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ESSENTIAL OIL PRODUCTION OF BASIL *OCIMUM CITRIODORUM***

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## IMPACT OF *SPIRULINA PLATENSIS* AND *STEVIA REBAUDIANA* ON GROWTH AND ESSENTIAL OIL PRODUCTION OF *BASIL OCIMUM CITRIODORUM*

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### ABSTRACT

The constituents of essential oils isolated by diethyl ether of the *in vitro* explants of *Ocimum citriodorum* cultured on alternative media containing green algae spirulina and stevia plant powder or filtrate were examined by GC-MS. The *Spirulina platensis* green algae were added in culture medium powder at (0.5, 1.0 and 2.0 g/l) and powder filtrated at 2, 4 and 6 ml/l from concentrations 5, 10 and 15 %, respectively. Also, *Stevia rebaudiana* dry leaves were added powder at 0.5, 1.0 and 1.5 g/l and powder filtrated 5, 10 and 15 ml/l from concentrations 0.05, 0.1 and 0.15 %, respectively. FTIR and XRD were examined for both materials using as alternatively medium. The explants that were cultured on media containing spirulina filtrate at 4 ml/l and 6 ml/l scored survival 100 % and shootlets length 0.84 and 0.92 cm; shootlet number 1.98 and 2.33; leaves number 8.66 and 2.60, respectively. The explants cultured on media containing stevia filtrate 5 and 10 ml/l also gave the best results but the media turned to browning (++) and (+++), respectively. A total of 12 components were identified accounting for 94.52 % of the oils of *O. citriodorum*. The oil contained, as main components, geranial (31.24 %), Linalool (8.29 %), Estragole (4.98 %), neral (25.71%) and Neryl acetate (8.857 %).

### 1. INTRODUCTION

Tissue culture is use to micro propagate rare plants and other biotechnological studies. The tissue cultures tools are a high coast comparatively with other original propagation methods especially culture media, PGRs and vitamins. So, the natural products produced from plants, algae, yeast ...etc., were used as a source of minerals, carbohydrates, vitamins and sometimes growth regulators (Saha, *et al.*, 2010).

The green algae have many nutritional values because it's content of a wide range of essential nutrients, such as provitamin, minerals, proteins, polyunsaturated fatty acids like gamma-linolenic acid and sulfated polysaccharides (Abd El Baky, *et al.*, 2003 and Colla, *et al.*, 2007).

*Stevia rebaudiana* (Bertoni) is the wild source from northeastern Paraguay, and today it is cultivated around the world and little area of Egypt. The leaves extraction of stevia naturally contains a mixture of 8 sweet diterpene glycosides: stevioside, steviolbioside, rebaudiosides (A, B, C, D and E) and dulcoside A (Geuns, 2003). Stevioside is present with an average of 4-20% in the dry matter of the plant leaves, which primarily depends on cultivar characteristics of plants and basic agricultural techniques (Brandle and Rosa, 1992; Geuns 2003). Specifically, green leaves contain a greater amount of chlorophyll A

and B located in the chloroplasts of plant cells. The precursors of steviol glycosides were synthesized in chloroplasts of the cell so, the tissues without chlorophyll pigment do not contain or contain only minor amounts of the sweet steviol glycosides (**Brandle and Rosa, 1992; Singh and Rao, 2005; Braz de Oliveira et al., 2011**). Moreover, when the leaves drying process the structure of chlorophyll is changing and as the main consequence occurs the change in color from green to brown. This color change ultimately affects the change of color during the extraction and purification process of sweeteners (**Abou-Arab et al., 2010**). Sweetness i.e. stevioside concentration in the plant leaves is the highest just before flowering of plant, and the plants should be harvested as soon as flowering starts or before the first frost (**Brandle and Rosa, 1992**). Some food additives using the leaves of stevia and a sweetener in food products. Sometimes, on the production of stevia present as: a green powder obtained by grinding of dried green leaves (**Mishra et al., 2010**), a white powder obtained by depigmentation process of green powder (**Brandle and Rosa, 1992**) and a solution obtained by different extraction methods of stevioside and rebaudioside A from green powder (**Abou-Arab et al., 2010**).

Lemon balm (*Ocimum citriodorum*), a member of the Lamiaceae family (formerly Labiatae), is one of the important medicinal plant species. Essential oil, as well as any other plant based natural ingredients, have been the beneficiary of legal, regulatory, and consumer preference as the result of a shared opinion on food safety (**Burdock and Wang, 2017 and Wandersleben, et al., 2018**). The lemon balm has been used for different medicinal purposes as tonic, antispasmodic, carminative, diaphoretic, surgical dressing for wounds, sedative-hypnotic strengthening of the memory, and relief of stress-induced headache. In the modern pharmacology uses its value is in the management of mild to moderate Alzheimer's, against migraine and rheumatism, antitumel and antioxidant activities (**Moradkhani et al. 2010**). In addition, lemon balm essential oils have been shown to possess antiviral and antimicrobial activity (**Allahverdiyev et al. 1994; Dikbaş et al. 2010**). Similarly, the lemon-scented leaves add flavor to jellies, liqueurs, fruit salads, and cold drinks (**Bahtiyarca and Cosge 2006**).

The aim of the present work was to produce a nutrient growth media using *Spirulina platensis* filtrate and/or stevia filtrate with a high content of mineral, vitamins, polysaccharides and evaluate their effect on in vitro growth characters and essential oil contents.

## 2. MATERIAL AND METHODS

### 2.1 Basil lemon in vitro preparation

#### 2.1.1 Seed disinfection

The seeds were taken from Agrogreen Company in the growing season 2018 /2019 and were divided into groups each one contains more than 50 seeds. The seeds were washed in soap water using septol soap for 30 min and rinsed with running tap water for one hour. Then were transferred under laminar flow cabinet. Seeds were immersed in 70% ethanol solution (v/v) for 30 sec. and immersed in 10 % Clorox (commercial bleach containing 2.25% sodium hypochlorite) plus tween-20 (2 drops / 100 ml) as emulsifier for 15 with agitation under aseptic conditions and then rinsed in sterile distilled water for three times.

#### 2.1.2 Seed germination

The sterilized seeds were germinated on MS medium free hormone for four weeks and the seedlings were transferred on proliferation MS medium containing BAP at 2 mg/l as control medium.

### 2.2. MEDIA ALTERNATIVELY COMPOUNDS

#### 2.2.1 Source of *spirulina platensis*

The strain was maintained in 500 mL sterilized Erlenmeyer flasks containing 100 mL Zarrouk's

medium (Zarrouk, 1966) at  $30 \pm 2$  °C, pH 9 with continuous illumination using cool white fluorescent tubes (2500 Lux) and twice daily shaking by hand.

### **2.2.2 Isolation of *Spirulina platensis***

The culture would be picked up from the stock culture with the help of a needle and transferred to petri plates and culture tube containing Zarrouk's medium and incubated at 28°C for 30 days with (600 – 1600 lux) with a continuous light, 12 hrs per day (Sony *et al.*, 2008).

### **2.2.3 *Stevia rebaudiana* dry matter**

The dry leaves were collected from Medicinal and Aromatic Department, Hort., Researches Institute, A.R.C. and were powdered in an electrical blinder to complete homogenate and stored in the dry package to using in optimizing media.

## **2.3. OPTIMAL CONCENTRATIONS OF ALTERNATIVES**

### **2.3.1 *Spirulina platensis***

The green algae *Spirulina platensis* was drying and powdered in electronic blinder, the powder weighted at 0.5, 1.0 and 2.0 g/l and adding to the media as showing:

0.5 g/l *Spirulina platensis* powder + agar + 25 g/l sucrose

1.0 g/l *Spirulina platensis* powder + agar + 25 g/l sucrose

2.0 g/l *Spirulina platensis* powder + agar + 25 g/l sucrose

The mixture of algae was dissolved in distilled water with 25 g/l sucrose and stirring with electronic stir to complete homogenate, adjust pH at 5.7 and adding 7 g/l agar. The filtrate of green algae was prepared by boiling optimum weight (5, 10 and 15 %) of algae in 100 ml distilled water at 100 °C for 30 min. and filtered with Wittman filter paper No.1. The filtrate was diluted on culture media at 2 ml/l of 5%, 4 ml/l of 10% and 6 ml/l of 15%. After complete cooked all treatments dispensed in 200 ml glass jars at 15 ml and autoclaved at 121°C with 1.12 bar for 20 min.

### **2.3.2 *Stevia rebaudiana***

The dry leaves of stevia were blinded in an electronic blinder, after complete homogenate the powder was added to MS culture media without sucrose at 0.5 , 1.0 and 1.5 g/l and with 25 g/l sucrose at the same concentrations.

The filtrate of dry leaves was prepared by boiling optimum weight (0.05%, 0.1% and 0.15%) of dry leaves in 100 ml distilled water at 100 °C for 30 min. and filtered with Whatman No.1. The filtrate was diluted on culture media at 5 ml/l of 0.05%, 10 ml/l of 0.1 % and 15 ml/l of 0.15%. After complete cooked all treatments we dispensed it in 200 ml glass jars and autoclaved at 121°C with 1.12 bar for 20 min.

### **2.3.3 Combination of algae with MS salts**

The optimum weight of dry matter (2 g/l) of green algae was added to the MS (Murashige and Skoog, 1962) salts at combination with the following:

Dry matter 2 g/l green algae

Dry matter 2 g/l green algae + Sucrose

Dry matter 2 g/l green algae + Macronutrients + Sucrose

Dry matter 2 g/l green algae + Micronutrients + Sucrose

Dry matter 2 g/l green algae + Vitamins + Sucrose

Dry matter 2 g/l green algae + MS full + Sucrose

## **2.4 CHARACTERIZATION ALTERNATIVES**

The functional groups of green algae and stevia were detected to confirm active groups in each which

responsible for reaction with the main components and increase essential oil in leaves extract using Fourier Transform Infrared (FTIR) by (FTIR Perkin Elmer-Spectrum One) in the range between 400-4000 $\text{cm}^{-1}$

## 2.5 GAS CHROMATOGRAPHY–MASS SPECTROMETRY (GC-MS) ANALYSIS

The chemical composition was performed using Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25  $\mu\text{m}$  film thickness). The column oven temperature was initially held at 50 C and then increased by 5°C /min to 230°C hold for 2 min. increased line temperatures were kept at 250, 260°C respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 3 min and diluted samples of 1  $\mu\text{l}$  were injected automatically using Autosampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40–1000 in full scan mode. The ion source temperature was set at 200 °C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral database

## 2.6 DATA AND PARAMETERS

The treatment divided into three replicates, each replicate containing three explants in the jar. The experiment was designed by Complete Randomized Design (CRD) and using LSD at 5 % to compare the means of treatments. The data analyzed by Co-State software Version-4 (Co-Stat Graphics, 1999).

## 3. RESULTS AND DISCUSSION

### 3.1 *Spirulina platensis*

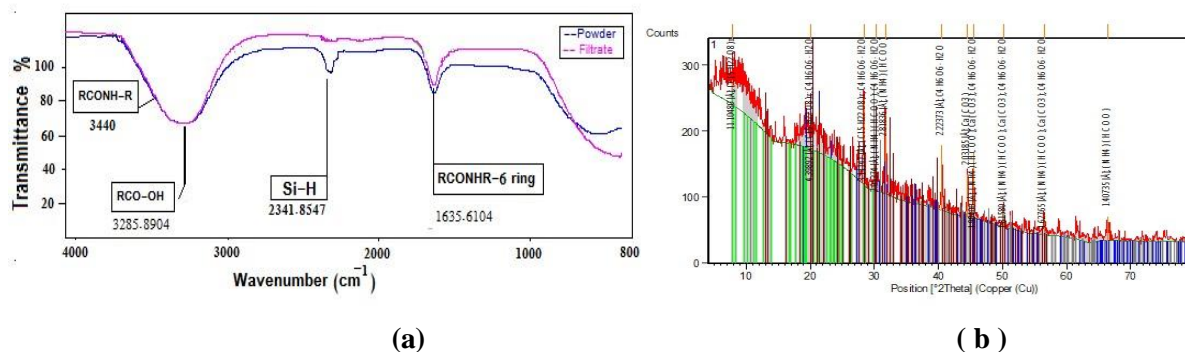
#### 3.1.1 Fourier transforms infrared (FTIR) spectroscopy

The FTIR spectrum shows a frequency ranges from 3500–3200 (s,b)  $\text{cm}^{-1}$  (Fig. 1 a) representing the O-H stretching vibration. The frequency ranges from, 3000–2850  $\text{cm}^{-1}$  peaks are representing in the C-H stretching vibration presence of alkenes. The frequency ranges from 3300–2500  $\text{cm}^{-1}$  peaks are representing aliphatic N-H stretching vibration presence of secondary amines (proline or lipids). The frequency ranges from 2260–2100  $\text{cm}^{-1}$  peaks are representing –C (triple bond) C– stretching vibration (ester alkynes). The following peaks 1680–1640  $\text{cm}^{-1}$  are present in the –C=C– stretching vibration present in the alkenes. These results are in agreement with that found by **Yang *et al.*, (2005)**, **Mao *et al.*, (2006)** and **Abd El Baky *et al.*, (2009)** which describing a symmetrical C-O-S vibration associated to a C-O-SO<sub>3</sub> group at 819  $\text{cm}^{-1}$  and a discernible shoulder at 857  $\text{cm}^{-1}$  was also due to the symmetrical C-O-S vibration. The FT-IR analyzed of *Spirulina* having high quantity of proteins, (**Dillon, 1995; Richmond, 1988 and Zarrouk, 1966**) vitamins, phycocyanin and antioxidants substances. The *Spirulina* algae using as a source of minerals and supplements plant with some minerals, vitamins, polysaccharides. Based on the systematical analysis of *Spirulina* contains in protein, lipid, carbohydrate, aliphatic (C-H), Carbonyl (esters and acid), Carbonyl Beta-Unsaturated Ketone amide (C=N), ester, symmetric C-H stretching vibration, halogen compounds (C-Cl).

#### 3.1.2. XR-Diffractions

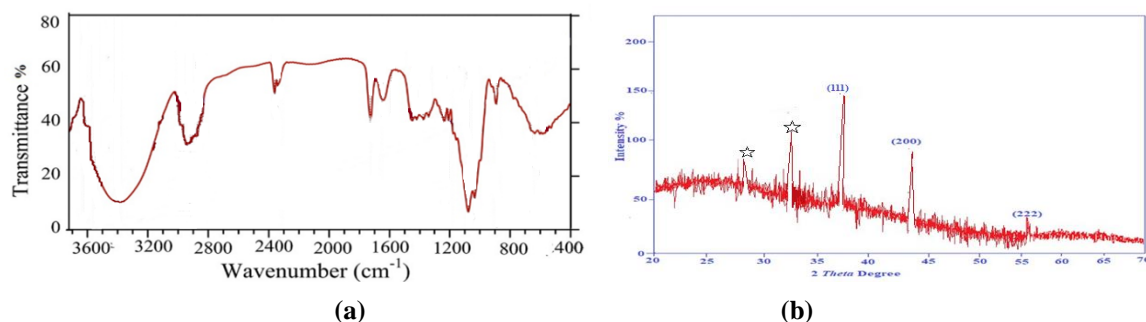
The XRD pattern of the microparticles (Fig. 1(b)) indicates a shape of typical amorphous in nature, but some crystalline zones were observed. On the other hand, XRD analysis of the *spirulina* showed intense peaks, corresponding to 2 $\Theta$  position 7.838, 11.57, 19.029, 19.45 and 21.49. Bragg reflection, based on the monoclinic structure of crystalline, with a lattice constant of a =11.76, b=15.31 and c=15.14 Å and  $\alpha = \beta = 90$  but  $\gamma = 106$  indicate to cellulose tripropionate, hydrogen tartrate hydrate, ammonium format and calcium carbonate, respectively. This shows that during the preparation of the

spirulina filtrate, the *S. platensis* structure was modified, and the crystalline zones disappeared.



**Fig. 1 (a) Fourier transforms infrared (FTIR) spectroscopy of *spirulina platensis* analysis and (b) XR-Defraction of green algae**

The XRD pattern, thus, clearly shows that the *Spirulina platensis* shows some crystalline for present inorganic compounds and amorphous peaks for present organic polymers. The unidentified crystalline peaks are also apparent in many works in which the XRD pattern includes the relevant 2 range (El-Sayed, *et al.*, 2005). The existence of the broad peak at  $2\theta = 25^\circ$ , attributed to an amorphous phase, is also reported (Kannan *et al.*, 2011)



**Fig. 2 (a) Fourier transforms infrared (FTIR) spectroscopy of stevia analysis and (b) XR-Defraction of stevia**

### 3.2. *Stevia rebaudiana* dry matter

#### 3.2.1. Fourier transforms infrared (FTIR) spectroscopy

FTIR spectra of several dried powder water stevia leaf filtrate were presented in fig. 2. Wide and intense absorption at  $3418\text{ cm}^{-1}$  corresponded to the stretching vibration of the OH bond (-OH stretching) and was associated with the presence of hydrogen bond. Absorption at  $2916\text{ cm}^{-1}$  was characteristic of stretching-CH  $\text{sp}^3$  bond Intense peaks at  $1736$  and  $1597\text{ cm}^{-1}$  corresponded to stretching vibrational-C=O bond. Bending vibrational of -CH bond was observed at  $1415$  and  $1385\text{ cm}^{-1}$ . Furthermore, high intense peaks at  $1030$  and  $1065\text{ cm}^{-1}$  corresponded to C-O derived from steviol glycoside and was characteristic absorption band of the glycosidic bond. Finally, peaks at  $891\text{ cm}^{-1}$  and  $814\text{ cm}^{-1}$  were recognized to bending vibration of =CH and =CH<sub>2</sub> bonds, respectively [28].

In this concern, Yilmaz (2011) using stevia leaves as a capping reagent(s) and molecules that responsible for the reduction of ions. The stevia leaf extracts FTIR measurements showed distinct bands in the  $1000\text{--}1750\text{ cm}^{-1}$  and  $2850\text{--}3000\text{ cm}^{-1}$  ranges and around  $3400\text{ cm}^{-1}$  on the FTIR spectra of the extract. Bending vibrations of acetylenic C-H deformation, aliphatic CH<sub>3</sub> deformation and rocking modes, NH<sub>2</sub> and N-H wagging modes of amines and olefinic cis-CH CH, as well as the out

-of plane bending vibrations of the alcoholic and phenolic O–H bonds may all contribute to the broadband between  $400\text{ cm}^{-1}$  and  $810\text{ cm}^{-1}$  in the spectrum of the extract. It should be noted that the IR spectrum of tamarind leaf **Shanker, et al., (2004)** and lemongrass leaf **Ankamwar, et al., (2005)** broth-reduced gold nanoparticles also reveal an absorption band at  $1599\text{ cm}^{-1}$  with a long tail at high-frequency side. Low-frequency side of the band at  $1590\text{ cm}^{-1}$ , which covers the  $1460\text{--}1590\text{ cm}^{-1}$  range could be ruled by the C–C vibrations in aromatic rings, amides and the scissoring mode of NH<sub>2</sub> in amines. The last two also contribute to the high energy side of this band and could be responsible for the weak band of the extract appearing at  $1650\text{ cm}^{-1}$ .

### 3.2.2. XR-D analysis

The XRD spectrum analysis of stevia dry leaves powder depicted in Fig. 3 reveals that amorphous and crystalline phases coexist. The crystalline peaks at  $2\Theta = 37.3^\circ$ ,  $44.7^\circ$  and  $56.3^\circ$  can be indexed as (111), (200) and (220) planes of CaO, respectively. The unassigned peaks at  $2\Theta = 28.6^\circ$  and  $33.70^\circ$  (denoted by \*) and the broad peak centred at  $2\Theta = 25^\circ$  in Fig. 3 are thought to be related to crystalline and amorphous organic phases, respectively, accompanying crystallized SNPs, as stated in the discussion of the TEM images in Fig. 1a and b. These unidentified crystalline peaks are also apparent in many works in which the XRD pattern includes the relevant  $2\Theta$  range (**Dwivedi, et al., 2010; Philip, et al., 2011 and Mallikarjuna, et al., 2011**). The existence of the broad peak at  $2\Theta = 25^\circ$ , attributed to an amorphous phase, is also reported (**Dwivedi, et al., 2010 and Mallikarjuna, et al., 2011**).

### 3.3. Optimum concentration with MS salts

The data presented in table (1) reported that the combination of natural compounds spirulina platensis and MS components individually, only adding powder at  $2\text{ g/l}$  with MS medium salts with sucrose is the best result of growth characters which scored survival 75 %, an average of shootlet no. 1.22; an average of shootlet length 3.22 and average of leaves no. 5.22 with no callus formation and phenolic compound observation and 25 % rooting compared with other treatments.

## 4. IN VITRO CHANGES

### 4.1. Media changes

According to Table (2) and Fig. (3) culture media was turned to brownish color after adding stevia leaves powder to culture media and the color was increased with increasing powder concentration  $0.5$ ,  $1.0$  and  $1.5\text{ g/l}$  to (+), (++) and (+++), respectively. Similarly, adding 4 and 6 ml/l of green algae to the medium gave a slightly brown color (+) and (++)). Also, using stevia filtrate at 10 ml and 15 ml in media containing sucrose gave browning color (++) and (+++) and the same effect found with those treated with 15 ml/l stevia filtrate to culture media without sucrose. Both of micro and macronutrients plus  $2\text{ g/l}$  green algae gave the positive effect of browning (+++) for each and this effect observed with vitamins.

### 4.2. Morphological changes

The explants of basil were cultured on MS media containing green algae at  $0.5$ ,  $1.0$  and  $2.0\text{ g/l}$  increased survival gradually to 25, 33.33 and 66.66 %, respectively with weak growth of other parameters shootlet no, shootlet length and leaves number (average of shootlet no. 1.12, 0.81 and 0.55); (average of shootlet length 1.10, 0.61 and 0.72 cm) and ( average of leaves no. 4.5, 2.42 and 2.01) although explants were survived few growth was observed and no callus or root formation. In contrast, the green algae filtrate adding to the culture media at 2, 4 and 6 ml/l gave the best growth forms, moreover 4 ml/l filtrate of green algae was recorded 100 % survival and average of leaves 8.55 with root formation at percentage 5.5 % without callus formation. As well, Increased volume of

filtrate to 6.0 ml/l increased average of shootlet no. to 2.33 and average of shootlet length 0.92 without formation callus of roots.

**Table (1) Effect of various concentrations and sources of nutrition compounds on the growth of basil explants by *in vitro* technique**

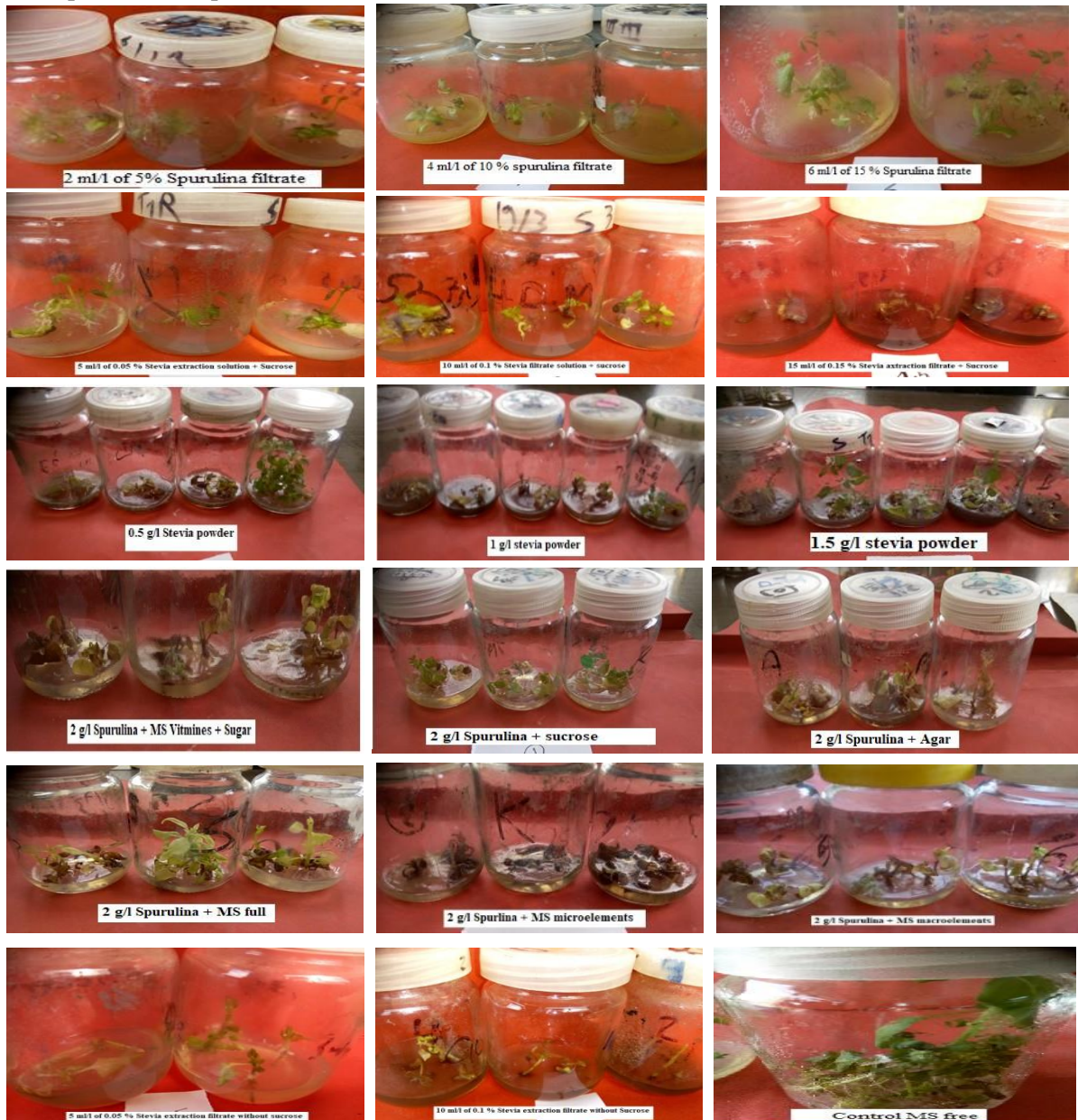
Nutrition treatments	Survival %	Shootlet No.	Shootlet Length	Leaves No.	Callusing %	Rooting %	browning media
<b>• Optimum concentration of algae</b>							
Algae 0.5 g/l + MS	25.00	1.12	1.10	4.50	0.00	0.00	-
Algae 1.0 g/l + MS	33.33	0.81	0.61	2.42	0.00	0.00	-
Algae 2.0 g/l + MS	66.66	0.55	0.72	2.01	0.00	0.00	-
2 ml/l Filtrate of 5% (w/v) + MS	88.88	0.55	0.75	2.50	0.00	11.11	-
4 ml/l Filtrate of 10% (w/v) + MS	100.00	1.98	0.84	8.66	0.00	5.50	+
6 ml/l Filtrate of 15% (w/v) + MS	100.00	2.33	0.92	2.60	0.00	0.00	++
LSD 5 %	9.0187	0.1671	0.7231	0.0412	NS	4.0912	
<b>• Optimum concentration of stevia</b>							
Stevia 0.5 g/l + Sucrose	11.11	1.12	3.55	2.10	6.66	0.00	+
Stevia 1.0 g/l + Sucrose	11.11	0.91	1.23	0.87	0.00	0.00	++
Stevia 1.5 g/l + Sucrose	25.00	1.03	1.16	2.30	0.00	0.00	+++
5 ml/l Filtrate of 0.05% (w/v) +sucrose	88.88	1.54	3.55	4.21	6.66	0.00	-
10 ml/l Filtrate of 0.1% (w/v) +sucrose	66.66	2.09	1.23	3.03	0.00	0.00	++
15 ml/l Filtrate of 0.15% (w/v) +sucrose	0.00	0.00	0.00	0.00	0.00	0.00	+++
5 ml/l Filtrate of 0.05% (w/v)	50.00	1.23	2.11	3.12	0.00	0.00	-
10 ml/l Filtrate of 0.1% (w/v)	25.00	1.52	2.41	2.12	0.00	0.00	-
15 ml/l Filtrate of 0.15% (w/v)	00.00	0.00	0.00	0.00	0.00	0.00	+++
LSD 5 %	6.0932	0.2563	0.4532	0.6031	2.198	NS	
<b>• Combination of algae with MS salts</b>							
Algae 2 g/l + Agar	22.22	1.00	0.52	2.00	0.00	11.11	-
Algae 2 g/l + Sucrose	33.33	1.12	1.61	3.70	0.00	22.22	+
Algae 2 g/l + MS+ Sucrose	75.00	1.22	3.22	5.22	0.00	25.00	-
Algae 2 g/l + Macronutrients + Sucrose	6.00	0.33	0.45	2.00	0.00	0.00	+++
Algae 2 g/l + Micronutrients + Sucrose	6.00	0.33	0.42	2.00	0.00	0.00	+++
Algae 2 g/l + Vitamins + Sucrose	0.00	0.00	0.00	0.00	0.00	0.00	++
LSD 5 %	9.023	0.2891	1.021	0.912	NS	7.2109	

+ light brown, ++ Moderate brown, +++ Deep brown

The stevia leaves containing about 3-7 % glycoside stevioside which containing three monosaccharides these monosaccharides were hydrolyzed after soluble in water and cooked in media. The browning formed after cooked the media and autoclaving at high concentration 1.5 g/l and 1.0 g/l but disappeared at low concentration 0.5 g/l. The situation is different when applying leaves filtrate. In this case, hydrolysis of glycosides obtained after dissolved powder in water and filtration, so phenolic compounds were not formation with a low level of filtrate 5 ml/l without sucrose or with sucrose and 10 ml/l without sucrose compared with all stevia treatments.

The best growth characters were obtained for the medial containing 5 ml/l of stevia filtrate which scored 88.88 % survival, average of shoot no 1.54, average of shootlet length 1.55 cm and average of leaves no. 4.22 with few callus formation 5% following by applying 10 ml/l filtrate but

some phenolic compound formed in the media.



**Fig. 3** Photos of growth treatments of applying *Spirulina platensis* powder, *Stevia rebaudiana* powder and their filtrate in culture media individual or with MS media salts basil (*Ocimum citriodorum*)

#### 4.3. Essential oil changes

Chemical compositions of the essential oils of *Ocimum citriodorum* L. were given in Table 2 in the order of the retention times of the constituents. Eleven constituents were identified in *O. citriodorum* control explants, it was representing 91.373 % of the oil for all components and 42.21 % for the main components (Table 2) and (Fig.4). Myrcen (1.249%), linalool (8.29 %), geraniol (31.24%),  $\beta$ - Caryophellene (1.431%) these compounds were presented in control and all treatments except p-cymene were absent in control and present in all treatments. Moreover,  $\alpha$ - pinene (0.604%); 1,8-cineole (1.34%); Estragole (4.98%); Neral (25.71%); Nerol (5.608%); Neryl acetate (8.857%); Eugenol (1.157%); Methyl eugenol (4.042%) were presented in control only and disappeared from other treatments.

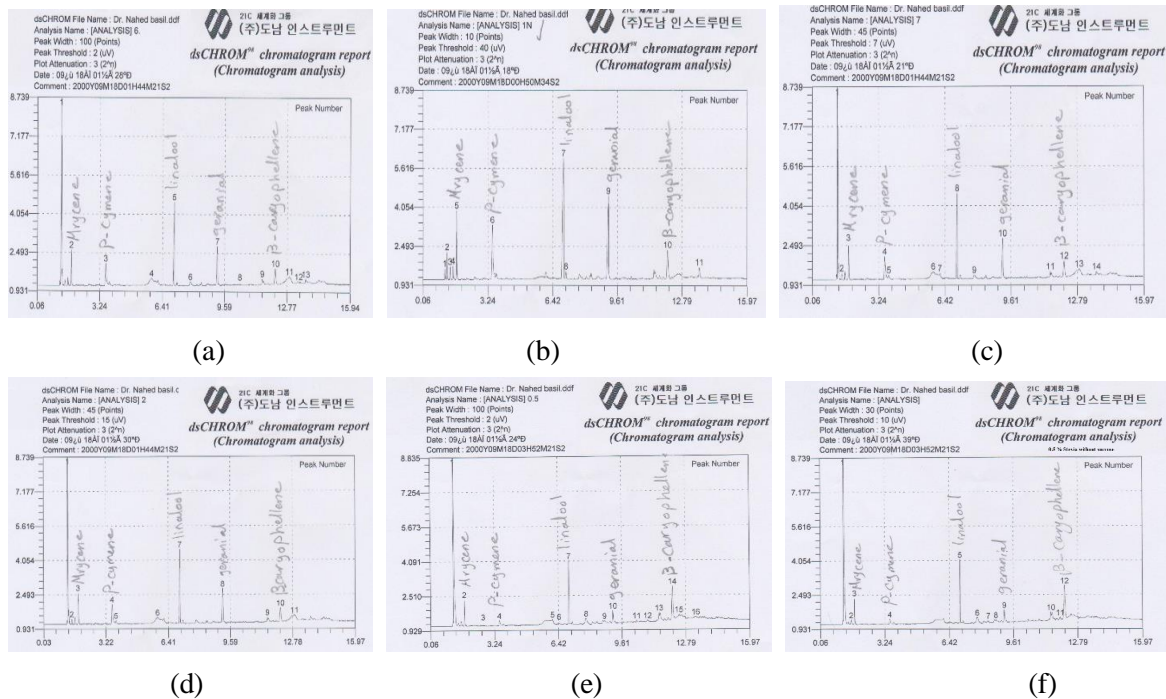
**Table (2). Effect of natural compound additives in culture media on essential oil components and leaves area of *Ocimum citriodorum***

No	compound	RT	Control	A	B	C	D	E	F
1	▪ <i>α-pinene</i>	3.643	0.604	nd	nd	nd	nd	nd	nd
2	▪ <i>benzaldehyde</i>	3.782	nd	nd	nd	nd	nd	nd	nd
3	▪ <i>Camphene</i>	3.981	nd	nd	nd	nd	nd	nd	nd
4	▪ <i>β-pinene</i>	4.011	nd	nd	nd	nd	nd	nd	nd
5	▪ <i>Sabinene</i>	4.589	nd	nd	nd	nd	nd	nd	nd
6	▪ <i>Myrcene</i>	4.873	1.249	2.809	2.347	6.976	9.105	8.139	8.048
7	▪ <i>1,8-cineole</i>	5.062	1.34	nd	nd	nd	nd	nd	nd
8	▪ <i>α-ocimene</i>	5.218	nd	nd	nd	nd	nd	nd	nd
9	▪ <i>p-cymene</i>	5.987	nd	2.589	1.110	12.72	6.386	6.072	9.879
10	▪ <i>γ-terpinene</i>	6.043	nd	nd	nd	nd	nd	nd	nd
11	▪ <i>3-carene</i>	6.122	nd	nd	nd	nd	nd	nd	nd
12	▪ <i>Linalool</i>	6.244	8.290	6.769	8.653	37.01	13.657	13.505	13.333
13	▪ <i>d-camphor</i>	6.456	nd	nd	nd	nd	nd	nd	nd
14	▪ <i>terpinene-4-ol</i>	7.091	nd	nd	nd	nd	nd	nd	nd
15	▪ <i>Estragole</i>	7.173	4.980	nd	nd	nd	nd	nd	nd
16	▪ <i>Neral</i>	8.065	25.71	nd	nd	nd	nd	nd	nd
17	▪ <i>Geraniol</i>	8.563	31.24	3.162	3.970	23.23	11.499	9.693	8.431
18	▪ <i>Nerol</i>	8.749	5.608	nd	nd	nd	nd	nd	nd
19	▪ <i>Neryl acetate</i>	8.973	8.857	nd	nd	nd	nd	nd	nd
20	▪ <i>Citral</i>	9.021	nd	nd	nd	nd	nd	nd	nd
21	▪ <i>Eugenol</i>	9.346	1.157	nd	nd	nd	nd	nd	nd
22	▪ <i>β-elemen</i>	9.812	nd	nd	nd	nd	nd	nd	nd
23	▪ <i>Methyl eugenol</i>	10.915	4.042	nd	nd	nd	nd	nd	nd
24	▪ <i>β-Caryophellene</i>	12.168	1.431	9.331	8.390	8.289	5.719	5.181	5.016
25	▪ <i>α-bergamotene</i>	13.190	nd	nd	nd	nd	nd	nd	nd
26	▪ <i>α-guaiene</i>	13.273	nd	nd	nd	nd	nd	nd	nd
27	▪ <i>α-humulene</i>	13.566	nd	nd	nd	nd	nd	nd	nd
<b>Fresh weight (g)</b>				0.28	0.275	0.673	0.441	0.382	0.389
<b>Third leaf top area (cm<sup>2</sup>)</b>			3.25	1.25	1.12	5.25	1.63	2.56	4.87
<b>% for main compounds</b>			42.21	24.66	24.47	88.225	46.365	42.86	44.707

C : control ; A : 0.5 g stevia with sucrose; B: 0.5 g stevia without sucrose; C: 1g stevia without sucrose; D,E and F : 2,4 and 6 ml/l algae filtrate

The essential oil from explants cultured on MS medium containing 0.5 g/l stevia powder with sucrose 25 g/l varied of essential oil contents of myrcene, *p*-cymene, linalool, geraniol and  $\beta$ -caryophellene which recorded the highest level 9.33%.

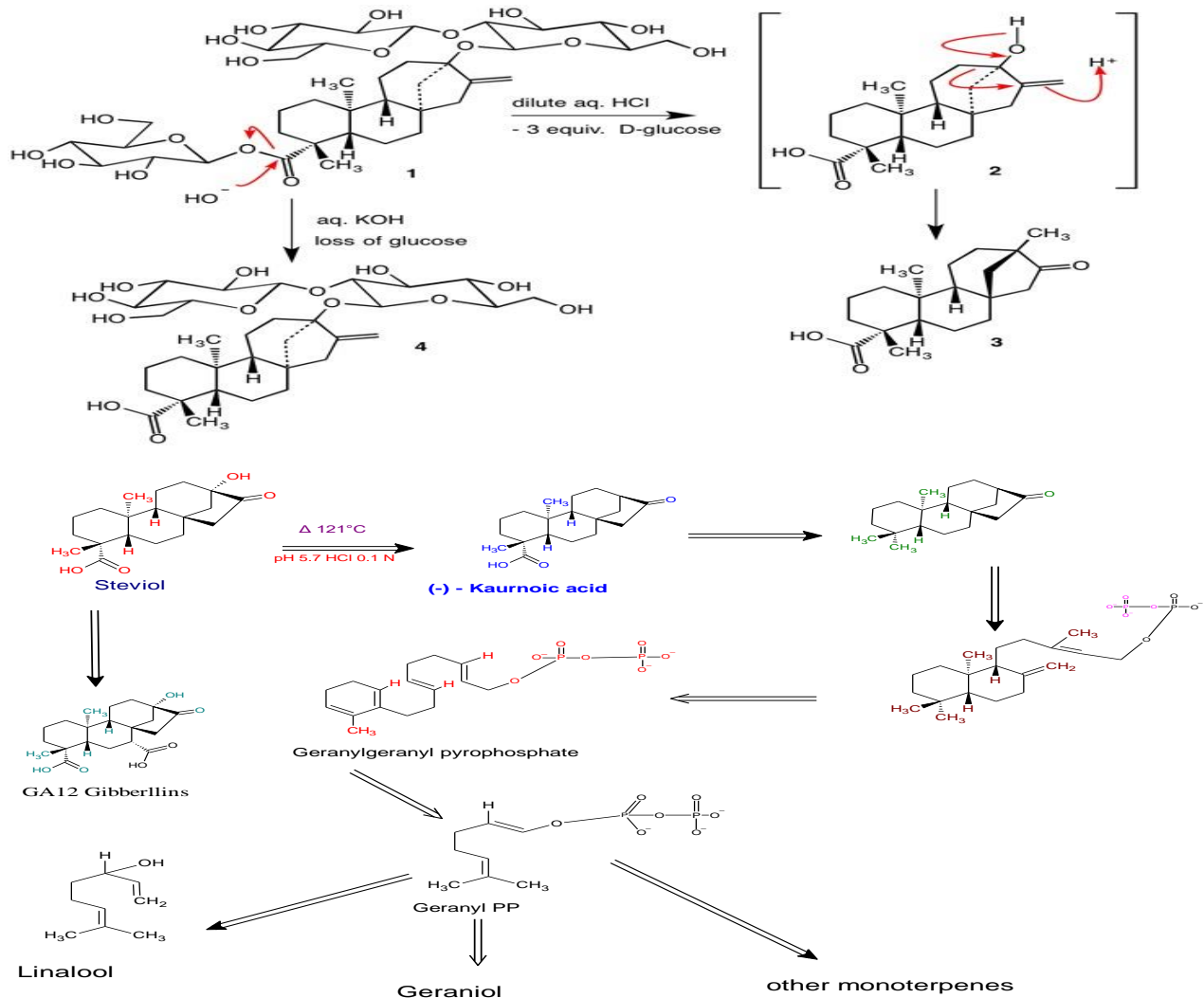
Also, increasing leaves area from 3.25 cm<sup>2</sup> for control to 5.25 cm<sup>2</sup> for explants cultured on media containing 1 g/l stevia without sucrose. These treatments gave the highest value of the main components of oil reached to 88.22 % of oil contents. The increasing leaves area gave the highest dry weight of dry matter 0.673 g.



**Fig. 4** GC-MS chromatogram showed the main components in *Ocimum citriodorum* explants cultured on media containing A : 0.5 g stevia with sucrose; B: 0.5 g stevia without sucrose; C: 1g stevia without sucrose; D,E and F : 2,4 and 6 ml/l alginate filtrate

The oil of *Ocimum spp.* was the subject of former studies (Akgül 1989; Baritiaux *et al.* 1992; Ravid *et al.* 1997; Martins *et al.* 1999; Keita *et al.* 2000). It was previously reported (Keita *et al.* 2000) that the oil of *O. basilicum* contained monoterpene linalool eugenol (10%) (69%), thymol (2%) and (E)- $\alpha$ -bergamotene (3%). Some compounds observed like linalool (45.7%), eugenol (13.4%), methyl eugenol (9.57%) and fenchyl alcohol (3.64%) were reported to be the main components of the previously analyzed materials as shown by (Akgül 1989). Khatri *et al.* (1995) who found that methyl chavicol (87.3%), linalool (5.4%), methyl eugenol (1.5%),  $\beta$ -caryophyllene (2.4%),  $\alpha$ -pinene (1.0%),  $\beta$ -pinene (0.8%), limonene (0.5%) and camphene (0.2%). Marotti *et al.* (1996) stated that a few compounds such as linalool, methyl chavicol and eugenol as main components of *O. basilicum*. On another hand, the major compounds observed were linalool and methyl chavicol according to Lachowicz *et al.* 1996.

The results published on the chemical composition of *Ocimum citriodorum* oil reveal that linalool (52.7%) represents the most important compound in the genus followed by eugenol (9.1%) and bornyl acetate (1.9%). In our search, we found different results, according to literature findings, as concerns the major compounds. The finding results differences may be probably due to different treatments, environmental and genetic factors, different chemotypes and the nutritional status of the plants as well as other factors that can influence the oil composition. The effect of stevia leaves at 1.0 g/l filtrate without sucrose increase linalool to the highest level (37.01 %) indicate to glycoside bonds was break by hydrolysis and release terpene molecule that breaking to isoprene units that increase essential oil components. These results show that *Ocimum citriodorum* are remarkably variable species. Certainly, the high quantities of methyl eugenol and geranyl acetate, respectively, making them a most interesting species from the economic point of view. Lawrence (1988) proposed several chemotypes based on the composition of the essential oils.



**Fig. 5 Non-enzymatic hydrolysis sugars from stevia and produce monoterpenes (suggested By Dr sheriff saied)**

There is a non-enzymatic mechanism for removing the three glucose moieties of stevioside **1**. Unfortunately, dilute mineral acid (HCL 0.1 N) hydrolysis not only removes the glucose entities but also effects Wagner-Meerwein rearrangement of the aglycone steviol **2** to isosteviol **3**.<sup>1</sup> While vinegar (5% aqueous acetic acid) is a weaker acid than hydrochloric acid, Also, heating that used in cooking media and sterilization process responsible to avoid the rearrangement of steviol **2**. Exposure of stevioside **1** with aqueous NaOH effects saponification of the ester group leading to steviobioside **4**. After removing 3 units of glucose from steviobioside steviol terpene was liberation and broken to acyclic polyterpenoid under high pressure and heat to Kauronic acid **3** this compound loss carboxylic group by decarboxylase enzyme and turn into Kaurene **2**. The cycle was open and bind by pyrophosphate group to form (-) – copalyl pyrophosphate that rearrangement to geranylgeranyl pyrophosphate (GGPP). Two units of geranyl removed phosphate group and isolate to form linalool and geraniol this two compound was observed in the GC -MS analysis which records 37.01 % linalool and 23.23 % geraniol with media supplemented with 0.1 % stevia filtration without adding sucrose to media. On the other hand, the growth of the explant cultured on MS media containing stevia filtrate or powder was improved more than algae because kauronic acid transformed to gibberellins that enhanced the plant growth.

### COMPETING INTERESTS

Authors have declared that no competing interests exist.

### REFERENCES

- Abd El-Baky Hanaa, El-Baz Farouk, El-Baroty, S. Gamal (2013). *Spirulina* Species as a Source of Carotenoids and A-Tocopherol and Its Anticarcinoma Factors. *Biotechnol.* 2: 222-240.
- Abd El-Baky Hanaa, El-Baz Farouk, El-Baroty, S. Gamal (2009). Potential Biological Properties of Sulphated Polysaccharides Extracted From The Macroalgae *Ulva Lactuca* L. *Acad J Cancer Research* 2: 1-11.
- Abou-Arab A.E., Abou-Arab A. A., Abu-Salem M. F. (2010). Physicochemical assessment of natural sweeteners steviosides produced from *Stevia rebaudiana* Bertoni plant. *African Journal of Food Science* 4 (5): 269- 281.
- Ahmad N., Fazal H., Abbasi B., Farooq S. (2010). Efficient free radical scavenging activity of *Ginkgo biloba*, *Stevia rebaudiana* and *Parthenium hysterophorus* leaves through DPPH (2,2-diphenyl-1-picrylhydrazyl). *International Journal of Phytomedicine*, 2(3): 231–239.
- Akgul A. (1989). Volatile oil composition of sweet basil (*Ocimum basilicum* L.) cultivating in Turkey. *Nahrung*, 33: 87–88.
- Allahverdiyev, A.; Duran N.; Ozguven, M. and Koltas, S. (1994). Antiviral activity of the volatile oils of *Melissa officinalis* L. against Herpes simplex virus type-2. *Phytomedicine*, 11: 657–661.
- Ankamwar, B.; Chaudhary, M. and Sastry, M. (2005). Gold Nanotriangles Biologically Synthesized using Tamarind Leaf Extract and Potential Application in Vapor Sensing. *Syn. React. Inorg. Met.* 35: 19-26.
- Bahtiyarca B.R. and Cosge B. (2006). The essential oil of lemon balm (*Melissa officinalis* L.) its components and using fields. *Journal of Faculty of Agricultural*, 21: 116–121.
- Baritoux, O.; Richard, H.; Touche, J. and Derbesy, M. (1992). Effects of drying and storage of herbs and spices on the essential oil. Part I. Basil, *Ocimum basilicum* L. *Flavour Fragr. J.*, 7: 267–271.
- Benzie, F. and Watchel-Galor, S. (2011). *Herbal Medicine: Biomolecular and Clinical Aspects*, CRC Press, USA (465) 30.
- Brandle, J.E. and Rosa, N. (1992). Heritability for yield, leaf: stem ratio and stevioside content estimated from a landrace cultivar of *Stevia rebaudiana*. *Canadian Journal of Plant Science* 72 (4):1263-1266.
- Braz-de Oliveira, A.J.; Correia Gonçalves, R.A.; Cantuaria Chierrito, T.P., Müller dos Santos M., Mera de Souza L., Gorin P.A.J. (2011). Structure and degree of polymerisation of fructo oligosaccharides present in roots and leaves of *Stevia rebaudiana* (Bert.) Bertoni. *Food Chemistry* 129 (2): 305–311.
- Burdock, G.A.; Wang, W.(2017). Our unrequited love for natural ingredients. *Food Chem. Toxicol.*, 107, 37–46.
- Chaidedgumjorn A, Toyoda H, Woo ER, Lee KB, Kim YS et al. (2002) Effect of (1→3)- and (1→4)-Linkages of Fully Sulfated Polysaccharides on their Anticoagulant Activity. *Carbohydrate Res* 337: 925-933.
- Colla LM, Reinehr CO, Reichert C, Costa JAV (2007) Production of Biomass and Nutraceutical Compounds By *Spirulina platensis* Under Different Temperature and Nitrogen Regimes. *Bioresource Technol* 98: 1489-1493.

Co-Stat Graphics, software ver. 4. 1999

- Dikbas N., Bagci E., Kotan R., Cakmakci R., Ozer H., Mete E., Erdogan G. (2010): Comparative antibacterial activities and chemical composition of some plants' oils against *Salmonella enteritidis*. *Research on Crops*, **11**: 118–124.
- Dillon, J.C., Phuc, A.P. and Dubacq, J.P. (1995). Nutritional value of the alga *Spirulina*. *World Rev. Nutr. Diet.* **77**, 32–46.
- Dwivedi, A.D. K. Gopal, (2010). Biosynthesis of Silver and Gold Nanoparticles Using *Chenopodium album* Leaf Extract. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, **369**, 27-33.
- El-Sayed, I.H., Huang, X and M.A. El-Sayed. 2005. Surface plasmon resonance scattering and absorption of anti-EGFR antibody conjugated gold nanoparticles in cancer diagnostics: applications in oral cancer. *Nano Lett.*, **5** (5):829-834
- Geuns, J. M.C. (2003). Safety of Stevia and stevioside. *Recent Res. Devel. Phytochem.* **4** : 75-88
- Jacobs M.B (1973). *The chemical analysis of foods and food products*; 3rd edition Robert Krieger Publishing Co., New York.
- Keita, SM., Vincent C., Schmit J.P., Belanger A. (2000): Essential oil composition of *Ocimum basilicum* L., *O. gratissimum* L. and *O. suave* L. in the Republic of Guinea. *Flavour Fragr. J.*, **15**: 339–341.
- Khatri, M., Nasir, M., Saleem, R. and Noor, F. (1995). Evaluation of Pakistani sweet basil oil for commercial exploitation. *Pakistan J. Sci. Ind. Res.*, **38**: 281–282.
- Kannan, N. and Subbalaxmi, S. (2011). Biogenesis of nanoparticles-a current prospective. *Rev. Adv. Mater.Sci.*, **27**, pp.99–114.
- Kim, I.; Yang, M.; Lee, O. and Kang, S. (2011). The antioxidant activity and the bioactive compound content of *Stevia rebaudiana* water extracts. *LWT – Food Science and Technology*, **44** (5): 1328-1332.
- Lachowicz, K.J., Jones, G.P., Briggs, D.R., Bienvenu, F.E., Palmer, M.V., Ting, T. and Hunter, M. (1996): Characteristics of essential oil from basil (*Ocimum basilicum* L.) grown in Australia. *J. Agr. Food Chem.*, **44**: 877–881.
- Lahariya A.K. and Rao J.T. (1979): *In vitro* antimicrobial studies of the essential oil of *Cyperus scariosus* and *Ocimum basilicum*. *Ind. Drugs*, **16**: 150–152. LAWRENCE B.M. (1985): A review of the world production of essential oil. *Perfum. Flavor.*, **10**: 2–16.
- Lawrence B.M. (1988): In: Lawrence B.M., Mookheyee B.D., Willis B.J. (eds): *Developments in Food Sciences, Flavors and Fragrances: a World Perspective*. Elsevier, Amsterdam.
- Mallikarjuna, K.; Narasimha, G; Dillip, B.; Shreedhar, C.; Sree-Lakshmi, B.; Reddy, B. and Raju, B. (2011). Green synthesis of silver nanoparticles using *ocimum* leaf extract and their characterisation, *Digest J. Nanomater. Biostruct.*, **6**(1):181–186.
- Mao, W.; Zang, X.; Li, Y. and Zhang, H. (2006). Sulfated Polysaccharides From Marine Green Algae *Ulva Conglobata* And Their Anticoagulant Activity. *J Appl Phyco* **18**, 9-14.
- Marotti, M., Piccaglia, R. and Giovanelli, E. (1996). Differences in essential oil composition of basil (*Ocimum basilicum* L.) Italian cultivars related to morphological characteristics. *J. Agr. Food Chem.*, **14**: 3926–3929.
- Martins, A.; Salgueiro, L.; Vila, R.; Tomi, F., Caniguel, S., Casanova, J., Proença, D. Cunha, A. and Adzet T. (1999). Composition of the essential oils of *Ocimum canum*, *O. gratissimum* and *O. minimum*. *Planta Med.*, **65**: 187–189.

- Mishra P., Singh R., Kumar U., Prakash V. (2010). *Stevia rebaudiana*- A magical sweetener. Global Journal of Biotechnology & Biochemistry 5 (1): 62–74.
- Mitchell, H.L. (2006). Sweeteners and Sugar Alternatives in Food Technology, Blackwell Publishing Ltd., Oxford, UK (412) 341-347.
- Moradkhani H., Sargsyan E., Bibak H., Naseri B., Sadat-Hosseini M., Fayazi-Barjin A., Meftahizade H. (2010): *Melissa officinalis* L., a valuable medicine plant. A review. Journal of Medicinal Plants Research, 4: 2753–2759.
- Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant, 15: 473-497
- Nie, X, Shi, B, Ding, Y. and Tao, W (2007) Preparation of a Chemically Sulfated Polysaccharide Derived From *Grifola Frondosa* and Its Potential Biological Activities. Inter J Biologi Macromolecules 39: 228-233.
- Philip, D. C. Unni, S.A. Aromal, V.K. Vidhu, (2011). *Murraya Koenigii* leaf-assisted rapid green synthesis of silver and gold nanoparticles. Spectrochim. Acta A 78 (2): 899 - 892.
- Ravid, U., Putievsky, E., Katzir, I. and Lewinsohn E. (1997). Enantiomeric composition of linalool in the essential oils of *Ocimum* species and in commercial basil oils. Flavour Fragr. J., 12: 293–296.
- Riaz, M., Khalid, M.R., Hanif, M. and Chaudhary, F.M. (1994): Extraction and GC/MS analysis of the essential oil of *Ocimum basilicum* . Pakistan J. Sci. Ind. Res., 37: 362–364.
- Richmond, A. (1998). Spirulina. In: Borowitzka MA, Borowitzka LJ, editors. Microalgal biotechnology. Cambridge: Cambridge University Press, 85–119.
- Saha, S. P.D. Ghosh and C. Sengupta (2010). Efficient method for micropropagation of *Ocimum basilicum* l. Indian J. Plant Physiol., Vol. 15, No. 2, (N.S.) pp. 168-172.
- Sahelin, R. and Gates, D. (1999). The Stevia Cookbook, Avery, New York, USA (181): 2-29.
- Shanker, S.S. Rai, A. B. Ankamwar, A. Singh, A. Ahmad and M. Sastry (2004) Biological synthesis of triangular gold nanoprisms. Nat Mater. 3(7):482-488.
- Shukla, S., Mehta A., Bajpai V., Shukla S. (2009). *In vitro* antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert. Food and Chemical Toxicology 47 (9): 2338–2343.
- Singh, S. and Rao, G. (2005). Stevia: The herbal sugar of 21st Century. Sugar Technology, (71): 17–24.
- Sony, B., Trivedi, U. and Madamwar, D., A. (2008). Novel Method of Single Step Hydrophobic Interaction Chromatography for the Purification of Phycocyanin from *Phormidium fragile* and its Characterization for Antioxidant Property. Bioresource Technology, 99, No. 1, 188.
- Thomas J., Glade M. (2010). Stevia: It's not just about calories. The Open Obesity Journal 2: 101–109.
- Wandersleben, T.; Morales, E.; Burgos-Díaz, C.; Barahona, T.; Labra, E.; Rubilar, M.; Salvo-Garrido, H. (2018). Enhancement of functional and nutritional properties of bread using a mix of natural ingredients from novel varieties of flaxseed and lupine. Food Sci. Technol., 91, 48–54.
- Yilmaz Mukremin , H. Turkdemir, Mehmet Akif Kilic, Bulent Ulug. (2011). Biosynthesis of silver nanoparticles using leaves of *Stevia rebaudiana*. Fuel Process. Materials Chemistry and Physics 130(3):1195-1202
- Yang J, Du Y, Huang R, Wan Y, Wen Y (2005) The Structure Anticoagulant Activity Relationships of Sulfated Lacquer Polysaccharide Effect of Carboxyl Group and Position of Sulfation. Inter J

Biologi Macromolecules 36: 9-15.

Zarrouk. C. 1966, Contribution a l'étude d'une Cyanobacterie: Influence de Divers Facteurs Physiques et Chimiques sur la Croissance et la Photosynthese de *Spirulina maxima* (Setchell et Gardner) Geitler. Ph. D. Thesis. University of Paris, France.

تأثير سبيرولينا بلاتينسيس وستيفيا ريبوديانا على النمو وإنتاج الزيوت العطرية للرياحان

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تم فحص مكونات الزيت الطيار المستخلصة بواسطة إيثيل الإيثر من النباتات الناتجة من مزارع الانسجة للرياحان الليمونى المستزرعة على بيئات تحتوي على مسحوق أو راشح من طحلب الاسبيرولينا او نبات الاستيفيا بواسطة GC-MS. تمت إضافة طحلب سبيرولينا بلاتينسيس في بيئة زراعة الانسجة بجرعات (0.5 ، 1.0 و 2.0 جم / لتر) والراشح بتركيز 10 ، 15 ، 5 % على التوالي. أيضا ، تمت إضافة الأوراق الجافة لنبات الاستيفيا في صورة مسحوق في 0.5 و 1.0 و 1.5 غرام / لتر والراشح بتركيز من 0.05 ، 0.1 و 0.15 % ، على التوالي حيث تم اضافته حجم 2 ، 4 ، 6 مل / لتر من راشح الاسبيرولينا و 5 ، 10 ، 15 مل / لتر من راشح الاستيفيا . تم فحص FTIR و XRD لكلا من المواد المستخدمه كاضافات للبيئة. سجلت الاجزاء النباتيه المتكشفة المنزرعة على البيئات التي تحتوي على ناتج ترشيح الاسبيرولينا بمعدل 4مل / لتر و 6 مل / لتر على قيد الحياة بنسبة 100% ومتوسط نمو الفريعات 0.84 و 0.92 ؛ ومتوسط طول الفريعات 1.98 و 2.33 ومتوسط عدد الاوراق لكل فريعه 2.60 و 8.66 ، على التوالي. أعطت نمو نباتي متطور على بيئات النمو التي تحتوي على راشح الاستيفيا 5 و 10 مل / لتر أيضا أفضل النتائج ولكن تحولت بيئات النمو إلى اللون البني (++) و (+++) ، على التوالي. تم الحصول على 12 مكوناً أساسياً يمثل 94.52% من زيوت *O. citriodorum*. يحتوي الزيت ، كمكونات رئيسية ، جرانيال (31.24 % ) ، لينالول (8.29 % ) ، استراجول (4.98 % ) ، نيرال (25.71 %) و نيتيل أسينات (8.857 %).



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