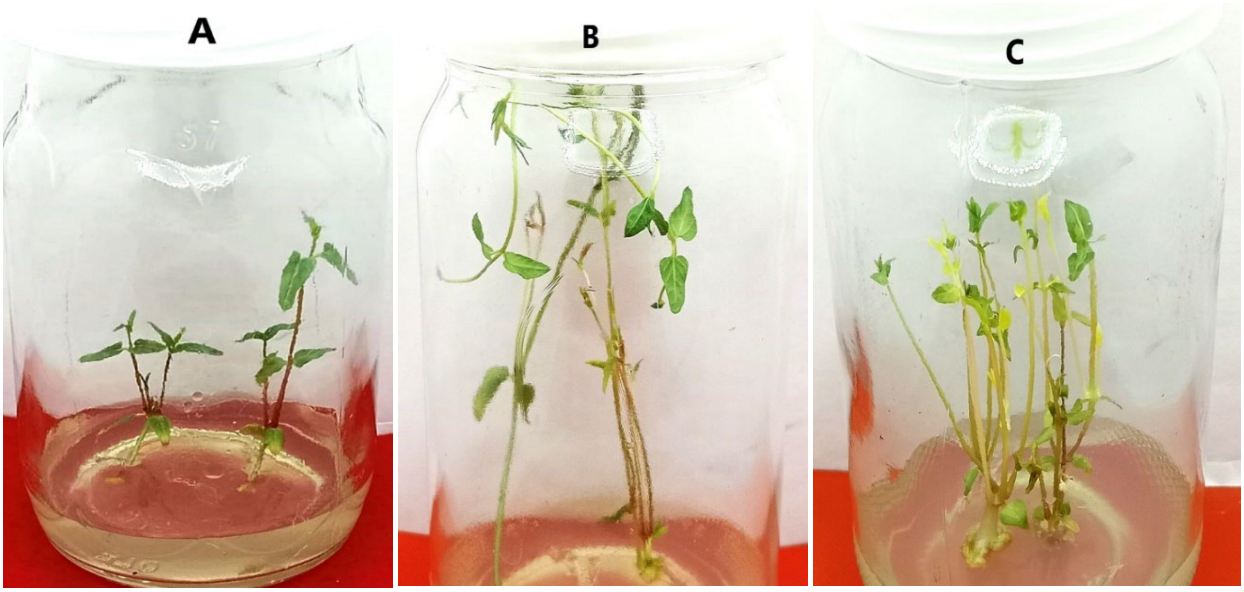

Figure 5. Effect of different concentrations of *M. aeruginosa* extract mg/L after 21 days on growth parameters of *cynanchum acutum. In vitro.* Where (A) control MS+0.00 algal extract, (B) MS+ 4mg/l algal extract, and (C) MS+ 12mg/l algal extract.

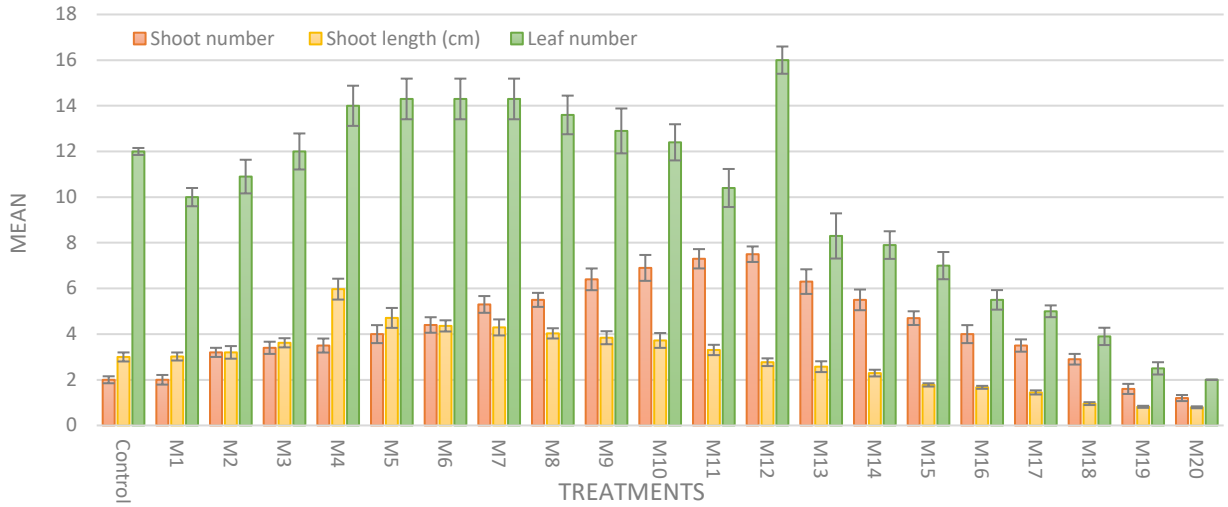

Figure 6. Effect of different concentrations of MS ± crude extract of *M. aeruginosa* after 3 weeks on different growth parameters (shoot number, shoot length, and leaf number) of *Cynanchum acutum* L. plantlets.

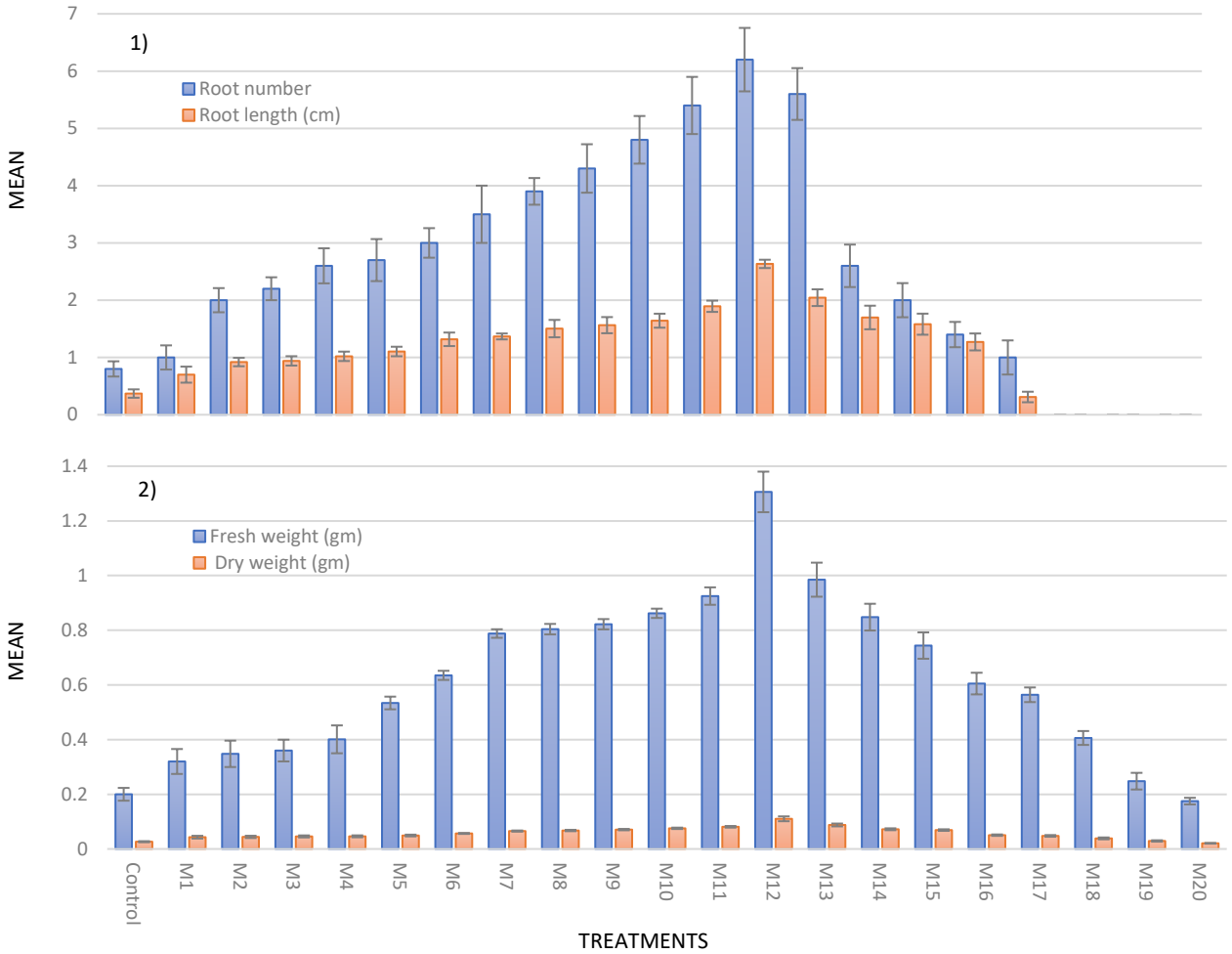

Figure 7. Effect of different concentrations of MS + crude extract of *M. aeruginosa* after 3 weeks on (1): root number and length (cm), (2): fresh and dry weight (gm) of *Cynanchum acutum L*. plantlets.

Figure 8. Effect of different concentrations of *H. pluvialis* extract mg/L after 21 days on growth parameters of *cynanchum acutum. In vitro.* Where (1) control MS+0.00 algal extract, (2) MS+ 2 mg/L algal extract, and (3) MS+5 mg/L algal extract.

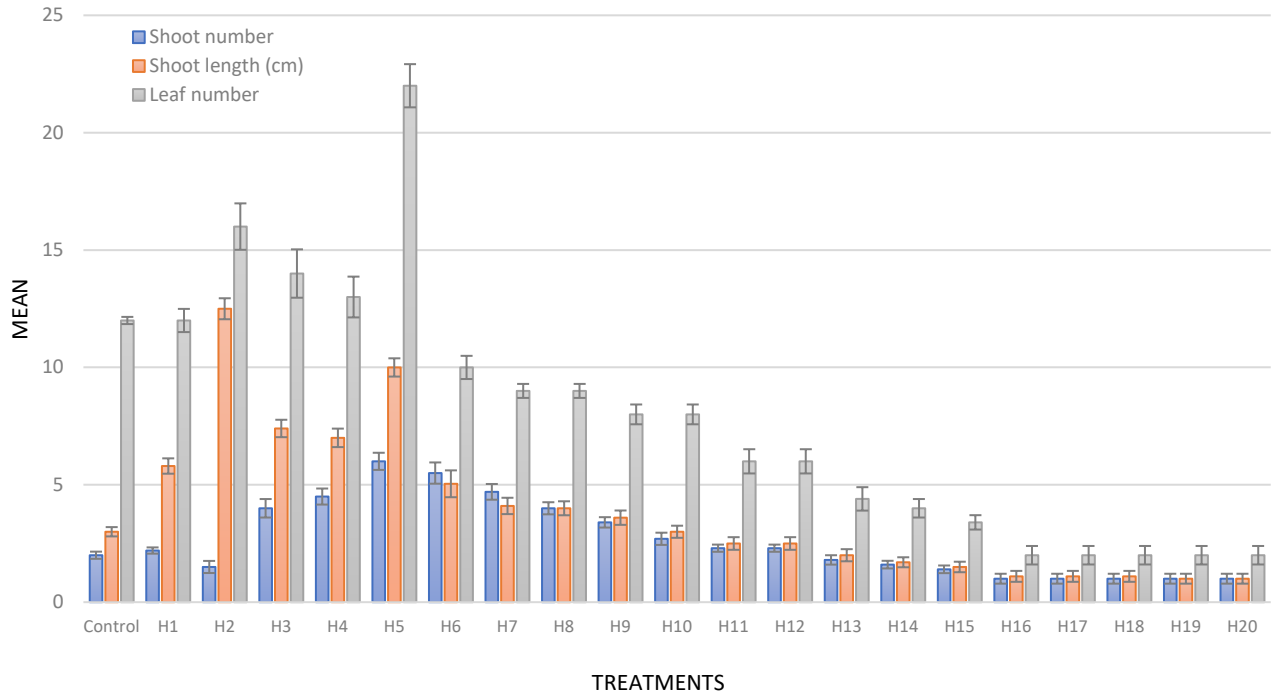

Figure 9. Effect of different concentrations of MS + crude extract of *H. pluvialis* after 3 weeks on different growth parameters (shoot number, shoot length, and leaf number) of *Cynanchum acutum* L. plantlets.

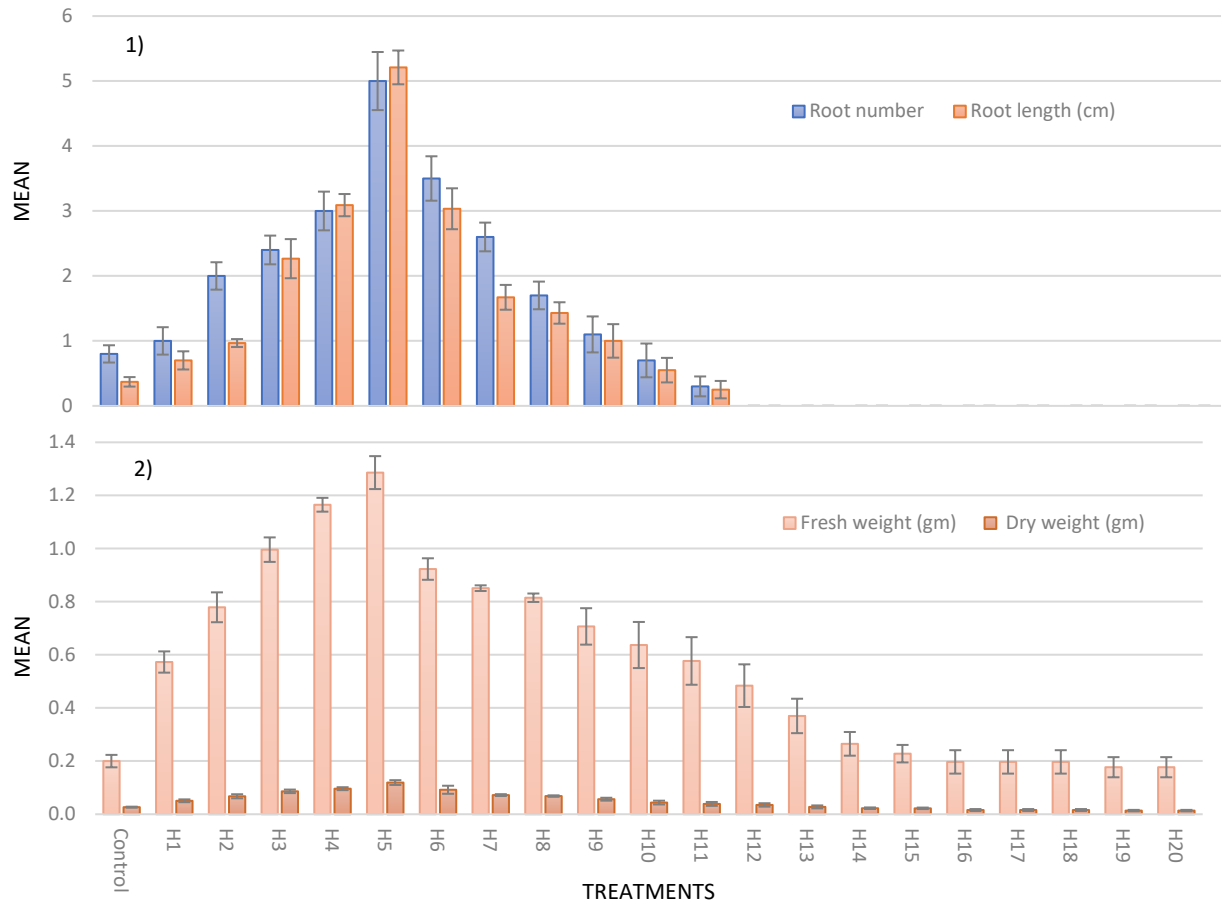

Figure 10. Effect of different concentrations of MS + crude extract of *H. pluvialis* after 3 weeks on (1): root number and length (cm), (2): fresh and dry weight (gm) of *Cynanchum acutum L* plantlets.

Our results in using *M. aeruginosa* were in complete agreement with (Grzesik et al., 2017) as they proved that independently of the chemical fertilization, *Microcystis Sp.* more effectively than *Anabaena sp.* and *Chlorella sp.* in increased willow (*Salix viminalis L.*) height, total shoot length, fresh, dry weights and its physiological performance in comparison to control, while (El-Sheekh et al., 2014) said that *Microcystis aeruginosa* caused a notable decrease in root, shoot lengths, number of roots, fresh and dry weights, leaf area and pigment contents of *Z. mays L.* This difference may be due to the use of algal cells in the death phase, according to (El-Sheekh et al., 2010), there are more alkaloids and total phenolic compounds in the cell-free media during the death phase than in the log phase, which could explain the algal cell-free medium's inhibitory effects. Additionally, phenolic compounds obstruct several essential functions, including protein and chlorophyll synthesis, respiration, mineral uptake, and photosynthesis, in our study we used the algal extract in the stationary phase, so *Microcystis aeruginosa.* had a less harmful effect and had beneficial benefits on the plant.

We also concur with (El-Sadek & Ahmed, 2022), who discovered that the application of blue-green algae (*Spirulina platensis*) extract had a noticeable impact on the *Capparis Cartilaginea* plant's growth parameters (shoot number, shoot length, and roots). Also, (Ibrahim et al., 2018) demonstrated in their study on the date palm Phoenix dactylifera that the irrigation treatment of the biomass water extract (20%) of blue-green algae (*Oscillatoria tenuis*) also improved vegetative and root growth and increased the percentage of acclimatization success, which may be attributed to plant hormone-like substances produced by algae. The study also showed a significant increase in plant survival rate, plant height, and the number of leaves on the plant. Moreover, (Abd El Moniem & Abd-Allah, 2008) concluded that all

growth parameters (Leaf area, shoot length, and number of leaves/shoot) improved by using the green algae *Chlorella vulgar.*

Determination of Phenolic and Flavonoid Compounds Present in the Mother Plant and Plant Produced *in vitro* by Using Media Combined with Algal Extract

Table 4 and Figure 11 present the values of phenolic and flavonoid compounds in the mother plant, Control plantlets on MS free from plant growth regulators, and plantlets produced *in vitro* harvested from the medium with *M. aeruginosa* extract which present the best multiplication and highest in (M12), and (M4) respectively, in comparison with those media contain *H. pluvialis* extract (H5), (H2). HPLC analysis showed the presence of nineteen important phenolic and flavonoid substances. Since we used all parts of the plant in the analysis, many compounds appeared, unlike previous studies where only the seeds or leaves were used. Some of these compounds were not present in the mother plant, while their production was stimulated once the plant was grown *in vitro*, whether on a hormone-free medium or in the presence of the algal extract such as Ellagic acid 335 µg/g, in control treatment compared to nil in the mother plant, while only the *H. pluvialis* extract stimulated the synthesis of apigenin, kaempferol, and hesperetin at 4.65, 36.4, and 26.64 µg/g, respectively. Regarding rutin reported in M.P. at 6.52, but when *M. aeruginosa* extract was used, its concentration increased to 192 µg/g. In the same way, Caffeic acid was reported in M.P. at 295 µg/g while, its concentration increased to 420 µg/g with *H. pluvialis* extract. Concerning cinnamic acid, it was observed in M.P. at 4.73 µg/g, whereas with *H. pluvialis* extract, it was 14.4 µg/g.

As it is clear, cultivation of the plant *in vitro* whether in the presence of an algal extract or not, has stimulated the production of active substances at a higher rate, and sometimes even the production of substances that were not originally present in the mother plant. Our findings concur with (Ahmad et al., 2020) findings, which clearly showed that the addition of a higher concentration of GA (2.0 mg/L) is the best option for enhancing the biosynthesis and production of phenolics in *S. rebaudiana.*

Furthermore, we agreed with (Park et al., 2017) who stated that GA. at specific concentrations increases the accumulation of certain flavonoids, such as rutin and catechin, as well as total phenolic components in common buckwheat sprouts. Also, (Karalija et al., 2017) found that shoots grown in cytokinin-containing media generally had higher concentrations of phenolics than shoots grown in a PGR-free medium. Additionally, they observed that all cytokinins used in *Knautia sarajevensis* shoot cultures

Table 4. HPLC Analysis for phenolic and flavonoid content of methanol extract of (M.P) Mother plant, (Control) MS+0.5 mg/L BA, (M_4) Explant on MS + 4mg/L *M. aeruginosa* extract. (M_{12}) Explant on MS + 12ml/L *M. aeruginosa* extract. (H_2) Explant on MS + 2ml/L *H. pluvialis* extract. (H_5) Explant on MS + 5ml/L *H. pluvialis* extract.

Ph. & flav. compounds	M.P (µg/g)	Control (µg/g)	*M. aeruginosa* (µg/g)		*H. pluvialis* (µg/g)	
			M_4	M_{12}	H_2	H_5
Gallic acid	2333	718	597	878	647	597
Chlorogenic acid	428	134	92.8	89.9	66.3	46.4
Catechin	854	21.9	92.6	110	111	123
Methyl gallate	134	85.3	20.1	21.9	6.54	4.36
Caffeic acid	295	97.1	43.4	356	420	374
Syringic acid	168	53.3	68.9	74.6	65.3	36.9
Pyro catechol	77.7	29.2	0.00	0.00	0.00	0.00
Rutin	6.52	38.9	192	179	159	191
Ellagic acid	0.00	335	16.9	6.67	74.9	85.8
Coumaric acid	495	0.00	172	155	148	119
Vanillin	130	14.7	107	94.0	103	83.2
Ferulic acid	237	40.5	110	119	160	112
Naringenin	276	169	16.7	26.5	8.61	11.0
Daidzein	150	47.8	32.1	13.28	14.7	0.00
Querctin	96.5	7.98	9.64	0.00	2.49	0.00
Cinnamic acid	4.73	7.63	0.00	0.00	14.4	0.00
Apigenin	0.00	0.00	0.00	0.00	4.65	0.00
Kaempferol	0.00	0.00	0.00	0.00	36.4	0.00
Hesperetin	0.00	0.00	0.00	0.00	26.6	0.00

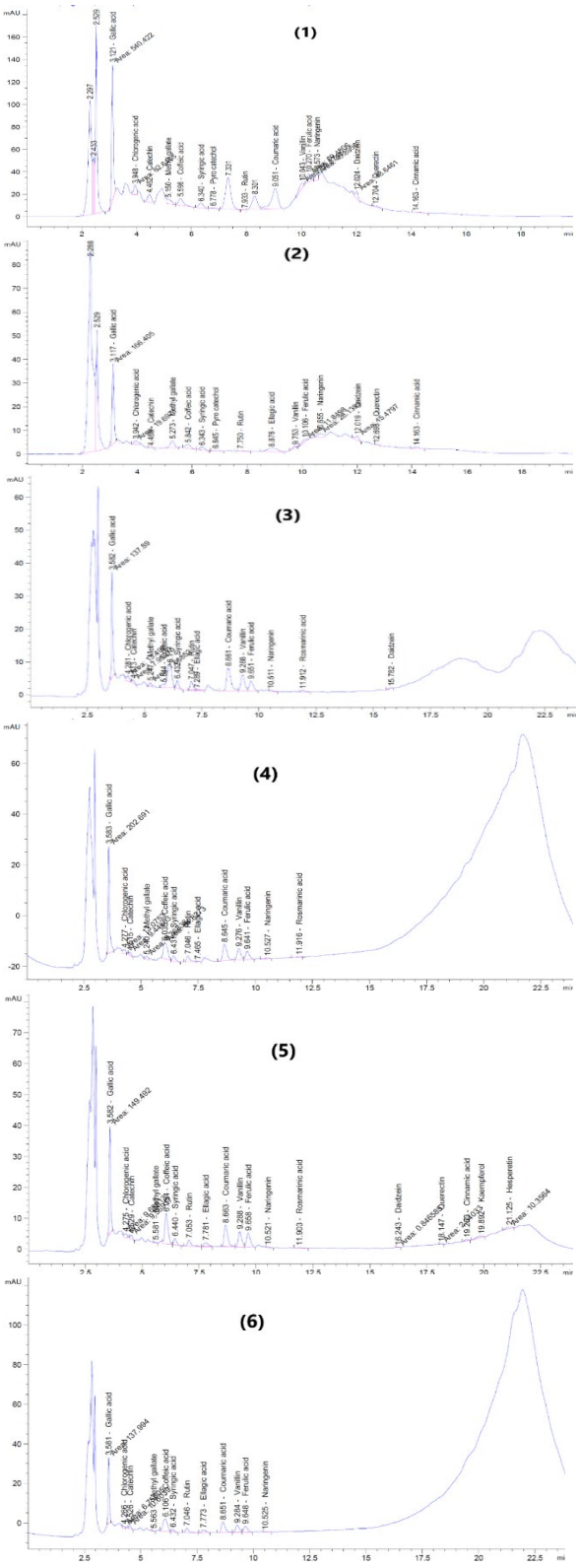

Figure 11. HPLC Analysis for phenolic and flavonoid content of methanol extract of (1) Mother plant. (2) Explant on MS+ 0.5 mg/L BA. (3) Explant on MS+ 4ml/L *M. aeruginosa* extract. (4) Explant on MS+12ml/L *M. aeruginosa* extract. (5) Explant on MS+ 2ml/L *H. pluvialis* extract. (6) Explant on MS+ 5ml/L *H. pluvialis* extract.

had higher flavonoid content, with variations depending on the concentrations used. Also, according to (Sardoei et al., 2014), extracts of *C. officinalis* showed an increase in phenolic compounds when the concentration of GA was raised.

CONCLUSION

From the results of this study, we can conclude that using algal extract, whether from green or blue-green algae, as a green alternative to expensive synthetic plant growth regulators is remarkably positive and efficient on all plant growth parameters and, enhances increasing the important plant secondary metabolites. Therefore, we recommend doing more research on other algae species to detect more growth regulators.

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