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Vinblastine and Vincristine Alkaloids Production From Callus of *Catharanthus roseus* (L.) G. Don under Some abiotic factors

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Abstract

This experiment was conducted in Faculty of Science labs, Kufa University, carried out during 2013-2014 to study many experiments to induce callus tissues from leaves of (*Catharanthus roseus* (L.) G. Don *in vitro* using MS medium supplemented with Dichlorophenoxy actic acid (2,4-D) at difference concentrations (0.5, 1, 1.5) mg/l with the interaction of (BA) benzyl adenine at concentrations of (0.5, 1, 1.5) mg/l. Identically callus fresh weight, then use it in the next experiments and investigate the effect of abiotic factors (drought and sucrose) on vinblastine and vincristine production from callus tissue, and leaves of intact plant. This study include determination the catalase activity, proline and total soluble carbohydrates content as responsible to the variety of the stresses in callus as well as quantities and qualities determination of vinblastine and vincristine using TLC (Thin layer chromatography) and HPLC(high performance liquid chromatography). Results reveled that the highest fresh weight of callus (3.276) g was for (1mg/l 2,4-D + 1mg/l BA) combination treatment. However, the results showed that the additions of abiotic factors to the callus cultural medium cause difference results in fresh and dry weights of callus in accumulation phase, reducing the fresh and dry weight in all treatments expect sucrose 40 g/l treatment which gave increase in fresh and dry weight. Also, this results showed that all treatments increase total soluble carbohydrates, proline content and catalase enzyme activity with significant difference compared with control treatment and significant increase of alkaloids production with the superiority of (vinblastine and vincristine) content in Callus with abiotic factors stresses than the content in control treatment(without any factor).

Key Words: Culture media, callus, CAT, proline, vinblastine , vincristine

Introduction

For a long time medicinal plants are considered an important source of medicine value. We have relied on medicinal properties of plant species (Cragg and Newman ,2005) . There are 25% of drugs are derived from plants as a natural sources. 85% of traditional medicines involve the use of plant extract which contains bioactive compounds, these natural compounds also referred to as secondary metabolites, with wide diversity in chemical structures .They are economically important to human due to their multiple applications, such as Pharmaceuticals ,flavors, fragrance ,insecticides, dyes, food additives and toxins(Zhao *et al*., 2005).

These compounds are very useful for many living organisms and can make during development stages or in special conditions and have relation with physiological state of plants and environmental state . These plants produce these compounds to defense against unsuitable conditions (Verpoorte *et al*.,2002).

Catharanthus roseus (L.) G.Don. belongs Apocynaceae family extensively investigated medicinal plants comprises of 8 species (C. *roseus, C.coriaceus, C. lanceus, C.ovalis, C.pusillus, C.scitulus,C. trychophyllusand C. longifolius*)(Gupta *et al*.,2007).This plant known in trade as Vinca and is a pan tropical species occurring chiefly in India and Madagascar and now oriented in wide areas of world. In Iraq cultivated as a garden Plant, known (cat eyes) with rosy and whitey color (Semysim,2012) .The plant is known to produce more than 200 important compounds (mainly terpene indole alkaloids) the anticancer, vinblastine and vincristine in low amounts and depends on the physiological and development stage of the plant and in most cases has not been economically feasible (Zhuo and Verpoorte ,2007).These chemicals are often less, produced in very small amounts(less than 1% of total carbon) (Cragg and Newman,2005). A total of 1000 kg of leaves and stems materials is required to produce 1g and 20 mg respectively of produced (Taha *et al*.,2009). It is very important that the utilization of medicinal plants in traditional medicine was found to be effective ,cheap, and particular (Vander *et al*.,2004).These valuable alkaloids are produced in very small quantities within the aerial parts of plant. The high cost of isolating of the drugs has led researchers to use plant tissues culture technique to increase the alkaloids contents of *C. roseus in vitro*. The valuable antineoplastic alkaloids vinblastine and vincristine extracted from *C. roseus* have long been target of researchers trying to increase their production. The (TIAs) pathways are not only regulated tissues specifically and developmentally but are also affected by external biotic and abiotic factors(Facchini and St-Pierre, 2005).

These plants produce these compounds to defense against unsuitable conditions. Terpine indole alkaloids are responsible to abiotic factors. Tissues culture considered important technique to produced many essential compounds and to increase and induce draught tolerance. There are many useful properties obtained from tissues culture such as good products , rapidly of chemicals isolation , products in laporatory are similar to that in natural, avoiding all the harmful compounds which products beside others, and the ability to produce large quantities of important compounds by tissues culture (Facchini،2001). In this study we followed a variety strategies in tissues culture technique of *c. roseus,* inducing callus formation and increase vinblastine and vincristine production using abiotic factors. sucrose (40 ,50 , 60) g/l in MS medium as a sucrose stress and PEG (5 ,10 .15) % in MS medium as draught stress .

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Materials and methods

preparation of leaves and callus tissue for alkaloids extraction

C. roseus plants were collected from green houses of Agriculture college /Babylon university in January 2014. leaves of plant was extracted by Soxhelet (Continuous extraction) for 24 hours using ethanol 80 % (Harborne,1980) .Extraction was concentrated to dryness by rotary evaporation. However, Alkaloid compounds were extracted from callus tissues, according to AL-Hattab (2000) by taking 500 mg from dry callus and crushed well in mortar casserole, after drying wet callus in oven on 40 C for 24 hr with ethanol 80 % and ether 20 % at 1/4(v/v) ratio.

Detection of vinblastine and vincristine alkaloids by (TLC)

Silica gel plates (precoated) were used in 0.25 mm thickness and dimensions of 20 * 20 cm and placed in electric oven at a temperature of 120 C and left to cool , then placed raw leaves, callus extraction spots and standard solutions for alkaloids vinblastine and vincristine each alone by drops using (ammonium hydroxide: water: acetone) 25 % at ratio(3:7:90) used as separate solution for alkaloids separation, the separate spots were limited by eyes and by UV-light on 254 wave length (Al-Hatami,2006). For detection of separate alkaloid compounds by estimate R*f* as the following :

$$Rf = \frac{\text{Distance of compound}}{\text{Distance of solvent}}$$

Quantity and quality determination of vinblastine and vincristine in raw leaves extracts by (HPLC)

HPLC conditions were :Nucleosil 5 C18, column (250 * 4.6 mm ,5 um). Samples were eluted with (methanol/ acetonitril/ 0.025 M ammonium acetate/triethylamine by 15:40:45:0.1 , volume), at 1 ml/min., monitored at 280 nm (Zhao *et al*., 2001).

Standard solutions for calibration curves

The solutions were prepared by dissolve 1mg from pure vincristine in 9 ml HPLC grade water to obtain on 100 ug/ml and by serial dilutions to obtain on 10 , 1 ug/ml and then by the same method for vinblastine sulfates compounds concentrations in samples were estimated as the following :

$$\text{Alkaloid} = \frac{\text{Relative area for sample}}{\text{Standard Relative area for standard}} \times \text{alkaloid concentration in sample}$$

Medium preparation and explants sterilization

The leaves of *C. roseus* were washed by tab water and liquid soap several times to remove dust and dirt and transferred to laminar air flow cabinet for surface sterilization by submerged in ethanol 70 % with shaking for 30 second then washed three times by distilled water and were sterile by sodium hypochlorite 6 % for 20 minutes then washed three time by distilled water and prepared for culture. (MS) medium was used for Callus induction in the form of packets with weight of 4.4 grams, which are used for preparation of a liter from tissues culture medium . Sugar was added at 30 g/L and agar at 8 g / L , and control PH on 5.8 ± 2 then transferred to autoclave for sterilization on 121 C and pressure 1.04 kg/cm2 for 20 minutes and left till cool, and used for next experiments.

Callus inducing phase

The sterilize leaves were cut to parts with 1cm² area nearly ,cultured on MS medium supplemented with plant growth regulators 2,4-D in concentrations (0.5 ,1, 1.5) mg/l and BA in concentrations (0.5 ,1 , 1.5) mg/l as nine interaction, and transferred to growth chamber under suitable conditions on 25±2 C and photoperiod 16/8 hr. light/darkness on 1000 lux light intensity, for knowing the optimum comprise to form callus. After 40 days ,fresh weight was taken.

Accumulation phase : Preparation of abiotic factors

Drought treatments

PEG 4000 at (5 , 10 ,15)% were added to the MS in accumulation phase with the best treatment of plant growth regulators for callus induced, pH was exacted on 5.8 before agar adding, placed in sterile glasses tubes and transferred to autoclave for sterilization, left to cool, transferred to cabinet for planted callus 250 mg on MS medium , then transferred to growth chamber at the same previous conditions

Carbohydrates treatments (sucrose)

Sucrose at concentrations (30 , 40 , 50, 60) g/l were added to the MS medium in accumulation phase with the best treatment of plant growth regulators in the sterile glasses tubes, pH exacted on 5.8 before agar adding ,transferred to autoclave for sterilization left to cool, then transferred to cabinet for planted 250 mg callus on it, then transferred to growth chamber at the same previous conditions. .

Callus fresh and dry weights determination

The fresh weight was taken after 40 days complete growth of callus in accumulation phase by sensitive balance under sterile conditions. then the samples were dried for all treatments in oven on 40 C till the constant weight , and the dry weights were taken.

Catalase enzyme activity determination

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Catalase enzyme activity determination in leaves and callus extraction in inducing phase and callus extraction in accumulation phase for all treatments according to methods of (Aebi,1983).Determination the catalase activity at 240 nm by Uv-spectrophotometer.

$$\text{Catalase activity (unit)} = \frac{\text{Abs/min} * \text{Reaction volume}}{0.001}$$

Abs = the different between the first absorption and the second.

Min = reaction time. 2.4 ml = reaction volume . 0.001 = constant.

Proline determination :

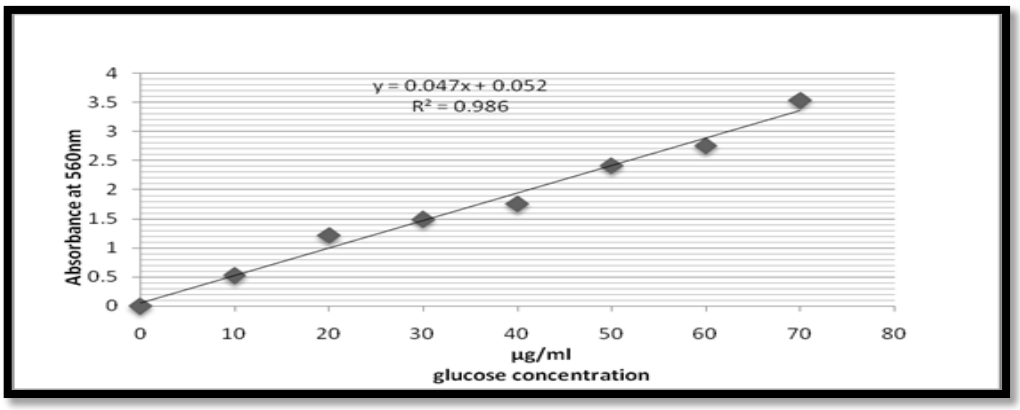
According to Bates *et al* (1973) method ,proline was determined in leaves and callus extraction in inducing phase and accumulation phase. The red toluene layer was reading by Uv- spectrophotometer at 520 nm while blank sample consist of 5ml toluene ,then wave length for variety concentrations of proline were measured for standard solution .Proline concentration calculate from the following :

$$\text{proline /g} = \frac{\text{Ug proline/ml} * \text{ml toluene} / 115.5 \text{ ug /u mole}}{\text{G sample} * 5}$$

Ugproline = proline concentration from standard curve.
Ml toluene = toluene volume = 5 ml . 115.5 ug/umol = constant
G sample = dry plant weight = o.5 g
Calibration curve for pure proline was prepared by use (2,4 , 6 , 8)% concentrations.

Total carbohydrates determination

Harbert *et al* (1971) method was used for determination of total carbohydrates in dry leaves powder and callus in inducing phase and callus in accumulation phase by phenol- sulfuric acid method for all treatments . Calibration curve for carbohydrates was prepared by use (20 ,40 , 60 , 80) ppm from pure glucose Fig(1).



Fig(1) : Calibration curve of glucose concentrations .

Quantity and quality determination of vinblastine and vincristine in callus using (HPLC)

HPLC technique was used by smart line, Knauer, system. 20 ul from extraction was injected in the column using constant and mobile phase according to Zhao *et al*(2001) vincristine and vinblastine were determination in callus cultures in accumulation phase for all treatments of abiotic factors .**Experimental design**

Statistical analysis was according to factorial complete random design for interaction treatments and just complete random design for others .10 replicates are used for each treatment and treatments mediums were tested by (L.S.D) the least significant design on improbability level 5 % according to system of SPSS (Levesque, 2007).

Results and discussion

Detection of vinblastine and vincristine in the leaves extraction and raw callus extraction by (TLC)

The result shown in Table(1)and figure (2 D&C) explained the presence of six compounds when examined by eyes and by Uv- light ray ,at 254 nm wavelength .R*f* values on TLC plates are (25,30,60,75,85,95) in leaves extraction and (25,35,65,75,85,95) in raw callus extraction. The results showed that R*f* for vinblastine and vincristine figure (2 A&B) (95 , 85) respectively are equal to two of raw and leaves extraction and they are another values with different values of R*f* to vinblastine and vincristine and not identified due to they are not standard solutions (Al-Hatamy,2006 and Smesim , 2012). Dragendorff reagent gave positive detection(Orang spot) on silica gel plates.

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Table (1) Detection of vinblastine and vincristine in leaves and callus using (TLC)

Extract	Compound number	Visible	Color under UV light	R*f*
Vinblastine(standard) A	1	white	yellow	0.95
Vincristine(standard) B	1	white	yellow	0.85
Leaves extract C	1	yellow	light yellow	0.25
	2	yellow	Light yellow	0.30
	3	yellow	Light yellow	0.60
	4	yellow	Light yellow	0.75
	5	brown	yellow	0.85
	6	brown	yellow	0.95
Callus extract D	1	yellow	Light yellow	0.25
	2	yellow	yellow	0.35
	3	yellow	Light yellow	0.65
	4	yellow	Light yellow	0.75
	5	brown	yellow	0.85
	6	brown	yellow	0.95

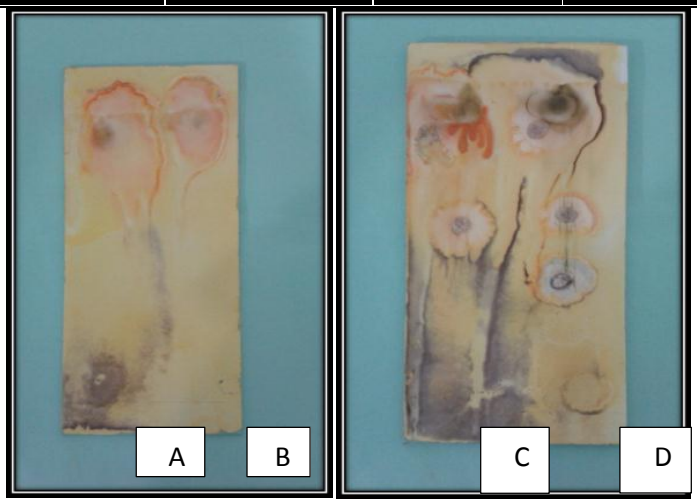
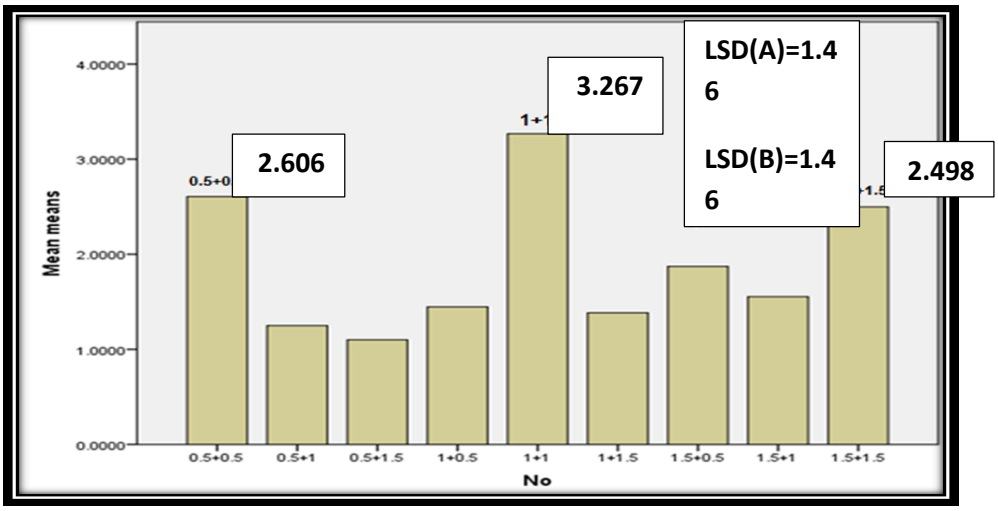


Fig (2)(TLC) Detection of vinblastine and vincristine in leaves and callus extract. (A .vincristine) (B. vinblastine) (C. Callus extract) (D. Leaves extract)

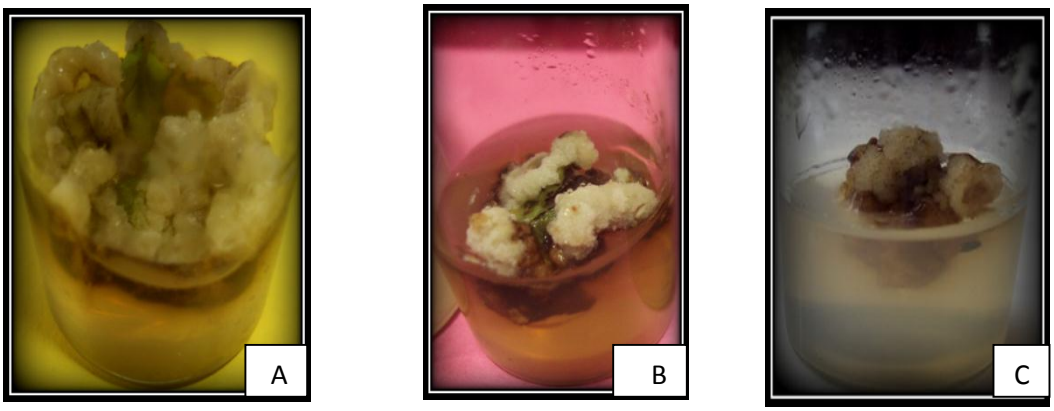
Effect of hormone combinations on callus fresh weight (g)

The results Fig(3) refer to the highest fresh weight (3.276) g was for (1mg/l 2,4-D + 1mg/l BA) combination treatment, Fig(4A) ,followed by (2.606) g for (0.5 mg/l 2,4-D+ 0.5 mg/lBA) combination treatment Fig(4B) and (2.498) g for (2mg/l 2,4-D

+ 2 mg/l BA) combination treatment ,Fig(4C) with significant difference among all treatments



Fig(3) Fresh weight for hormone combination of callus (g). LSD of auxins 0.146 and of cytokinin 0.146 and for interaction 0.0 84.



Fig(4) A: callus of (1mg/l 2,4-D+1mg/l BA) combination. B : callus of (0.5mg/l 2,4-D+ 0.5 mg/l BA) combination . C callus of(2mg/l 2,4-D+ 2 mg/l BA)

Results indicated that combination treatments between auxins and cytokinins is very important to obtain of the highest quantities from fresh weight of callus to use in the new experiments . The adding of growth regulators to MS medium have significant effects of callus inducing and have physiological effects and biological activities of division and growth of plant cells (Davies, 2004). Carew and Krueger (1977) indicated that 2,4-D and BA combinations are important to increase and inducing callus in C. roseus . Namdeo (2007) refer to callus inducing by combination of 4.8 umol 2,4-D with 4.6 umol kin which gave the highest results. Ethbaieb (2010) indicated that the best combination for callus inducing in *C. roseus* was 2 mg/l auxin with 2 mg /l cytokinin . These results may be due to equilibrium state between auxins and cytokinins as well as important in the induction (George *et al*., 2008).auxins effects directly of cell expansion through increase some enzymes activities which response of wall elasticity

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and increase permeability. The cytokinins are important for cell division due to induce biosynthesis of some important proteins and for RNA formation. The callus growth in tissues culture technique depend on ability of cells division to form new cells these can calculate by determination of fresh weight and contents of cells (Davies, 2004).

Effect of abiotic stress on callus fresh and dry weights (g)

The results in Table(2) showed that the additions of abiotic factors to the callus culture medium in accumulation phase cause reduce the fresh and dry weight in all treatments expect sucrose 40 g/l treatment which gave increase in fresh and dry weight (3.543, 0.440) g without any difference significant compared with control treatment (3.267 ,0. 326) g respectively (without any factor). Followed by drought treatments (PEG 5% ,PEG 10% , PEG 15%) which reduce fresh and dry weights to (2.128 ,1.889 , 1.699) g and (0.208 , 0.193 , 0.160) g respectively with significant difference compared with control treatment which gave fresh and dry weights (3.267 , 0.326) g respectively.

Table(2) Effect of abiotic stress on fresh and dry weights of callus(g)

Treatments	Fresh weight/g	Dry weight/g
Control	3.267	0.326
Sucrose 40 g/l	3.543	0.440
Sucrose 50 g/l	2.722	0.399
Sucrose 60 g/L	2.492	0.276
PEG5%	2.128	0.208
PEG10%	1.889	0.193
PEG15%	1.699	0.160
LSD(0.05)	0.331	0.039

Results refer to that additions of abiotic factors to the culture medium reduce the fresh and dry weights due to the negative effect of high stresses by drought and sucrose in culture medium which cause decrease in cells growth of callus exposed to these stresses (Radman*et al*, 2003), while the increase in fresh and dry weights in sucrose 40 treatment due to the stimulate of bioactivities for defense and adaptation to this concentration treatment and return back to important state of carbohydrates to build cells in this concentration(Orcutt and Nilson .,2000). Exposing of callus to these factors resulted high free radicals formation which have physiological effects cause reduce in the fresh and dry weights of callus cells(Cheeseman , 2006).The results agree with Xu and Huang (2010) who indicated that PEG treatments did not improve the plant regeneration ability in Maize tissues culture and cause decrease in fresh and dry weights of the callus in accumulation phase . Natas *et al* (2004) indicated that additions of 4% and 5 % sucrose to the culture medium stimulate secondary metabolites but reduce callus growth in Coleus blumet *in vitro*. The additions of PEG as drought stresses in all concentrations

reduce the fresh and dry weights due to the low in water absorption from MS medium and altered of water tension which important for transport and stimulate the nutrient materials as well as the less in activities of some important enzymes like alpha-amylase and beta- amylase which inhibited by drought treatments (Lotfi, 2010).

Catalase activity determination :

The results in Table (3) showed that the abiotic factors have different effects on catalase enzyme activity .The highest activity was for PEG 10 % and PEG 5% treatments (228.84 , 122.69) unit respectively with significant different compared with control treatments (without any factor) (36.97) unit .Followed by Sucrose 40 g/l and 50 g/l gave (35.21 , 36.33) unit respectively without significant different compare with control treatment. Plant extraction treatment (37.89) unit with significant different compared with control treatment . PEG treatments cause increase in catalase activity due to increase free radicals and H2O2 product by alternative catabolic processes inducing during the carbohydrates shortage caused by drought stress as well as breakdown of H2O2 to H2O and CO2 stimulate reaction of catalase to increase the water component in the dry cells (Cortento ,2010).Exposure to drought stresses cause common reactions in plant cells lead to cellular dehydration and osmotic stresses removal of water from cytoplasm to vacuoles (Alkula and Gokare ,2011). Baqir and Moslim, 2010 reported that addition of PEG 8000 5% to MS medium of two Grape varieties *Cardinal* and *Amber queen* increase catalase activity as natural mechanism to resistance water stress and to scavenge the free radicals production . Additions of sucrose, 40g/l and sucrose50 g/l have low effects on catalase activity and act as ready sources of energy but in sucrose 60 g/l treatment there was some osmotic stresses cause increase enzyme activity (Ashutoch*et al* 2012).

Total soluble carbohydrates determination :

Results in Table (3) showed that all treatments increase total soluble carbohydrates with significant different compared with control treatment (without any factor) (110 . 333) ppm . The highest value was for PEG 15% treatment(315.933) ppm . Sucrose treatments (40 , 50 , 60) g /l gave (165. 422 , 197.638 , 143 .430) ppm with significant different compared with control treatment (110 . 333) ppm . Plant extract treatment gave (122 . 329) ppm with significant different compared with control treatment too. These increases of total soluble carbohydrates as responsible and adaptation of stresses (Baqir and Muslim , 2011).

Analysis of polysaccharides in stresses condition cause increase in soluble carbohydrates as well as effect of stresses conditions one nzymes to form polysaccharides from simple sugar (Xu and Huang ,2010). These results agree with Baqir and Moslim ,2011 who indicated that the water stress using BEG treatments increase the total soluble carbohydrates indicated that this total soluble carbohydrates by salinity and osmotic stresses in *Populuseuphratica in vitro*.

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Proline determination :

Results in Table (3) showed increase in proline content as responsible to abiotic factors .The highest value was for PEG 15% treatment (1.146) umol / g d.w with significant different compared with control treatment. Extract of plant treatment (0. 357) u mol / g d.w without significant different compared with control treatment . Handa *et al* (1986) reported positive role for proline accumulation in adaptation of cells to change external water potentials .Obaid*et al* ; 2012 reported increase in proline as responsible to drought stress by PEG 6000 due to the deficiency of water content . The possible role of proline in membrane protection under stresses conditions drought and osmotic stresses. Proline accumulates under different conditions (Rashmi*et al* ; 2012) act as scavenger and protection agent against ROS which play important role in osmotic regulation and enzymes protection, , as well as stability of protein biosynthesis. proline increase under stress condition by deficit or analysis of protein and increase stability of plasmic membrane . Increase of protein considered protect adaptation by deficit of protiolytic activity act as regulator for different enzymes involved in protein formation. Proline act as save resource or relief for amino acids and proteins(Khatkar and Kuhad , 2000).

Table (3) : Catalase /unit , Total soluble carbohydrates/ *ppm* and proline /umol /d.w .

Treatments	Catalase/unit	Total soluble carbohydrates /*ppm*	Proline /u mol/g d.w
control	36.97	110.333	0.357
Sucrose 40 g/l	35.21	165.422	0.334
Sucrose 50 g/l	36.33	197.638	0.469
Sucrose 60 g/l	40.74	143.430	0.652
PEG 5%	122.69	113.527	0.525
PEG 10%	228.84	274.249	0.940
PEG 15%	53.79	315.933	1.146
Plant extraction	37.89	122.329	0.371
LSD	0.681	0.287	0.051

Effect of PEG 4000 treatments on vinblastine and vincristine production

PEG 15% Treatment Gave (48 .00 , 29.65) ug/g d.w of vinblastine and vincristine respectively with significant difference compared with control treatment with duplicate (29.44 , 16.94) folds for vinblastine and vincristine respectively table(4) and Fig(6) ,

PEG 10% gave(33.66, 27.87) ug/g(d.w) with duplicate(20.65, 15.92) folds for vinblastine and vincristine respectively with significant difference compared with control treatment (without any factor) followed by PEG 5% treatment (30.16, 26.45)ug/g(d.w) with duplicate (18.50, 15.11) folds for vinblastine and vincristine respectively .PEG treatments cause deficit in water component create different tensions between callus cells and MS medium as well as reduce the division and growth of cells, resulted to accumulation of these alkaloids as mechanism to defend and maintain against drought stresses (Zhange and Jain .,2003). Abiotic factors which also called elicitors may defined as materials which investigate or advance the biosynthesis of specific compounds when added in small amounts to a living cell system. In this way elicitation can be explained as the stimulated or important biosynthesis of compounds due to addition of trace amounts of elicitors (Radman*et al* .,2003 and Angelova*et al*., 2006).

Effect of sucrose treatments on vinblastine and vincristine production

Results in table (4) showed that sucrose 60 g/l treatment gave about (32.33 , 16.25) ug/g(d.w) for vinblastine and vincristine respectively with significant difference compared with control treatment with duplicate (19.83 . 9.28) folds for vinblastine and vincristine respectively, followed by sucrose 50 treatment (22.00 , 15.90) ug/g d.w with duplicate (13.49 ,9.08) folds for vinblastine and vincristine respectively with significant difference compared with control treatment(without any factor).Followed by sucrose 40 treatment (6.16, 4.25)ug/g(d.w) with duplicate (3.77 ,2.42) folds for vinblastine and vincristine respectively. Plant extract treatment (2.36 , 3.55) ug/g (d.w) for vinblastine and vincristine respectively. Ashutosh *et al* (2012) indicated that addition of sucrose 6% to MS medium of tissues culture of *C. roseus* decrease overall callus biosynthesis slightly, but increase of alkaloids content in compare with other concentration . AL-mufariji(2010) referred to increase of sucrose concentrations in callus medium cause increase in some secondary products. High sucrose concentration considered source of osmotic stress which used widely in tissues culture for increase active compounds Elkahoui *et al*(2013. The important of adding sucrose to MS medium come from photosynthesis of plant tissues depending on addition sucrose, but in high concentrations of sucrose resulting stress increasing and low in product in primary metabolites compounds then affecting the secondary metabolites (Ameen and Mohammed ,2010).

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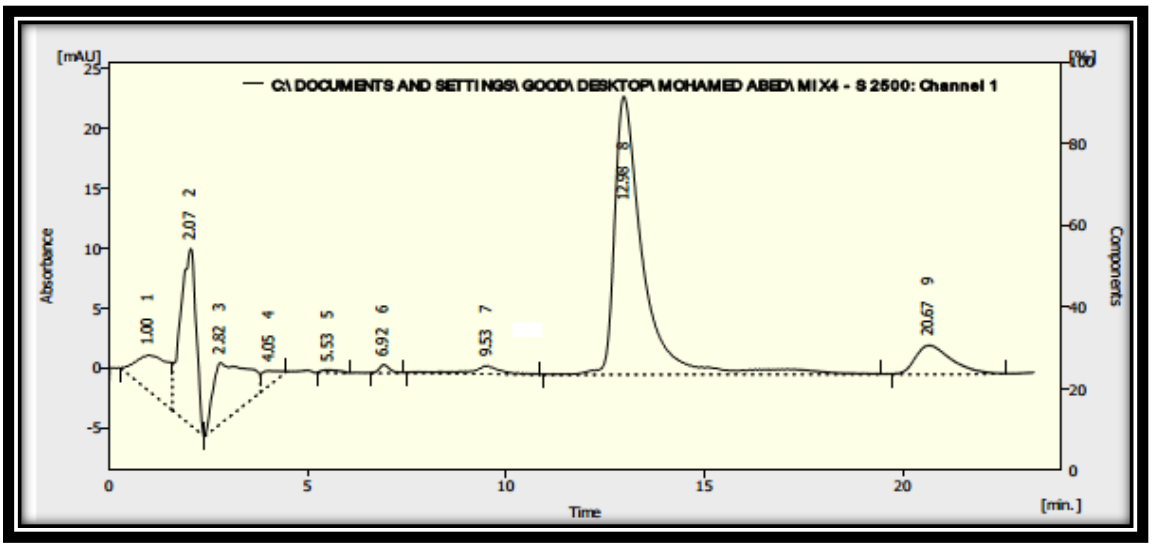
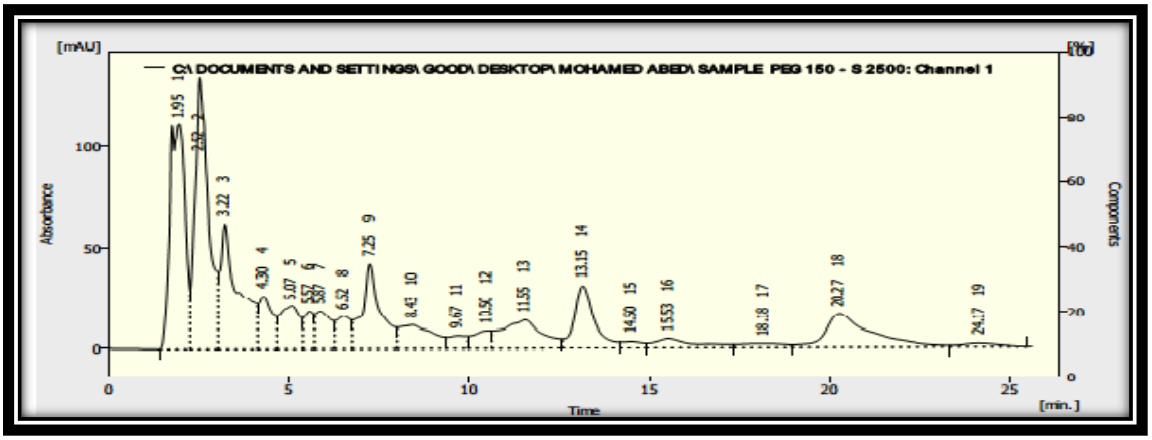


Fig (5) Retention time(min) and Peak Area for standard curve of vinblastine and vincristine by (HPLC) .



Fig(6): Peak area of PEG15% treatment by (HPLC).

Table(4) Effect of abiotic factors on vinblastine and vincristine alkaloids product from callus of *C. roseus* by (HPLC).

Treatments	Vincristine ug/g d.w	Vinblastine ug/g d.w
control	1.750	1.630
Sucrose 40g/l	4.250	6.160
Sucrose 50 g/l	15.900	22.000
Sucrose 60g/l	16.250	32.330
PEG 5%	26.450	30.160
PEG10%	27.870	33.660
PEG 15%	29.650	48.000
LSD(0.05)	0.163	0.155

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