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Enhancement of secondary metabolites production from callus of Catharanthus reseus (L.)G.Don through elicitation with seaweed extract

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Abstract :

This study was conducted in the postgraduate laboratory at the Biology department of education college at Al-Iraqia university, which aimed to induce callus from the leaves of the Catharanthus roseus (L.)G.Don Plant on Murashige and Skoog medium (MS) supplemented with different concentration of Thidiazuran (TDZ) (0.0,0.5, 1.0, 1.5, 2.0 mg.L-¹) and 2,4-dichlorophenoxy[MF1] -acetic acid (2,4-D) (0.0,1.0,2.0,3.0,4.0 mg. L-¹).The optimum combination was selected, which was 1.0 mg.L-¹ TDZ+ 2.0 mg.L-¹ 2,4-D five weeks of culture basied on the highest fresh weight of callus (739.3 mg).The present study included the multiplication of callus on different concentrations seaweed extract Ascophyllum nodosum L.(0.0,0.5,0.1,1.5,2.0 ml.L-¹) , where the concentration 1.5 ml.L-¹ is significant over the rest of the concentrations with the highest average fresh weight of 951.3 mg with It also gave a significant increase in the relative growth rate of callus reached to 12.6 mg.Day-¹, Alkaloids (Catharanathine, Vindoline,Vincristine and Vinblastine) were estimated in the callus growing on all concentrations using in the HPLC technique, the experiments were conducted to Duncan's multiple test , significant differences among means were determined under level of probability (0.05).

Keywords:Catharanthus roseus, Callus , *Ascophyllum nodosum* , Plant Tissue Culture, Alkaloids.

تحفيز إنتاج القلويدات من كالس نبات عين البزون وتحفيزها بمستخلص الطحلب البحري

آية حافظ زامل ، أ.د سهام عبدالرزاق سالم ، أ.م.د زبيدة عبداللطيف إسماعيل

مستخلص

اجريت هذه الدراسة في مختبر الدراسات العليا في قسم علوم الحياة/ كلية التربية / الجامعة العراقية بهدف استحثاث الكالس من اوراق نبات عين البزون Catharanthus roseus(L.)G.Don على وسط MS مجهزاً بمنظمي النمو TDZ (0.0، 0.5، 1.0، 1.5، 2.0) ملغم.لتر-¹) و 2,4-D (0.0، 1.0، 2.0، 3.0، 4.0) ملغم. لتر-¹) اختيرت التوليفة المثالية والتي كانت 1.0 ملغم.لتر-¹ TDZ+ 2.0 ملغم.لتر-¹ 2,4-D بعد خمسة أسابيع من الزراعة اعتمادا على أعلى وزن طري للكالس تم الحصول عليه من بين المعاملات الأخرى والذي بلغ 739.3 ملغم .كما تضمنت الدراسة تنمية الكالس على تراكيز مختلفة من المحفز الحيوي مستخلص الطحلب البحري Ascophyllum nodosum.L (0.0 و5.0 و1.0 و1.5 و2.0 ز.لتر-¹ (حيث تفوق التركيز 1.5 مل.لتر-¹ معنويا على بقية التراكيز بأعلى معدل وزن طري بلغ 951.3 ملغم. كما أعطى زيادة معنوية في معدل النمو النسبي للكالس بلغت 12.6 ملغم.يوم-¹ ، قدرت القلويدات Vinblastine و Vindoline و Vincristine و Catharanathin في الكالس النامي على جميع المعاملات باستعمال تقنية HPLC وصممت التجارب وفق اختبار دنكن متعدد الحدود، وحددت الفروقات المعنوية بين المتوسطات تحت مستوى احتمال (0.05).

الكلمات المفتاحية: نبات عين البزون، الكالس، مستخلص الطحلب البحري، زراعة الأنسجة النباتية، القلويدات.

Introduction

The rose periwinkle or Madagascar periwinkle Catharanthus roseus (L.)G.Don Plant belongs to the family Apocynaceae, is an evergreen perennial herbaceous plant with abundant branches, sometimes up to 100 cm long .It's leaves are oval or oblong in shape, opposite, and its flowers are rose- periwinkle with reddish eye. Seeds are small brown to black in color and are the main source of plant reproduction (2). The plant is medically famous because it contains more than 100 alkaloids including Vincristine and Vinblastine that are used to treat cancer diseases (19).During their growth, plants synthesize many metabolic compounds that use in growth and development, which are known primary metabolic compounds. Whearas, other compounds that are not directly involved in the growth of plants but are considered defensive compounds against various diseases or adaptive means to different environmental conditions, which are known secondary metabolites, which are of medical value (16)(11). medical compounds are extracted from plants, this requires the cultivation these plants at large areas. This process is very expensive. Therefore,researchers attention turned towards tissue culture technology،Tissue culture technology is one of the biotechnologies that has played and continues to play an important role in serving humans, especially in the field of propagation of several types of plants in huge numbers, free of pathogens and similar to the mother plant in a relatively short time and at any time of the year (4). Interest in the technique of plant tissue culture and its adoption as an alternative to traditional cultivation in the production of medicinal compounds has increased, and plant growth regulators increase the levels of secondary compounds and their accumulation in the plant (8).

The production of active compounds from the C.roseus plant was carried out by callus cultures , as previous studies have shown the success of this method using various types and concentrations of plant growth regulators, represented by auxins and cytokines (13). The path of callus formation from a piece of plant tissue grown on a nutrient medium can be divided into three stages: stimulation, division and differentiation as growth regulators control the emergence and development of callus as they have an ac-

tive role in regulating the processes of cell division, cell elongation and differentiation of callus tissue (1). The marine alga Ascophyllum nodosum is a rock alga that is widespread in abundance on the northeast coast of North America and the northwest coast of Europe (14). The extract of this alga is used in conventional cultivation applications to stimulate growth as a rich source of bioactive compounds such as fluorotannin, multiple carbohydrates, mannitol, laminarin and fucoids (6). Seaweed is one of the brown algae prevalent in the North Atlantic, used in the form of an extract for agricultural purposes and is considered an antioxidant because it contains alpha-tocopherol (10).Therefore, the current study relied on use seaweed extract as a bio elicitor in plant tissue culture to stimulate the growth of C.roseus callus and the production of some medical active alkaloids from it.

Materials and Methods

Experiments of the current study were carried out in the laboratory of postgraduate at the biology departmentof education college at Al-Iraqia university from October 2022 to May 20 23.

Fresh leaves were collected from C. roseus plant. These samples were transferred to the laboratory and placed under running water for 60 minutes and then sterilized with mercury chloride (HgCl2) at a concentration of 0.1% for 5 minutes. Then itThenthet were washed with sterile distilled water three times for a minute each time, after which the leaves were cut to a lengthof1cm.

The explants were cultured in a MS medium explants supplemented with 0.0,0.5,1.0,1.5,2.0 mg.L-^{1} of TDZ and 0.0,1.0,2.0,3.0,4.0 mg.L-^{1} of 2,4-D for callus induction, Data were recorded five weeks after cultivation, the fresh and dry weight of callus were calculated and the best combination of plant growth regulators was determined, 1.0 mg.L-^{1} TDZ+ 2.0 mg.L-^{1} 2,4-D , callus was subcultured on concentrations of seaweed extract Ascophyllum nodosum (0.0,. 0.5 , 1.0, 1.5, 2.0 ml.L-^{1}) Data were recorded three weeks after subculturing and the fresh weight of callus was measured to find out the effect of the algae extract on the growth and multiplication of the callus .The relative growth rate of callus was measured according to the following equation (3):

The relative growth rate of callus (mg.day-1) =weight at the appointed time-primary weight/primary weight.

Growning of plant cultures:

The explants were cultured in glass vials with a screw cover and containing of 10 ml of MS nutrient medium at PH 5.7 and sterilized with an autoclave at a temperature of 121 ºC under a pressure of 1.05 Kg. cm-¹ for 15 minutes. Then,Cultures were transferred to the growth room with a maximum illumination of 1000 lux and a daily sequence of 8 h of darkness and 16 h of light at a temperature of 2± 25 °C.

Measurement of antioxidant activity using the compound Diphenyl-2-picryl-hydrazyl (DPPH).

The callus samples(treated with concentrations of seaweed extract and control) and leaves sample of the parent plant were dried separately in an electric oven for 24 h under a temperature of 70 ° C, and then grind with a mortar and pestle.

Following the method of (15) to measure the ability of callus extract to oxidize DPPH mixed the extracts of samples of the parent plant and callus of C.roseus in an amount of 0.4 ml (3) mL(0.1 mm) of methanol DPPH solution with shaking for one minute, then mixture was incubated for 30 minutes in the dark at 37 °C.The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. The control absorbence was measured by replacing the plant extract with methanol and the percentage of oxidation of DPPH was calculated according to the following equation:

Antioxidant activity (%): control sample- treatments / control sample *100.

Isolation and Detection of Alkaloids using HPLC :

Extracts were prepared from the leaves of the parent plant and the growing callus on the seaweed extract by taking a weight of 3 g of each sample after drying them in the electric oven at a temperature of 45 ºC , then the dry samples were crushed with a mortar to obtain a fine powder to dissolve it in sulfuric acid (H2SO4) at a concentration of 3% at room temperature for two hours. The solution was filtered via filter papers (with a diameter of 2.5 micrometers), then the supernatant was taken and added to it 2.0 ml of ammonium hydroxide (NH4OH) at a concentration of 25% and pH=9.5) , then loaded into the separation column where the alkaloids were separated

with a solution of methylene chloride (CH2CL2) (6 ml.gm-^{1} of the suspended extract).The extract was evaporated to dry using a stream of liquid nitrogen with bubbles, then 1 ml of methanol was added to it and 20 micrometers were withdrawn from it for analysis using HPLC . .

Alkaloids were detected and estimated according to the method of (9), where the HPLC apparatus of the Shimadzu 10 AV-LC Germany type was used for the purpose of determining the retention time and the peak area for both standard compounds (Vindoline, Vincristine, Vinblastine, and Catharanathine) and the solutions of the samples under study, where the column separation of type C18 250 × 4.6 mm and a particle diameter of 5 micrometers), the mobile phase consists of acetic acid acetic acid (5%) and cinnamic acid and a flow rate of 1 ml.min-^{1} and read UV at a wavelength of 275 nm andatatemperatureof30ºC.

Table (1) shows the sequence of standard compounds, the time of their retention, the peak area and the concentration of the standard solution . The alkaloids above were measured in the treatments samples and then the beak area of the sample was compared with the known beak area of the standard compounds. The concentrations of the required compounds were calculated according to the following equation.

Sample concentration (mg.ml-^{1}) =Sample peak Area / standard compound peak area ×standard solution concentration × dilution coefficient.

Table (1) : The sequence of standard compounds of alkaloids, retention time, the peak area and concentration.

Sequencing	The standard of compounds	Time of retention (minute)	Peak area (Microvolt)	Standard solution concentration(25 microgram.ml-^{1})
1	Vindoline	2.780	101791	25
2	Vincristine	3.862	116074	25
3	Catharanathine	4.952	100773	25
4	Vinblastine	6.012	118236	25

Statistical Analysis

The data experiments included in the study were analyzed statistically and conducted to Duncan's multiple test for significant at probability level of 0.05 (7).

Results and Discussion:

The results of table (2) showed the effect of interaction between plant growth regulators TDZ and 2,4-D in the rate of fresh and dry weights of the callus of C.roseus plant. The highest rate of fresh weight of the callus was 739.3 mg at the interaction of 1.0 mg. L^{-1} TDZ + 2.0 mg. L^{-1}. 2,4-D (Table2) (Fig-1). Whereas the lowest fresh weight of the callus was 143.4 mg at 4.0 mg.L^{-1} of 2,4-D .white the control treatment did not respond to form of callus.

The results also showed that there were significant differences between the interaction of TDZ and 2,4-D in the dry weight rate (mg) of callus. The highest dry weight rate was 76.2 mg 1.0 mg.L^{-1} TDZ + 2.0 mg.L^{-1} is 2,4-D , and the lowest dry weight of the callus (23.0 mg) at 2.0 mg.L^{-1} of 2,4-D. The control treatment, no weight was given to the callus. This is consistent with (5) where they found that TDZ increased the dry and soft weight rate of Stevia plant.

Table (2): Effect of concentrations of TDZ and 2,4-D and their interaction in the rates of fresh and dry weight (mg) of callus induced from the leaves of the C.rouse plant five weeks after culturing on MS medium .

TDZ (mg.L-1)	2,4-D (mg.L-1)	Fresh weight (mg)	Dry weight (mg)
0.0	0.0	0.0 h	0.0 e
0.0	1.0	322.2 e	41.0 bcd
0.0	2.0	159.3 g	23.0 bc
0.0	3.0	206.6 f	32.0 bcd
0.0	4.0	143.4 g	29.0 bcd
0.5	0.0	273.2 f	44.0 bcd
0.5	1.0	431.2 d	51.2 ac
0.5	2.0	356.2 e	49.0 bcd
0.5	3.0	334.5 e	35.0 bcd
0.5	4.0	643.3 b	66.3 a
1.0	0.0	446.1 d	60.2 a
1.0	1.0	514.3 c	56.0 ac
1.0	2.0	739.3 a	76.2 a
1.0	3.0	300.5 e	40.0 bcd
1.0	4.0	501.7 c	65.2 a
1.5	0.0	251.3 f	54.0 ad
1.5	1.0	388.1 e	61.1 a
1.5	2.0	298.2 f	46.1 bcd
1.5	3.0	277.0 f	36.0 bcd
1.5	4.0	605.9 b	70.3 a
2.0	0.0	561.6 c	68.0 a
2.0	1.0	476.8 d	51.0 ac
2.0	2.0	436.8 d	51.1 ac
2.0	3.0	338.5 e	40.0 bcd
2.0	4.0	280.1 f	43.4 bcd

*Numbers with similar characters within the same column do not differ significantly from each other according to the Duncan's multiple test ($p < 0.05$).

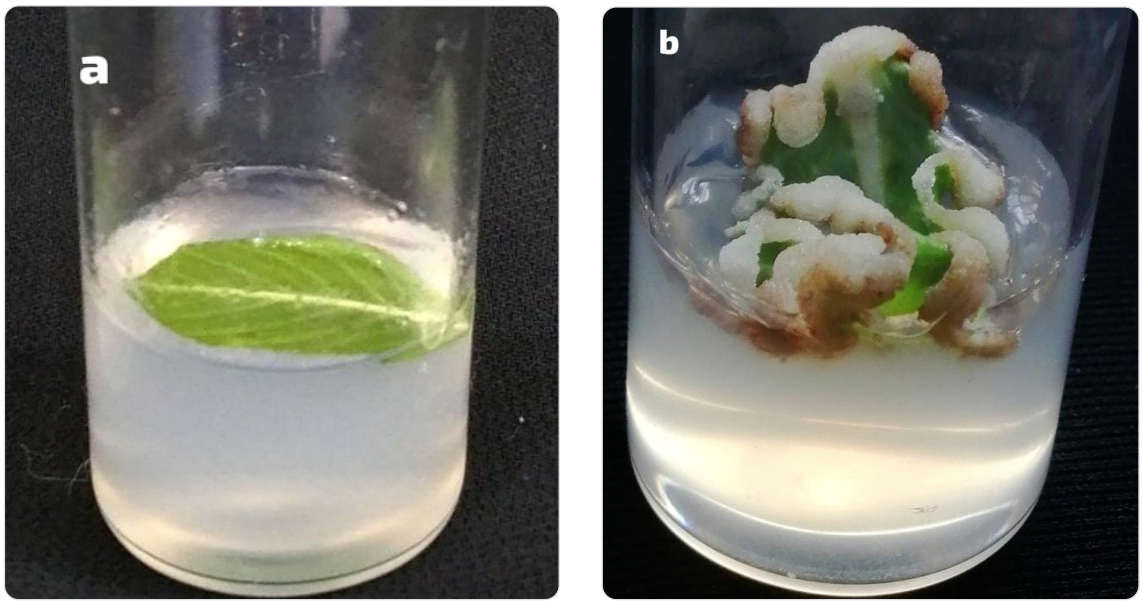

Figure (1): Effect of TDZ and D-2,4-D in interaction (1.0 mg.L - ¹ TDZ+2.0 mg L-¹ 2,4-D) on callus induction from the leaves of the C.roseus plant A – cultivation of Explant on the MS medium supplied with growth regulators B-callus induced on optimum interaction TDZ and 2,4-D .

The effect of seaweed extract (Ascophyllum nodosum) as bio elicitor on the growth and multiplication of the callus of the C.roseus plant.

Results of the effect of different concentrations of seaweed extract on the growth and multiplication of callus indicated that the highest rate of fresh weight was at 951.3 mg with the concentration 1.5 ml. L-¹. (Table 3)(Figure-2).Whereas the concentration of 0.5 ml.L-¹ gave the lowest rate of fesh weight (871.1 mg) that did not differ significantly from the control(863.6 mg). . Interesting, results indicated no significant differences among the different concentrations of seaweed extract on the relative growth rate of callus . for example, the concentration 1.5 ml.L-¹ gave a the highest relative growth rate(12.6 mg.day-1), which not differ significantly with control (9.4 mg.day-¹)(Table 3). Results of the effect of seaweed extract concentrations on the antioxidant activity using DPPH radical revealed that the antioxidant activity elevated with increased concentrations. However, there were not significant differences across all seaweed extract concentrations toward antioxidant activity (Table 3).

Table(3) : Effect of different concentrations of seaweed extract (Ascophyllum nodosum) on the growth , multiplication and antioxidant activity of C.roseus callus cultured on MS medium and supplied with the optimum interaction of growth regulators (1.0 mg.L-1 TDZ + 2.0 mg.L-1 2,4-D) after three weeks of culture

Ascophyllum nodosum extract (ml.L-1)	Fresh weight (mg)	Relative growth rate of callus (mg.day-1)	Antioxidant activity (%)
0.0	863.6 c	9.4 ac	34.2 a
0.5	871.1 c	5.7 bc	36.4 a
1.0	895.0 c	8.4 ac	36.7 a
1.5	951.3 a	12.6 a	36.9 a
2.0	912.6 b	9.8 ac	36.8 a

*Numbers with similar characters within the same column do not differ significantly from each other according to the Duncan's multiple test ($p < 0.05$).

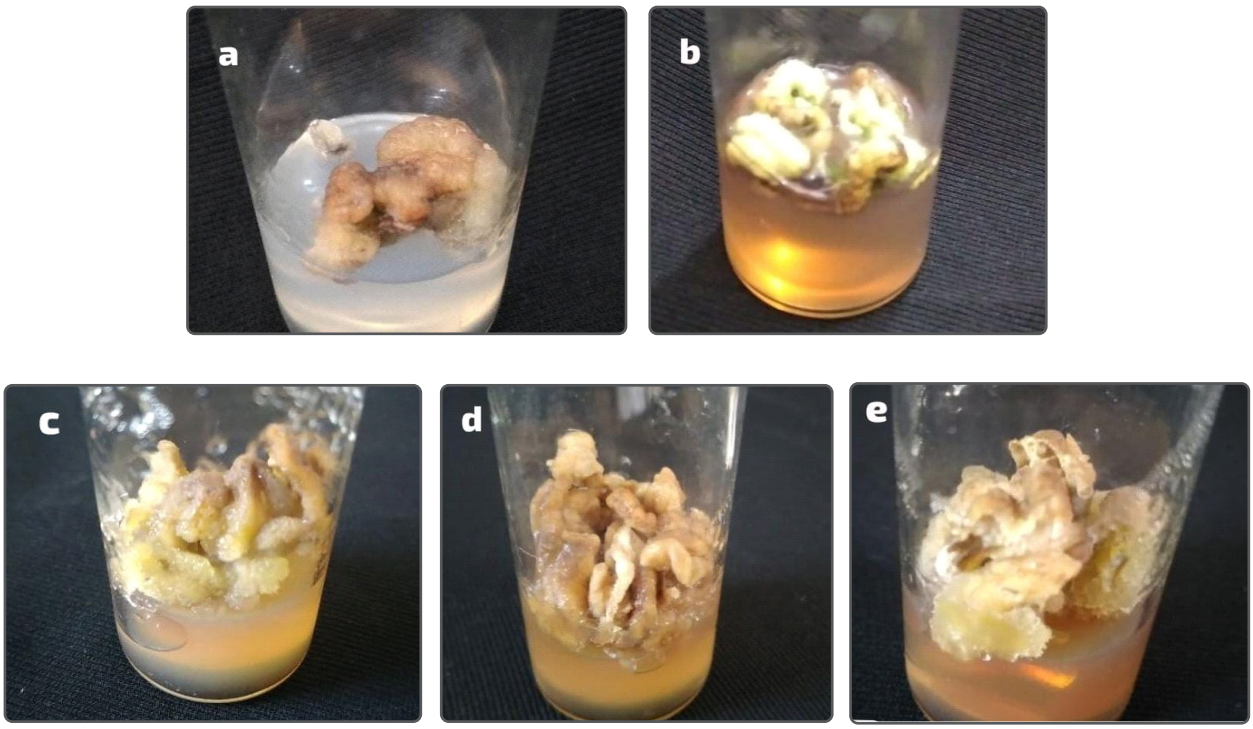

Figure (2): The effect of seaweed extract concentration on the growth and multiplication of callus of the plant Periwinkle. (a) Control . (B) 0.5 ml.Liter-1 . (C) 1.0 ml. Liter-1. (D) 1.5 ml.Liter-1 . (E) 2.0 ml. Liter-1

The effect of seaweed extract on the callus content of alkaloids :

Table (4) shows the positive effect of seaweed extract on the callus content of alkaloids found in the Periwinkle plant. Results showed that there were significant differences in the rate of alkaloids. Results noted that the original sample had low amounts of all the alkaloid compounds Vindoline (235.35 micrograms. Grams-1 dry weight), Vincristine (204.1 micrograms.Grams-1 dry weight), Catharanathine (483.04 micrograms.Grams-1 dry weight), Vinblastine (114.83 microgram.Gram-1 dry weight), while the comparison sample (recoreded an increase in concentrations of alkaloid compounds. A possible explanation for this might be the role of growth regulators in stimulating the synthesis pathways of active compounds in medicinal plants. Another possible explanation for this is that the addition of seaweed extract might be led to a significant accumulation of the alkaloid compounds with an increase in its concentration in the callus growth medium to reach the highest values of all four compounds at concentrations of 1.5 and 2.0 ml.L-1. the figures show the retention time(min) and the area under the curve for all standard vehicles and samples.

Table(4) : Effect of seaweed extract (*Ascophyllum nodosum*)concentrations on the callus content of alkaloids.

Study samples	Vindoline (microgram. gm-1- dry weight)	Vincristine (microgram. gm-1 dry weight)	Catharanathine (microgram.gm-1 dryweight)	Vinblastine (microgram. gm-1 dry weight)
Parent plant sample	235.35 e	204.1 e	483.04 e	114.83 e
Control sample	1114.06 d	702.27 c	1396.79 c	757.92 c
Ascophyllum nodosum 0.5 ml.L-1	1340.07 b	469.95 d	1297.11 d	609.74 d
Ascophyllum nodosum 1.0 ml.L-1	1292.54 c	707.72 c	1453.65 c	816.49 b
Ascophyllum nodosum 1.5 ml.L-1	1642.75 a	1093.70 b	1639.57 b	847.34 a
Ascophyllum nodosum 2.0 ml.L-1	1616.36 a	1226.41 a	1760.02 a	805.75 b

* Numbers with similar characters within the same column do not differ significantly from each other according to the Duncan's multiple test ($p < 0.05$).

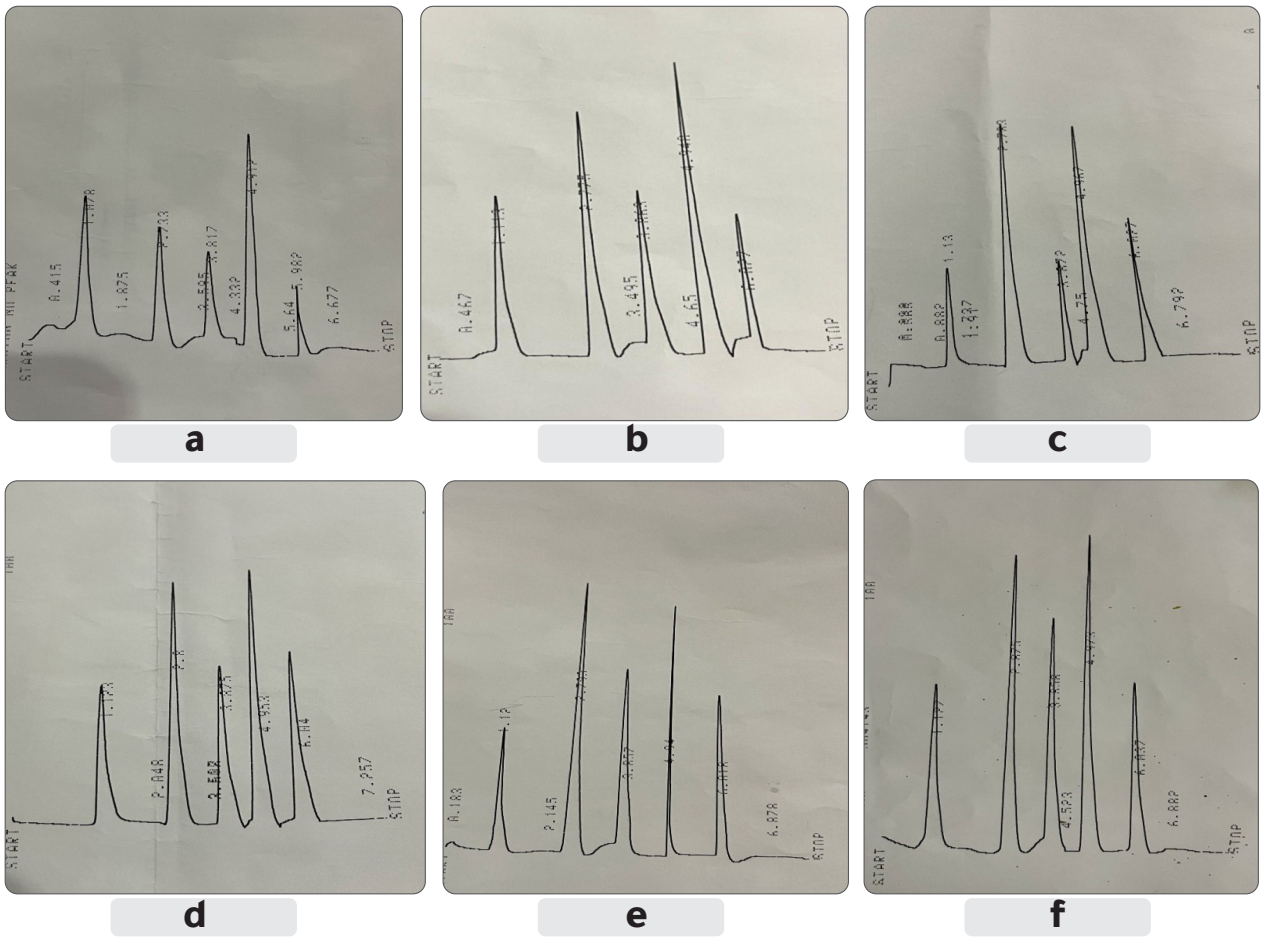

Figure (3): Alkaloid curves in the HPLC apparatus for callus growing on seaweed extract. a- Parent Plant . b- Control sample. c- Seaweed concentration of 0.5 ml.Liter-1 .d- Seaweed concentration of 1.0 ml.Liter-1. e - Seaweed concentration of 1.5 ml.L-1. f- Seaweed concentration of 2.0 ml.L-1.

Several reason could explain the different response of cells to callus formation. Firstly, the disparity in the concentrations of growth regulators inside the cells. Scondly, the external addition of growth regulators lead to a balance between the internal levels of hormones which lead to induce the division and callus formation (17). (18) used Growth Regulators 2,4-D and BA to produce effective compounds from the chia plant out of Vivo . Results noted that the different concentrations of seaweed extract (Ascophyllum nodosum) ,which considered one of the best media to induce callus growth, led to increase the effective antioxidant compounds in callus. Results of this the current study are consistent with (12) who tested the medicinal plant

Withania somnifera in the presence of different concentrations of seaweed extract. These results also reflect those of (10) who found that the addition of nano seaweed extract has helped in the growth and multiplication of fenugreek callus and increased its content of secondary metabolites.

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