NOVEL APPLICATION OF SPIRULINA PLATENSIS EXTRACT AS AN ALTERNATIVE TO THE EXPENSIVE PLANT GROWTH REGULATORS ON *CAPPARIS CARTILAGINEA* (DECNE.)

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ABSTRACT

Capparis cartilaginea is an important medicinal and industrial plant with various bioactivities compound, *C. cartilaginea* which is difficult to propagate in normal conditions. For establishment and multiplication, MS medium treated with crude extract *of Spirulina platensis* as a source of phytohormones was achieved. Growth parameters of calli revealed that the greatest shoot length (4.08 ± 0.86) and leaf number per shootlet (8.40 ± 1.14) on MS medium supplemented with 4mg/L of algal extract. The phenolic content was measured by the Folin–Ciocalteau method. Results of this study showed that application of crude extract *Spirulina platensis* at 4mg/L significantly increased the content of phenolic compounds in C. *cartilaginea* (1.01 mg/g dry mass). HPLC analysis of phytohormones of methanol extract of *S. platensis* recorded the contents of gibberellins, kinetin and adenine as 241.6, 150.2 and 140.6 ppm respectively.

Keywords: *Capparis cartilaginea*, *Spirulina platensis*, phytohormones and phenolic compounds

Introduction

Microalgae can be used as bio-fertilizer or feed food in agriculture and aquaculture. Bioactive compounds with attractive properties are abundant in microalgae, including pigments, carbohydrates, vitamins, proteins and lipids, of which the potential economic substances in public health, the food industry, and the medical field are astaxanthin, β-carotene, chlorophylls and polysaccharides (**Cardozo *et al*., 2007**).

Cyanobacteria can play a crucial role in the sustainable agriculture that contributes to the soil fertility, crop growth and yield and improvement of the environmental quality **(Singh *et al*., 2016 and Osman *et al*., 2016).** *Spirulina platensis* can be used as a rich source of macro- and micronutrients for plants for example vitamins, amino acids, polypeptides, phytohormones (gibberellins, auxins, cytokinins), **(Bhowmik *et al*., 2010; Osman *et al*., 2016 and Nawrocka *et al*.,2017**).

The conservation of natural population of plants recently has the priority for maintaining biological diversity. The first priority is for rare and endangered species. *Capparis cartilaginea* is *Capparis* species from family Capparaceae which are part of the Egyptian plant diversity and are economically important with pharmaceutical value **(Hegazi *et al*., 2011)**. *Capparis* species are difficult in their normal propagation as they

are poor in seed germination **(Ben Salem *et al*., 2001; Yildirim and Bayram, 2001 and Elmoiegy *et al*., 2011**), also have a great problem in rooting percentages whether propagation by cutting or *in vitro* (**Ben Salem *et al*., 2001 and Hegazi *et al*., 2011)**. All plant parts of *Capparis cartilaginea* contain medicinal substances have important biological activity including the antibacterial, antitumor, antioxidant, antifungal, insecticidal, anti-inflammatory and cytotoxic activities **(Moharram *et al*., 2018).**

Due to the importance of the *Capparis cartilaginea* as a source of active substances which can be used for several medicinal and other purposes, also due to its difficulties in propagation in normal conditions, the present work was carried out to study the effect of indigenous phytohormones in the algal extraction of *Spirulina platensis* on *Capparis cartilaginea* through different morphological parameter and phenolic compound analysis.

Microalgal extracts which containing phytohormones, might be a new prospect of phytohormones applications in crop productions, increasing opportunities of microalgae valorization (**Lu and Xu, 2015** and **Tarakhovskaya., *et al* 2007**).

Materials and Methods

The present study was conducted during 2020-2021, and the experiment was carried out in Plant Tissue Culture laboratory, Botany and Microbiology Department, Faculty of Science (girls), Al-Azhar University, Egypt.

1- Plant material.

The seeds of C. *cartilaginea* were obtained from Wadi Elnasab of South Sinai.

2- Explant sterilization.

The seeds were washed under tap water for 3-4 hours followed by soaking under Laminar Air Flow Hood using 15% commercial Clorox containing 5.25% sodium hypochlorite for 10 min **(Ibenthal, 2010).**

3- Culture media and culture condition.

Surface sterilized seeds were cultured in half strength Murashige and Skoog (MS) with vitamins medium **(Murashige and Skoog, 1962**) supplemented with, 3% sucrose and 5.0 g/L agar. The medium pH was adjusted to 5.8 before autoclaving for 20 minutes at 121°C. The culture jar were incubated in a growth room maintained at 22±2°C, illuminated with Phillips TLM 40W/33RS fluorescent lamp providing 4000 Lux light intensity for 16 hrs. a day.

Six weeks old stem segments were excised from the *in vitro* seedlings **(Fig 1)** and transferred to a 200 mL jar with 30 ml of strength MS medium supplemented with, 3% (w/v) of sucrose and 5 g/L agar and 8 different concentrations of algal extract (AE) **(table 1)**. Each treatment consisted of 5 Pyrex plant tissue culture tube each with one explant. The incubation conditions were as described for the *in vitro* seedlings above. After 21

days of culture, the number of shoots, plant height, number of leaves and rooting strength were recorded. The control plantlet (T_1) and the best treatment for morphological parameter(T_5) were collected and dried at room temperature, then active compounds were detected with Liquid Chromatography (HPLC) analysis, antioxidant have been tested.

Table 1: Different treatments of algal extract (AE)

Treatment	Algal extract concentration (mg/l)
T_1 (Control)	**MS+ 0**
T_2	**MS +1**
T_3	**MS+2**
T_4	**MS+3**
T_5	**MS+4**
T_6	**MS+5**
T_7	**MS+6**
T_8	**MS+7**

Fig (1). Seedling from germinated seed

4- **Cultivation condition and algal mass production**.

Spirulina platensis was obtained from Algal Biotechnology Unit, National Research Centre, Egypt. *Spirulina platensis* was subjected to mass production in different photobioreactor types with a final capacity of 1200-1500 liter. Harvesting was done when culture reached about 1.0 $g.l^{-1}$ (fresh weight) as mentioned by **Khan *et al*., (2015)**.

5- **Preparation of algal extract**.

The extraction of algal sample was done using methanol. 10g of algal powder were extracted in 250 ml of methyl alcohol solvent for 24 hrs. Then the soaked samples were filtered and concentrated under reduced pressure using rotary evaporator. Dried residue was re-dissolved with sterilized distilled water made up to 100 ml as stock algal extract then, at different concentration 1,2,3,4,5,6 and 7mg/L added in the media for *in vitro Capparis cartilaginea* propagation under sterile condition.

6- **Extraction of Phytohormones**

The acidic ethyl acetate-soluble fraction was used for determination of acidic hormones such as indole-3-acetic acid (IAA), gibberellin (GA), whereas the basic fraction was used for determination of cytokinin (e.g. benzyl adenine BA) **(Shindy and Smith, 1975; Chen, 1990).** HPLC analysis was carried out according to **Baydar and Ulger, (1998) and Wasternack and Parthier, (1997).** Sample was prepared as illustrated below in steps:

I. 10 gm fresh plant sample were taken and cut to small pieces in beaker containing 30 ml 80% methanol.
II. Cover the beaker with aluminum foil and keep in freezer for 24 h.

III. Filter with filter paper and combined the methanolic layer in amber bottle.
IV. Add 30 ml 80% methanol, cover the beaker and keep in freezer for 24 h then Filter with filter paper and combined the methanolic layer in amber bottle.
V. Discard the residue then evaporate methanol in water bath at 50°C then filter.
VI. Discard the residue then adjust pH of aqueous layer to 8.6 with 1% NaOH.
VII. Extract 3 times by ethyl acetate (equal volume) in separating funnel and combine aqueous layer.
VIII. Discard ethyl acetate (upper layer), then adjust pH of aqueous layer to 2.8 with 1% HCl.
IX. Extract 3 times by ethyl acetate (equal volume) in separating funnel and combine ethyl acetate (upper layer).
X. Apply to HPLC analysis.

Data for HPLC Analysis is mentioned in **fig 4**. Peak identification was performed after 20µl injection volume using Thermo Dionex Ultimate 3000 (apparatus model), C18 (Column), Methanol: Acetonitrile (mobile phase as 50:50 v/v) and 1ml/ min. (flow) throw 15 min. Comparing the retention times with pure standards (Sigma-Aldrich, Deisenhofer-Germany) and the concentrations were calculated from integrated areas of sample and the corresponding standards.

Total phenolic content and phytohormones analysis using HPLC were carried out according to **Kelly *et al*. (1995)** at the Desert Research Center, Egypt.

7- Determination of total phenolic content.

Total phenolic compounds were colorimetrically estimated using Folin Ciocalteu reagent (**Agbor *et al*., 2014**). Methanol extract of the calli or suspension cells (prepared as described above) was mixed with 1 ml of 95 % ethanol, 5 ml of distilled water and 0.5 ml of 50 % (v/v) Folin-Denis reagent. The extract was replaced by 1 ml methanol for the blank. After 5 min, 1 ml of 5 % (w/v) $Na_2 Co_3$ was mixed with samples and tubes were incubated in the dark for 10 min. Absorbance was measured at 725 nm. The total phenolic content was given based on the standard curve which was prepared using standard gallic acid at 0-20mg/ml.

8- Statistical analysis

The experimental design used was randomized complete blocks with three replications. Statistical analyses were carried out using IBM SPSS (SPSS Inc; IBM Corporation, NY, USA) Statistics Version 25 (2017) for Windows. Data were tested for a normal distribution by Shapiro- Wilk's; R test **(Shapiro and Wilk, 1965; Razali and Wah, 2011**). Data were subjected to ANOVA with a P-value of <0.05 being considered statistically significant. The treatment means were compared by least significant difference post-hoc test as reported by **Snedecor and Cochran (1994)**, with a P-value of <0.05 being considered statistically significant **(Alaa *et al*, 2021**).

Result and discussion

1-Multiplication rate and morphological parameter of *Capparis cartilaginea* Decne. after 21 days.

The germinated shoots obtained from seedlings and after growth to a length of about 2–3 cm was used as secondary explants for multiplication experiments. There were notable effects of using different concentrations of algal extract on the multiplication rates of *Capparis cartilaginea* as presented in (Table 2) and (Fig 2).

Table (2): *In vitro* effect of different algal extract (*S. platensis*) concentrations on shoot induction of *Capparis cartilaginea* Decne. after 21 day from stem segment culture.

Treatments	Algal extract concentration (mg/L)	Shoot number per explant (mean SE)	Shoot length (cm) (mean SE)	Leaf number per shootlet (mean SE)
T_1 (Control)	0	1.80 ± 0.45 ^{ab}	1.35 ± 0.95 ^{c}	4.60 ± 0.55 ^{a}
T_2	1	3.00 ± 0.71 ^{d}	0.54 ± 0.27 ^{a}	7.00 ± 1.22 ^{c}
T_3	2	2.00 ± 0.00 ^{bc}	1.41 ± 0.48 ^{c}	6.60 ± 0.65 ^{bc}
T_4	3	2.60 ± 0.89 ^{cd}	1.23 ± 0.46 ^{bc}	6.85 ± 1.27 ^{c}
T_5	4	1.60 ± 0.89 ^{ab}	4.08 ± 0.86 ^{d}	8.40 ± 1.14 ^{d}
T_6	5	1.00 ± 0.00 ^{a}	1.24 ± 0.32 ^{bc}	4.60 ± 0.89 ^{a}
T_7	6	1.20 ± 0.45 ^{ab}	1.28 ± 0.61 ^{bc}	6.50 ± 1.00 ^{bc}
T_8	7	1.20 ± 0.85 ^{ab}	0.64 ± 0.27 ^{ab}	5.30 ± 1.54 ^{ab}

SE ¼ standard error; S ¼ treatment number; means with different superscripts in the same column differ significantly

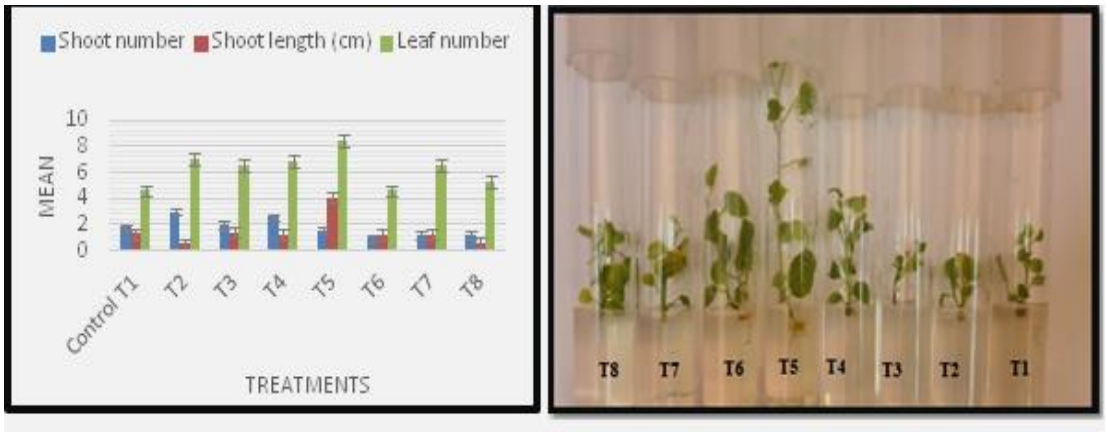


Fig (2). Effect of different concentrations of MS + crude extract of *Spirulina platensis* after 21 days on growth parameters (shoot number, shoot length and leaf number) of *Capparis cartilaginea*.

In this regard, using algal extracts as biostimulants of plant growth is a natural solution that guarantee the growth of food vegetables/fruits without any chemical residues in

addition, these natural products provide improved crop quality with full respect for human health and the environment (**du Jardin, 2015; Povero *et al*., 2016 and Michalak *et al*, 2016**). The treatments differed in their effects and the greatest number of shoots (3.00 ± 0.71) formed upon T_2 treatment on MS medium containing 1mg/L AE which was significantly differ from control treatment. On the other hand, the greatest shoot length (4.08 ± 0.86) and leaf number per shootlet (8.40 ± 1.14) resulted on T_5 treatment on MS medium supplemented with 4mg/l of algal extract which was greatly significantly different all over whole treatments. In this respect, it was found that the response of tomato plants cultivated in greenhouse were treated with the aqueous extract (spray) of the brown algae show a significant improvement in the growth of the tomato plant (**Baroud *et al*, 2021**). These algae extract contain vitamins, cytokinins, amino acids, auxins and acid abscissa which affect the cellular metabolism of the treated plants, resulting in increased growth with the yield of the cultures (**Crouch and Van ,1993 and Stirk *et al*., 2004).**

2- Effect of algal extract (AE) on rooting.

As shown in **Fig 2** all treatments have week to negative effect on enhancement of rooting except T_5 at which rooting was very healthy and strong. Similar data was reported recently by **(Baroud *et al*., 2021**). As well as rooting problem for C. *cartilaginea* recorded by **(Hegazi *et al*., 2011)**. **Fig 3** demonstrate that highest rooting of *C. cartilaginea* was obtained on MS medium supplemented with 4mg/L of algal extract compared with control This result harmony with **Hassanein *et al*., (2008)** who found that Poor rooting of *Capparis cartilaginea* on MS medium.

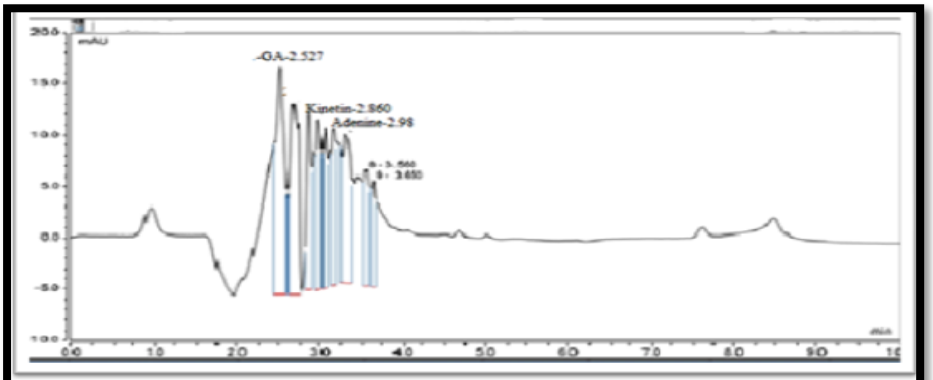
Fig (3). Rooting of *C. cartilaginea* on T5 containing MS + (4mg/L) of crude extract of *Spirulina platensis* after 21 days from culture.

The negative effect of the control media without algal extract on the rooting of shoots of this studied plants is supported the important role of *S. platensis* as a super supplement source for phytohormones in enhancing root initiation.

3-HPLC analysis of phytohormones of methanol extract of *Spirulina platensis*

Data shown in **Fig 4** indicate that the gibberellins, kinetin and Adenine contents determined for *Spirulina platensis*. The contents of GA, Ki and Ad. were observed 241.6, 150.2 and 140.6 ppm, respectively. Our result agree with **Amin *et al* ., 2009** indicated that phytohormones including indole acetic acid ,cytokinin and gibberellin from aqueous extract of *Spirulina* .

The positive effect of GA3 on the elongation of C. *cartilaginea* is in agreement with the results obtained by **Figueiredo *et al*., (2001)** who mentioned that GA3 was necessary for the elongation of shoots of *Rollinia mucosa* (Jacq.)



Peak Name	Retention Time(Min)	Area mAUmin	Height mAu	Relative Area %	Relative Height %	Amount ppm
G A	2.527	2.927	22.169	23.82	15.61	241.616
Kinetin	2.860	1.252	17.236	10.19	12.14	150.156
Adenine	2.987	1.125	16.681	9.07	11.75	140.570

Fig (4). HPLC Analysis for phytohormones of methanol extract of *Spirulina platensis*

Gibberellins regulate seed germination, stem elongation, leaf expansion and flower and seed development (**Davies,** 1995).

The obtained results were agree with **Amin and Omar, (2002)** who found that cyanobacterial exudates of *Nostoc piscinale* and *Anabaena fertillissima* as biotic natural source of vitamins and phytohormones led to maximum fresh and dry weight of callus tissue as the medium under investigation prepared without the biotic source. *Spirulina platensis* at 5% (high) concentration level showed significant effects in the aspects of morphological parameters such as seed germination percentage, radicle length, coleoptile length and epicotyls length and biochemical parameters,(**Sivalingam, 2020**).

The stimulatory responses of *Capparis cartilaginea* calli may be related to that *S. platensis* itself is a super supplement source for vitamins and phytohormones This result was highly supported by **Osman *et al*. (2010),** who reported that the cyanobacteria play a major role in the seed germination by secreting phytohormones like auxins, cytokinins and gibberellins.

4- Determination of total phenolic content.

Fig (5) presents the values of total phenolic content in plantlets produced *in vitro* harvested from the medium conferring the best multiplication (T5) in comparison with control one in order to determine total phenolic using Folin Ciocalteu reagent **(Agbor *et al.*, 2014**). The highest value of total phenolic was recorded for the plantlet on T5 medium (1.01 mg/g dry mass (DM)), while the corresponding value for the control extract was 0.76 mg/g DM. In general, the addition of AE enhanced the accumulation of phenolics more than the finding in the control one which grow without AE.

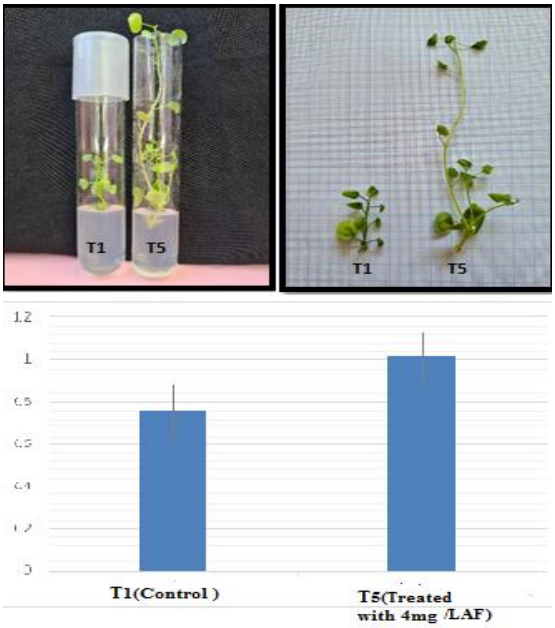


Fig (5). Total phenolic content in treated plant with 4 mg/L AE compared with those of control plant.

The results demonstrate that, the total phenolic content in treated plant with 4 mg/L of algal extract was higher than that of the control could be of interest as a primary source of naturally occurring bioactive substance of algal extract. Our results are in agreement with the finding of **Anastasia *et al.*, (2012)** who reported GA3, IAA and kinetin significantly increased specific phenolic compounds (gallic acid and rutin) in lentil plants.

Ali., *et al* (2014) showed that application of GA3 at first and second stages significantly increased the content of phenolic compounds in *C. officinalis* (61.96 and 62,74 mg gallic acid/100 g dry weight respectively), while at third stage decreased the phenolic compounds.

Declaration

The authors declare that they have no conflict of interest in the publication and that the submitted manuscript is originally, unpublished and not under simultaneous consideration by another journal.

Abbreviations

BAP	6-Benzylaminopurine
GA3	Gibberellic acid
HPLC	High Performance Liquid Chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-buteric acid
NAA	α-Naphthalene acetic acid
TDZ	Thidiazuron
2, 4-D	2, 4-Dichlorophenoxy acetic acid
MS	Murashige and Skoog

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تطبيق جديد لمستخلص *Spirulina platensis* كبديل لمنظمات نمو النبات باهظة الثمن على *Capparis Cartilaginea* (Decne.)

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- يعتبر نبات *Capparis cartilaginea* من أهم النباتات التي لها فوائد طبيه و صناعيه عديده نظرا لأنه يحتوي علي العديد من المواد الفعاله ولكنه من الصعب اكثاره تحت الظروف الطبيعيه ولهذا كان الهدف من البحث هو اكثار النبات معمليا في بيئه MS مع اضافه مستخلص طحلب *Spirulina platensis* بتركيزات محتلفه والذي يعتبر كمصدر للهرمونات النباتية.
- أظهرت النتائج ان أعلي معدل لنمو النبات عند تركيز 4 مجم / لتر من مستخلص الطحلب على وسط MS حيث وصل المجموع الخضري (4.08 ± 0.86) بينما سجل عدد الأوراق لكل فرع (8.40 ± 1.14)
- تم قياس المحتوى الفينولي بطريقة Folin-Ciocalteau. أظهرت نتائج هذه الدراسة أن استخدام مستخلص *Spirulina platensis* عند 4 ملجم / لتر أدى إلى زيادة معنوية في محتوى المركبات الفينولية في النبات (1.01 مجم / جم كتلة جافة).
- سجل تحليل HPLC لمستخلص الطحلب وجود العديد من الهرمونات النباتية وهي Gibberellins و kinetin و Adenine التي سجلت 241.6 و 150.2و140.6 . جزء في المليون على التوالي التي كان لها تأثير كبير في نمو النبات مقارنه بالنبات الطبيعي.

كلمات مفتاحية :- نبات القبار- طحلب الأسبيرولينا – الهرمونات النباتية – المواد الفينولية