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Elicitation of alkaloids production from callus cultures of *Catharanthus roseus* (L.) G. Don using yeast extract

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Abstract :

This study was conducted in the Laboratory of higher studies at the Department of Biology - College of Education - Al-Iraqia University, which aimed to induce Callus from the leaves of *Catharanthus roseus* (L.) G. Don plant on MS medium that equipped with growth regulators TDZ (0.0,.0.5, 1.0, 1.5, 2.0 mg.L^{-1}) and 2,4-D (0.0,1.0,2.0,3.0 and 4.0 mg. L^{-1}).The typical combination was chosen, (1.0 mg.L^{-1} TDZ + 2.0 mg.L^{-1} 2,4-D) after five weeks of culture depending on the highest rate of fresh weight of the callus (739.3mg) obtained among other treatments. The study was also included the development of callus on different concentrations of the yeast extract (0.0 , 100,200,300 ml.L^{-1}), where the concentration of 100 mg.L^{-1} was significant overcame on the rest of the concentrations with the highest average fresh weight of 967.7 mg. It also gave a significant increase in the relative growth rate of callus reached to 10.3 mg.day $^{-1}$. Alkaloids (Vinblastine, Vindoline,Vincristine, Catharanathin) were estimated in the callus using HPLC technique . The study experiments were designed according to a complete randomized design (CRD) with ten replicates , and the averages were compared using the least significant difference (L.S.D) and Duncan's multiplied test under the probability level of 0.05.

Key words :*Catharanthus roseus*(L.)G.Don, Callus, Yeast extract, Plant tissue culture, Alkaloids.

تحفيز إنتاج القلويدات من مزارع الكالس في نبات عين البزون *Catharanthus roseus* L.) G.Don) باستخدام مستخلص الخميرة

آية حافظ زامل د. سهام عبدالرزاق سالم د. زبيدة عبداللطيف إسماعيل
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مستخلص

أجريت الدراسة الحالية في مختبر الدراسات العليا التابع لقسم علوم الحياة/ كلية التربية –الجامعة العراقية حيث هدفت إلى استحثاث الكالس من اوراق نبات عين البزون Catharanthus roseus (L.)G.Don على وسط MS مجهز بمنظمي النمو TDZ (0.0 و0.5 و1.0 و1.5 و2.0 ملغم.لتر–¹) و2,4–D (0.0و0.5 و1.0 و1.5 و2.0 ملغم.لتر–¹). واختيرت التوليفة المثالية (1.0 ملغم.لتر–¹ +2.0 TDZ ملغم.لتر–¹ 2,4–D) بعد خمسة أسابيع من الزراعة اعتمادا على أعلى وزن طري للكالس (739.3 ملغم) تم الحصول عليه من بين المعاملات الأخرى.كما تضمنت الدراسة تنمية الكالس على تراكيز مختلفة من المحفز الحيوي مستخلص الخميرة yeast extract (0.0 و100 و 200 و 300 ملغم.لتر–¹) حيث تفوق التركيز 100 ململغم.لتر–¹ معنويا على بقية التراكيز بأعلى معدل وزن طري بلغ 967.7 ملغم. كما أعطى زيادة معنوية في معدل النمو النسبي للكالس بلغت 10.3 ملغم. يوم–¹ . قدرت القلويدات (Vincristine,Catharanathine , Vindoline ,Vinblastine) في الكالس المستحث في باستعمال HPLC، وصممت تجارب الدراسة وفق التصميم العشوائي الكامل بعشرة مكررات، وقورنت المتوسطات باستخدام اختبار دنكن متباين الحدود تحت مستوى احتمال 0.05 .

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

Introduction

The plant *Catharanthus roseus* (L.) G. Don considered to be one of the species of the Oleander family (Apocyanaceae), which is an evergreen perennial plant with abundant branches and sometimes reaches a length of 100 cm. Its leaves are oval or oblong in shape and flowers are in the form of an eye, so it is called locally the cat eye, and its seeds are small in size, black in color and is a source of its reproduction [1]. It is a floral plant, which is native to the island of Madagascar and then spread widely all over the world. the plant is also famous for its medicinal value, producing about 130 alkaloids, the most important of which is vincristine and vinblastine, which are used in the treatment of types of cancers and diabetes[2], in addition, its roots contain the alkaloids serpentine, ajmalicine, vindoline and catharanathine, which are used in the treatment of blood diseases [3].

These substances are medically important and are produced in small quantities depending on the physiological status of plant [4]. Plant tissue culture technology represents an effective source for the production of secondary metabolites in a short time [5] by producing large numbers of plants at the lowest possible cost and for a short period of time. Some of the factors that help in the success of tissue culture for medically important plants are the type of plant and its part necessary for cultivation and chemical components in the nutrient medium in addition to physical conditions [6].

Researchers usually resort to adding natural plant extracts to the nutrient medium for the development of plant tissue cultures instead of manufactured chemicals because these extracts contain natural growth regulators and thus greatly help to stimulate the growth of plant tissues, such as yeast extract, orange juice, and others [7]. Yeast extract is a safe biotic elicitor that plays an important function in plant growth because it is rich in essential nutrients, especially cytokinins, which increase plant growth [8].

Materials and methods

Preparation of the planting medium and cultivation of plant tissue

The MS nutrient medium [9] was used in a powder form from HI Media Ltd. to induce callus from the leaves of the *Catharanthus roseus* plant, where 4.9 g of MS powder medium was weighed and 30 $g.L^{-1}$ of sucrose

was added to it., which is an important source of carbon and energy, the components were mixed well without heat first to dissolve the components well, then growth regulators TDZ and 2,4-D were added to the mixture and then the volume was supplemented to a liter with distilled water, and the medium pH was adjusted to 5.7 ± 0.1 with a solution of 1.0N of hydrochloric acid (HCl) or sodium hydroxide solution (NaOH). Agar (Agar-Agar) was added to the medium in a concentration of 7 $g.L^{-1}$. Then, the medium was put on the hot plate magnetic stirrer and heated to boiling point to melt the agar and homogenize the nutrient medium, then pour the medium directly into the Pyrex glass tubes at 10 ml per tube and closed with its own lid and sterilized in the autoclave at a temperature of 121 °C with a pressure of 1.5 Kg. cm^{-2} for 20 minutes. After that, the tubes containing the sterilized nutrient medium were removed from autoclave and left to cool at room temperature until they were used in culturing of explants.

Fresh leaves were taken from the young shoots of *Catharanthus roseus* plant and gently washed with liquid soap and water to remove suspended dirt and dust, left under running tap water in the conical flask for 30 min. to ensure good cleanliness, then the leaves were immersed in a solution of mercury chloride ($HgCl_2$) at a concentration of 0.1% for five minutes with constant stirring, then washed with sterile distilled water three times for a minute each time to remove traces of the sterile substance, then the leaves were transferred after sterilization to sterile petri dishes, so they were ready to be cultured on the MS medium, the cultures were incubated at a temperature of 25 ±2.0 °C, and illuminated with an intensity of 1000 Lux for 16h of light and 8h of darkness. The observation on cultures were daily recorded.

Callus induction

For the purpose of inducing callus from the leaves of *C. roseus* plant and to obtain the required amount of callus, the ideal combination of growth regulators was used(1.0 $mg.L^{-1}$ TDZ + 2.0 $mg.L^{-1}$ 2,4-D) in callus induction five weeks after culturing and used in yeast extract experiments, as different concentrations of yeast extract (0.0, 100, 200 and 300 $mg.L^{-1}$) were prepared and added to the MS medium containing the above ideal combination of growth regulators. The callus induced by the ideal combination was transferred to the culture medium containing yeast

concentrations for the purpose of growth and multiplication of the callus, and then according to the fresh weight of the callus after three weeks of culturing under the same conditions previously indicated by temperature and light.

Antioxidant activity test using free root 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

Callus (grown on different concentrations of yeast extract and control) and the leaves of the parent plant were dried separately in an electric oven for 48 hours under a temperature of 70°C, and then was ground with a ceramic mortar.

A method of [10] was followed to measure the ability of callus extract to oxidize the compound DPPH, where the extracts of samples of the plant and callus of the were mixed in an amount of 0.4 ml with 3. 6 ml (0.1 mmol) of methanol DPPH solution with shaking for one minute, then incubated the mixture for 30 min. in the dark at 37 °C, the absorbance of the resulting solution was measured at 517 nm using a spectrophotometer, the absorbance of the control 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 sample /control sample* 100

Isolate and Detection of alkaloids using HPLC :

Extracts were prepared from the leaves of the parent plant and the growing callus on the biotic elicitor (yeast extract), where a 3.0 g was taken from each sample after drying in the electric oven at a temperature of 45 °C, then the dry samples were crushed with ceramic mortar to obtain a fine powder to dissolve it in sulfuric acid (H_2SO_4) at a concentration of 3% at room temperature for two hours, then, the solution was filtered by filter papers (with a diameter of 2.5 µm), take the supernatant and add to it 2.0 ml of ammonium hydroxide (NH_4OH) 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 (CH_2Cl_2 at 6 $ml.g^{-1}$ from 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 µl were withdrawn from it for analysis using HPLC.

Alkaloids were detected and estimated according to the method of [11], where the HPLC apparatus of the Shi-

madzu 10AV-LC Germany type was used for the purpose of determining the retention time and an area of the peak area of both standard compounds (Vindoline, Vincristine, Vinblastine and Catharanathine) and the solutions of the samples under study, using the column separation (column type C18 250 ×4.6 mm and the diameter of the molecules 5 µm, the mobile phase consists of acetic acid acetic acid (5%) and cinnamic acid and a flow rate of 1 $ml.min^{-1}$ and UV reading was at a wavelength of 275 nm at a temperature of 30°C.

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

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

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

Sequencing	The standard compound	Time of retention (min.)	Peak area (µV)	Standard solution concentration (25 µg)
1	Vindoline	2.780	101791	25
2	Catharanathine	3.862	116074	25
3	Vincristine	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 [12].

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 callus induced from leaves of the *Chatharanthus roseus* plant, where the highest rate of fresh weight was 739.3 mg at the interaction of 1.0 $mg.L^{-1}$ TDZ + 2.0 $mg.L^{-1}$ 2,4-D compared to other interactions (Figure-1), whereas the lowest fresh weight was 143.4 mg at

the interactions 0.0 mg.L^{-1} TDZ + 4.0 mg.L^{-1} 2,4-D. The control treatment did not respond to form of callus.

Results of the same table showed that there were significant differences among the interaction of the concentrations of growth regulators TDZ and 2,4-D in the dry weight rate (mg) of callus, where the highest dry weight rate (76.2 mg) was achieved in the interaction of 1.0 mg.L^{-1} TDZ + 2.0 mg.L^{-1} 2,4-D, while the lowest dry weight of the callus (23.0 MG) was at the interaction of 0.0 mg.L^{-1} TDZ + 2.0 mg.L^{-1} 2,4-D. Control treatment did not give any weight of the callus.

Table (2): Effect of TDZ and 2,4-D interactions on the fresh and dry weight (mg) rates of callus induced from the leaves of the *C. roseus* 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 at a probability level of 0.05.

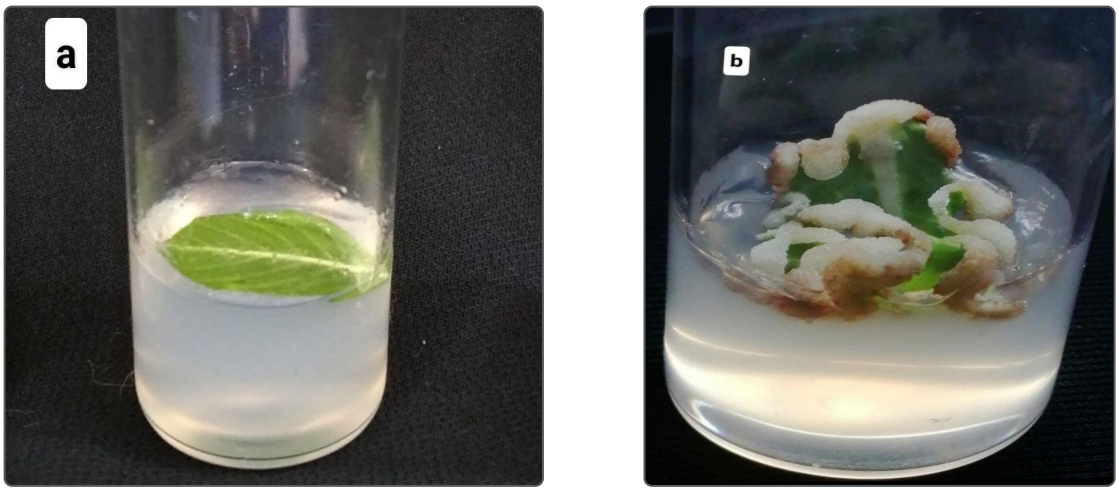

Figure (1): Effect of TDZ and 2,4-D growth regulators on callus induction from the leaves of the plant. a - Culturing of the explant on the MS medium equipped with growth regulators. b – callus induced on the optimal combination of growth regulators (1.0 mg. L^{-1} TDZ + 2.0 mg.L^{-1} 2,4-D).

Effect of yeast extract on growth and multiplication of callus of *C. roseus* growing on growth regulators in the optimal combination

The results of the statistical analysis in Table (3) and Figure (2) showed the effect of different concentrations of yeast extract on the growth and multiplication of callus induced from leaves of the *C. roseus* plant. The concentration of 100 mg.L^{-1} was significant on all concentrations by recording the highest average fresh weight of callus at 967.7 mg, while the control gave an average of 863.6 mg. Results also showed a decrease in fresh weight rates of callus as the concentration of yeast extract increases . Also, results of the same table indicated the effect of yeast extract concentrations on the relative growth rate of callus, with the highest rate reaching 10.3 mg. day^{-1} at a concentration of 100 mg.L^{-1}, but it was not a significant increase in comparison with the rest of the concentrations. From the same table also, results indicated the effect of yeast extract concentrations on the antioxidant activity using the free radical DPPH, where the activity increased by increasing various concentrations of yeast extract, but it was not significant for all concentrations.

Table (3): Effect of yeast extract concentrations on fresh weight rate (mg), relative growth rate (mg.day^{-1}) and antioxidant activity with the use of DPPH (%) of the *C. reseus* callus three weeks after culturing on the MS medium equipped with the optimal combination of growth regulators (1.0 mg.L^{-1} TDZ + 2.0 mg.L^{-1} 2,4-D).

Yeast extract (mg.L^{-1})	Fresh weight (mg)	Relative growth rate of callus (mg.day^{-1})	Antioxidant activity (%)
0.0	863.6 b	9.4 a	34.2 a
100	967.7 a	10.3 a	35.1 a
200	806.2 b	8.2 a	35.5 a
300	600.8 c	6.9 a	35.9 a

*Numbers with similar characters within the same column do not differ significantly from each other according to the Duncan's multiple test at a probability level of 0.05 .

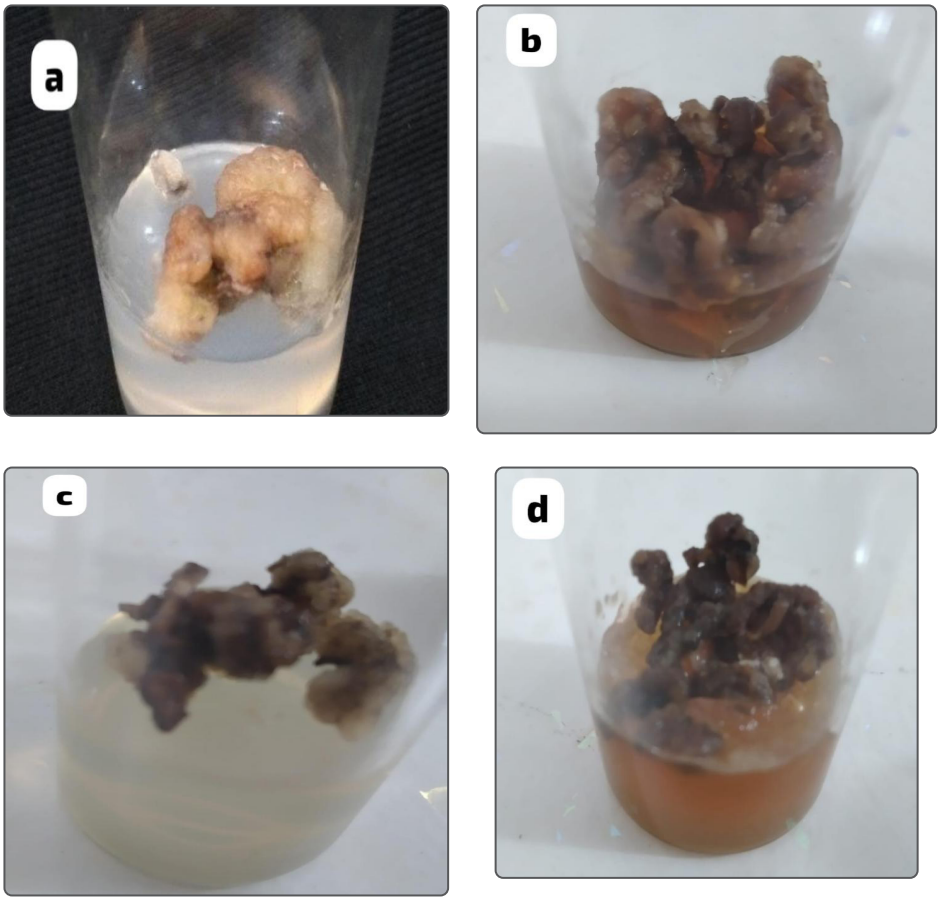

Figure (2): Effect of yeast extract concentrations on the growth and multiplication of callus. a – Control callus. b-Callus grown on concentration 100 mg.L^{-1}. C - Callus grown on concentration of 200 mg.L^{-1}. D-Callus grown on concentration of 300 mg.L^{-1}.

It is noted from the above results that the different concentrations of yeast extract have clearly affected the growth and multiplication of callus derived from leaves of *C. roseus* plant due to the fact that yeast extract is a natural source of each of niacin, thiamine, pyridoxine and riboflavin, in addition to containing growth regulators such as cytokinins that responsible for stimulating the process of cell division and elongation, which leads to a balance between vital and physiological processes and increasing the fresh weight and relative growth rate of callus [13].

The effect of yeast extract on the callus content of alkaloids

The results in Table (4) showed the significant differences in the increase in the rate of alkaloids under the influence of different concentrations of yeast extract, where it is noted that the original sample contained low amounts of all the alkaloid compounds under study (Vindoline 235.35 µg .g^{-1} dry weight, Vincristine 204.1 µg.g^{-1} dry weight, Catharanathine 483.04 µg.g^{-1} dry weight and Vinblastine 114.83 µg.g^{-1} dry weight), while the control sample (which included the optimal combination of growth regulators of 1.0 mg.L^{-1} TDZ + 2.0 mg.L^{-1} 2,4-D) gave marked increase in the concentrations of alkaloid compounds, indicating the role of growth regulators in stimulating the synthesis pathways of active compounds in medicinal plants, and the addition of yeast extract led to a significant accumulation of the studied 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 200 and 300 mg.L^{-1}. Figure (3) shows the retention time (min) and the area under the curve for both standard compounds and the samples under study.

Table (4): Effect of concentrations of yeast extract on *C. roseus* callus content of alkaloids three weeks after culturing on the MS medium equipped with the optimal combination of growth regulators (1.0 mg.L^{-1} TDZ + 2.0 + mg.L^{-1} 2,4-D).

The studied samples	Vindoline (µg.g^{-1} dry (weight	Vincristine µg.g^{-1} dry) (weight	Catharanthine µg.g^{-1} dry) (weight	Vinblastine µg.g^{-1} dry) (weight
Sample plant origin	235.35	204.10	483.04	114.83
Control sample	1114.06	702.27	1396.79	757.91
100	1133.70	757.90	1368.41	640.13
200	1592.88	1028.08	1417.85	673.80
300	1444.54	801.67	1462.31	757.48
P value	0.001	0.001	0.01	0.01

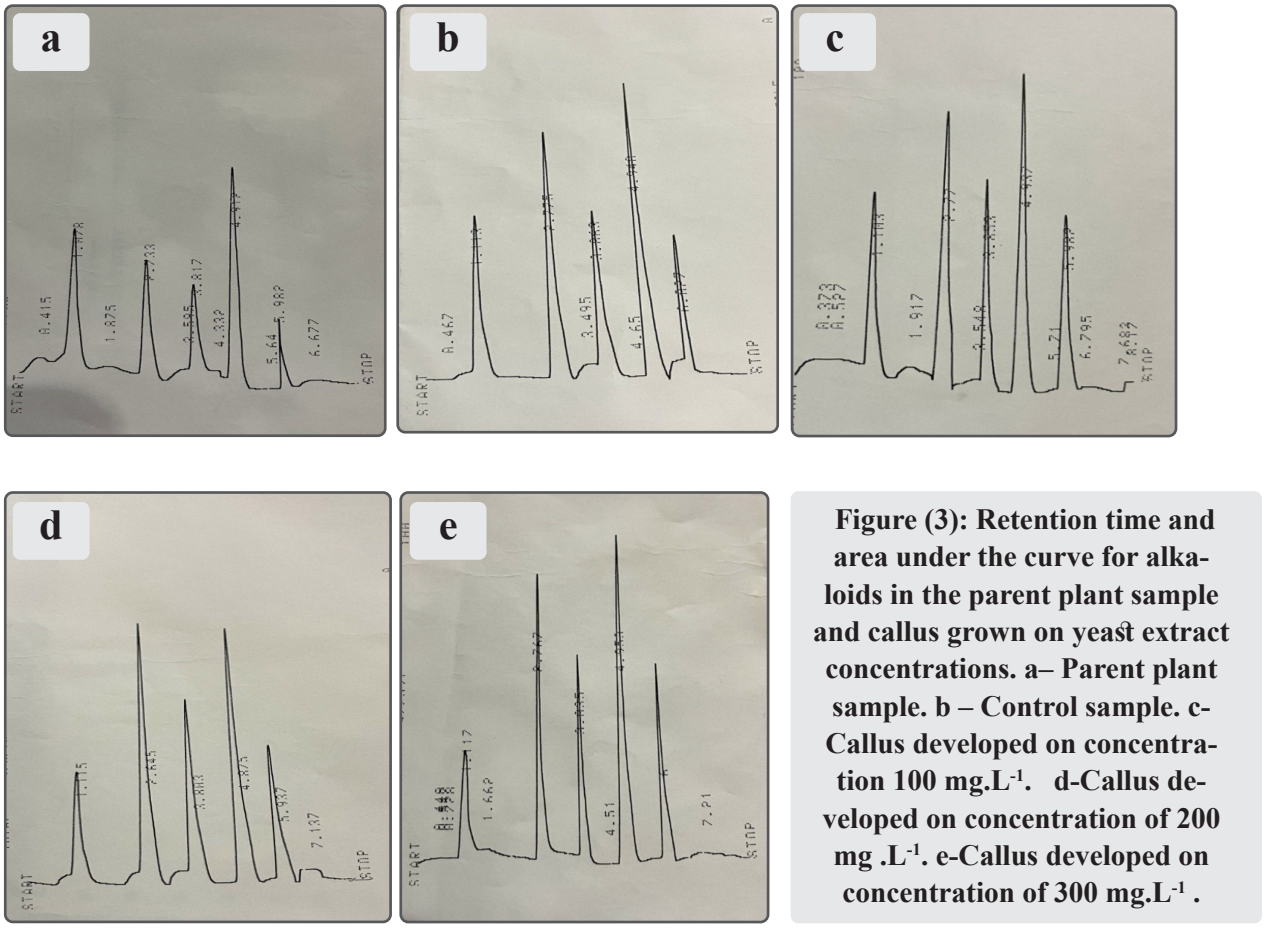

Figure (3): Retention time and area under the curve for alkaloids in the parent plant sample and callus grown on yeast extract concentrations. a– Parent plant sample. b – Control sample. c-Callus developed on concentration 100 mg.L^{-1}. d-Callus developed on concentration of 200 mg .L^{-1}. e-Callus developed on concentration of 300 mg.L^{-1} .

The reason for the different response of cells to callus formation is due to the disparity in the concentrations of growth regulators inside the cells and thus the external processing of regulators led to a balance between the internal levels of hormones inside the plant parts, which led to induction of division and callus formation [14]. This is consistent with [15] where the study was confirmed to obtain callus from the leaves of *C. roseus* plant when supplying the MS medium with different concentrations of growth regulator 2,4-D with silver nanoparticles and increasing the production of alkaloids in the induced callus. Also, this is consistent with [16] where the obtaining of callus from the leaves through using of the 2,4-D and BAP for inducing and multiplying of callus and increasing the production of alkaloids in it .The results also showed that different concentrations of yeast extract have a clear effect on the growth and multiplication of callus through a significant increase in the weight of soft callus in addition to its relative growth rate in addition to its significant effect on the antioxidant activity of the growing callus by increasing the concentrations of compounds with antioxidant effectiveness in callus. The results of this study are consistent with [17] in their study on the induction of black seed callus under the influence of different concentrations of yeast extract. Abadi, in his study [6] clarified the role of various concentrations of yeast extract and cytokinein 2ip in the growth of the embryonic callus of the *Phoenix dactylifera* L. date palm. The blond variety, where it was shown that the yeast extract had clearly affected the fresh weight of the callus. While these results did not agree with [18], who indicated in the results of their study on the ginger plant (*Zingiber officinale*) that the addition of different concentrations of yeast extract did not lead to a significant increase in fresh and dry weight.

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