Biochemical and Physiological Changes of Callus Growth and *t*-anethole Essential Oil Production from *pimipinella anisum L*. under Salinity Stress

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Abstract

This experiment was performed in faculty of Science labs, Babylon University, carried out during 2014-2015 for extraction, and Quantitative of essential oil *t*-anethole , from seeds , leaves and callus tissue of (*pimipinella anisum L*.). This study include sterilized anise seeds were germinated in free MS medium and callus induction from leaves (2cm pieces) by using same MS medium supplemented with plant regulator 2,4-Dichlorophenoxiactic acid (2,4-D) at different concentration (1, 2, 3) mg/l (induced phase). Callus tissue induced sub-cultured on MS medium with (BA) benzyl adenine at concentration of (1, 2, 3) mg/l and 2,4-D at concentration of 2 mg/l. Then, identically callus fresh weight sub-cultured on the same MS Medium supplemented with different concentration of NaCl (6, 9, 12 and 15Ds/m), Finally, comparisons study were made between *in vitro* and *in vivo* grown plant. Solution of control (*t*-anethole) used as standard. The content of *t*-anethole compound was done by using HPLC (high performance liquid chromatography), and compare of the quantity of *t*-anethole in callus with these content in seeds and leaves of mother plant. The result showed significant increased ($P<0.05$) of *t*-anethole production in callus tissue than the content in seeds and leaves of mother plant. Antioxidant enzymes activity like Catalase (CAT), were high in callus under different stresses conditions than in callus grown without stress. However, Proline was higher levels in callus under salt stress than in callus grown without stress.

Key Words: MS media, callus, CAT, proline, Anethole compound

الخلاصة

تم اجراء التجربة في مختبرات كلية العلوم / جامعة بابل خلال الفترة 2014-2015 لاستخلاص والكشف عن الزيت الاساسي الانيثول في اوراق وبذور وكالس نبات اليانسون (*pimpinella anisum* L.) . تتظمن الدراسة تعقيم بذور اليانسون وزراعتها على وسط MS (Murashinge and Skooge, 1962) خالي من منظمات النمو النباتية واستحثاث انسجة الكالس من الاوراق (2سم/قطعة) باستخدام وسط MS مزود بمنظم النمو ال 2,4-D (2,4-dichlorophenoxiactic acid) وبتراكيز مختلفة (3,2,1) ملغرام/لتر (طور النشوء) . اعادة زراعة الكالس على وسط MS حاوي على منظم النمو Benzyl adenine (BA) وبتراكيز مختلفة (3,2,1) ملغرام/لتر مع منظم النمو 2,4-D عند التركيز 2 ملغرام/لتر. بعد ذلك تم اعادة زراعة الكالس على وسط MS مزود بتراكيز مختلفة من NaCl (15,12,9,6) ديسيمنز/متر. تم تحديد الوزن الطري والجاف للكالس في جميع المعاملات. استخدمت تقنية الHPLC للكشف عن نوعية وكمية مركب الانيثول في اوراق وبذور وكالس نبات اليانسون من خلال استخدام محلول قياسي للانيثول ومقارنة كمية الانيثول في انسجة الكالس لجميع المعملات مع كميته في البذور والاوراق . اظهرت النتائج وجود زيادة معنوية ($P<0.05$) لمركب الانيثول في انسجة الكالس عند مقارنتها مع كمية المركب في الاوراق والبذور. تم قياس بعض المؤشرات الكيموحيوية في انسجة الكالس تحت الاجهاد وبدون اجهاد مثل فعالية انزيم الكتليز وكمية الحامض الاميني البرولين. حيث لوحظ زيادة في فعالية انزيم الكتليز في انسجة الكالس المعرض للاجهاد مقارنة مع انسجه الكالس غير معرضه للاجهاد. اما كمية البرولين فقد ازدادت مع زيادة الاجهاد الملحي.

الكلمات المفتاحية: وسط الMS , كالس, كتليز , برولين, مركب الانيثول.

Introduction

Plant essential oils as secondary metabolites characterized by low molecular weight and are include terpenoids compounds, and other aliphatic and aromatic constituents (Degenhardt *et al*., 2009). All plant organs have able to synthesis essential oils such as, fruits, leaves, stems, flowers, seeds and twigs and accumulated and stored

in cavities, glandular trichomes or epidermic cells, secretary cells and canals (Bozin *et al*., 2006). *P. anisum* is an annual grassy herb belonging to the Umbelliferae family, is one of the oldest medicinal plants, which grows in the Middle East, Spain, Egypt, and Eastern Mediterranean Region (Salehi, 2010).As medicine plant, *P.anisum* has been used as antifungal agent (Kosalec *et al*., 2005). *P.anisum* seeds extract is helpful in cancer treatment and prevention prostate cancer cell line [PC-3] (Kadan *et al*., 2013). Different experiments were performed to study *P.anisum* seeds extract effects and various properties such as antioxidant, analogesic, muscle relaxant and anticonvulsant activity. Aniseeds cause hypolipidemic and hypoglycemic effect and reduce lipid peroxidation (Shojaii and Fard, 2012). *P.anisum* which synthesis and accumulate the largest amount of essential oils include *t*-anethole (90%), γ-himachalene(2-4%), *p*-anisaldehyde (1%), methylchavicol (0.9-1.5%) (Rodrigues *et al*., 2003). Also, content, quality and its composition affected by environmental factors and soil type (Jevdjovic *et al*., 2012). *t*-anethole with Molecular formula as $C_{10}H_{12}O$ is the most common essential oil in aniseed.

Plant tissue culture technique is one field of biotechnology to investigate and enhance the production of many important medicinal secondary metabolites under controlled laboratory growth conditions and not depends on environmental changes or soil conditions. (Vijayasree *et al*., 2010). Salt stress is natural major conditions presented in water and soil, causes soil salinity which conceder as the major problems that faced the production plant, including the medicinal plants in many irrigation areas in the world, especially the arid and semi-arid regions (Jin *et al*., 2010). Salinity experiments were used extensively in the field of education and improvement of plants to understanding physiological effects of stress , as pointed out by many researchers the possibility of isolating communities from plant cells and cultured it on salt medium to give it tolerances and study the effect of salinity in various metabolic processes within the plant cells (Basu *et al*., 1997).The harmful effect of salinity on plant growth is caused by the replacement the growth medium osmotic potential, nutrient ion insufficiency and toxicity of specific ion (Luo *et al*., 2005). Plant tissue culture is carried out under controlled conditions that are considered of the most important techniques that are used to determine the plant's response to stresses (Lokhande *et al*., 2011). Increase the amount of production of some substances and compounds in plants as exposure to salt stress and this is one of the mechanisms by which plants resist saline stress, Examples of such materials are proline , catalase enzyme and carbohydrates (Mehr, 2013).

Material and methods

***P. anisum* seeds culture:** Seeds were collected from the Babil province market and were diagnosed in the lush College of Science /University of Babylon**.** M.S. medium (Murashige and Skoog, 1962) free form plant growth regulators were used in this experiment. A suitable amount of seeds washed with distilled water to eliminate dust and sterilized by immersing them in the solution of 20% sodium hypochlorite with Shake for 3-4 minutes. Washed with sterile distilled water for one minute at three times, and then add 70% ethyl alcohol and Shake for one minute and then washed with sterile distilled water for one minute at three times. (Awika and Rooncy, 2004). Sterilize seeds, in vitro germination was done by culturing three seeds in each tube containing 15 ml of

MS medium. Then incubated under light conditions (16 hours day and 8 hours night) at temperature of 24±2°C. Five weeks-old seedlings were used as a source of explants for callus induction.

Callus induction: Method of Hirata *et al*. (1990) used for the purpose of inducing callus from leaves of *P.anium*. Steriled leaves segments were cut off and cultured on MS supplemented with 2,4-D at concentrations (1, 2 , 3) mg/L and by one vegetarian portion of each tube at a rate of 12 repeater for each concentration of 2,4-D for the purpose of callus induction. Tubes were incubated in a growth champer at a temperature of 25 ± 2°C and intensity illumination of 1000 lux for 16 hours.

Determination of callus fresh (g) and dry weight (mg): Where fresh weight of callus induced from the leaves after 45 days was calculated for all concentrations of 2,4-D used an electric sensitive balance. callus fresh weight was placed in a glass Petri dishes by two dishes each containing 6 pieces of callus tissue for each concentration of 2,4- D, Callus dry weights were determined after dried of fresh callus in oven at 40°C for 28 hours and then get out and then calculated.

Accumulation phase: Growth regulator benzyl adenine (BA): After a period of 6 weeks of cultivation of anise leaves were selected developing callus on MS medium containing 2 mg/L 2,4-D. Callus tissue cut into parts up to 250 mg and sub-cultured again on the same MS media containing growth regulators 2,4-D at concentration of 2 mg/L and BA at concentrations of (1,2 and 3 mg/L) with same growth conditions. Callus cultured at 12 repeaters for each concentration of BA, and tubes were incubated in the incubator at a temperature of 25±2 and photoperiodic conditions of 1,000 lux for 16 hours.

Callus fresh (g) and dry weight (mg) determination: followed the same steps mentioned in determination of callus fresh and dry weight of callus in induction phase.

Salinity treatment: Preparation of salt solution: sodium chloride NaCl solution prepared by dissolving of 3 grams of pure NaCl in 50 ml of distilled water to make stock solution (Buringh, 1962) and was used in preparation of media with different concentrations of salinity to study the effect of salt stress on production and accumulation of secondary metabolites and some parameters.

Callus culture in salt media: MS media was contained 2mg/L 2,4-D and 2mg/L BA because it represents the best treatment of plant growth regulators. The electrical conductivity (EC) of media was altered by NaCl solution and measure by using EC-Meter device, media were prepared at different levels of salinity (6,9, 12 and 15) DS/m. Callus fresh and dry weight of the developing callus was measured after 5 weeks of culturing in MS medium.

Callus fresh (g) and dry weight (mg) determination: followed the same steps mentioned in determination of callus fresh and dry weight of callus in induction phase.

Alcoholic crude extract preparation: According to Harborne (1984) method used in preparation of an alcoholic extract of the seeds, leaves and callus of anise plant. Added 1 g of explant dry powder in a glass beaker containing 10 ml ethyl alcohol. And shaking with plate magnetic stirrer for 24 hours, and then filtered by use filter paper type watman (No.1). Then the filtrate centrifuged at 3000 r/min for 10 minutes and stored in the refrigerator for use at a later time.

Qualitative determination of *t*-anethole compound by TLC: Silica gel plates were used at thickness of 0.25 mm and dimensions of 20 × 20 cm as a stationary phase. Solvents system used was toluene/ethyl acetate (93:7) as a mobile phase with sulfuric acid as spry reagent for the separation of bioactive compounds in crude extracts of anise intact plant and callus at UV light in absorbance at 254 nm and calculated the relative flow values(*Rf*) (Najdoska *et al*.,2010).

Estimation of catalase enzyme activity: Catalase enzyme activities estimated by the method mentioned by Aebi (1974), Where this method depends on the amount of change in absorbance at 240 nm wavelength of hydrogen peroxide solution (30mM) and phosphate buffer solution (50mM) at PH= 7. Calculated the activity of catalase enzyme activity by the following equation:

$$\text{catalase activity(unit)} = \frac{\Delta\,\text{bs/min} \times \text{reactionvolume}}{0.01}$$

Where: $\Delta\text{bs} =$ absorbance difference within a minute 0.01 = constant,

Min = reaction time , 2.4 ml = reaction volume

Unit = 1 unit (U) is the amount of enzyme that catalyses the reaction of 1 µmol of substrate per minute

Estimation of proline content: Method of Bates *et al*. (1973) is used to estimate the amount of proline, through the addition of 0.1 ml of seeds, and callus extract with 2 ml of ninhydrin acid and glacial acetic acid, and then read the absorbance at a wavelength of 520nm.

Proline standard curve: Standard solution of proline was prepared at concentration 10µg/ml by dissolving 0.01gm in a small amount of distilled water and then completes the volume to one liter using distilled water. Prepared graded concentrations of standard solution of proline as (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4) µg/ml as shown in Fig (1). Then calculate amount of proline in samples by using proline standard curve through followed equation:-

$$\mu\text{ moles per gm freash weight} = \frac{\text{proline }\mu\text{g/ml} \times 5\text{ ml toluene}}{115.5 \times \text{G sample}}$$

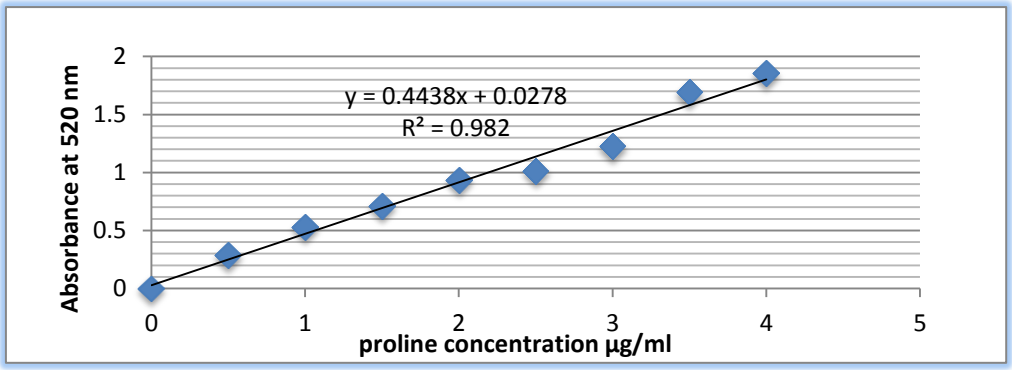

Fig (1) Proline standard curve at 520 nm Wavelength

Quantitative evaluation of *t*-anethole compound using HPLC technique:

HPLC technique was used in the estimation of the quality and quantity of *t*-anethole compound using a mixture solvents consisting of 80% methanol and 20% distilled water, which represents the mobile phase, while the stationary phase column was a C-18 (250 × 4.6mm, 5pm) particle size at 257 nm wavelength (Jurado *et al.*, 2006).Then injected 100 Micro liters of extract of seeds, callus induced , callus growing in accumulative phase and extracts of callus growing in different salt concentration. Then calculate of concentration of *t*-anethole in samples According to the following equation

$$Sample\ conc. = \frac{Sample\ area}{Standard\ area} \times Standard\ conc. \times Dilution\ factor$$

(Illiana and Alfermann, 1989)

Results and discussions

Effects of 2,4-D concentrations on callus fresh (g) and dry weight (mg): Different levels of growth regulator 2,4-D (1, 2, 3 mg / l) was studied in callus induction from leaves explants fig (2a) of *p.anisum*. The results indicate to the significant effect of 2,4-D concentrations added to the MS medium on the fresh and dry weight of callus induced , Where the concentration (2 mg/l) 2,4-D gave a higher rate of fresh weight reached to (1.85)g , and differed significantly with all treatment of2,4-D concentrations, While the lowest fresh weight was in the concentration of (1 and3 mg/l) 2,4-D reached to (0.97and 1.23)g respectively which differed significantly from all concentrations of 2,4-D as shown in in the table (1) and Fig (2b). Through the results in the same table is clear that the values of dry weight commensurate with the values of fresh weight of callus induced from the *p.anisum* leaves and in all concentrations of 2,4-D and was the best dry weight rate of induced callus recorded in MS medium with (2mg/l) 2,4-D which reached to (180)mg and differed significantly from all concentrations of 2,4-D. Perhaps the reason for the reduction of callus fresh weight rate at higher concentrations of 2,4-D(3mg/l) in callus growth medium to the role of auxin to stimulate the production of ethylene gas, which reduces cell division and growth rate (More,1982) or may be to the amount of endogenous auxin lost by cells to the medium (Al-Hatmy,

2006). Results in this study observed that 2,4-D at 2mg/l concentration conceder as the best medium for callus initiation from *p.anisum* leaves which uses in all treatments of accumulation phase. Nguyen *et al*. (1981) referred to that the 2,4-D has un induction effect on callus cells number as well as increase in cells volume due to increase of water content in cells,. These results are agreed with the results of Carew and Kruger (1977) and Yamada and Hashimoto (1982).

Table (1) Effect of 2,4-D concentrations on callus fresh and dry weight

Concentration of 2,4-D mg/l	Fresh weight	Dry weight
1	1.23	119
2	1.85	180
3	0.97	110
L.S.D. (0.05)	0.036	3.750

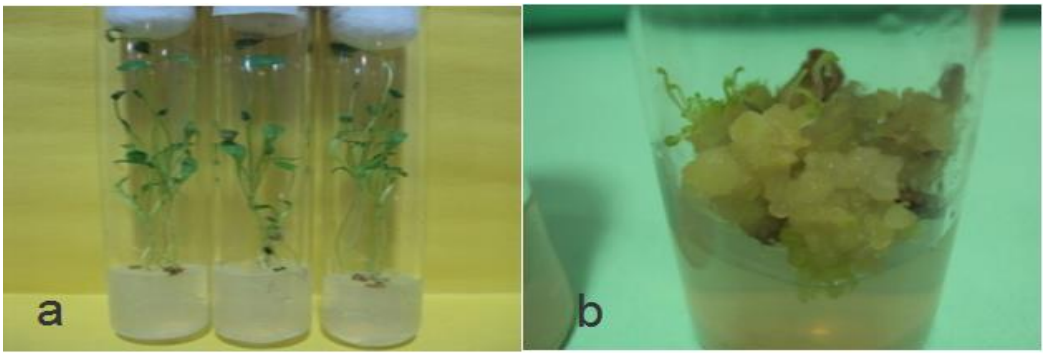

Fig (2) *a: P.anisum* seedling grown in MS
b: Callus grown in MS with 2mg/l 2,4-D

Effect of BA on callus fresh (g) and dry(mg) weight : Results at table (2) indicate to presence significant effect of BA concentrations on fresh weight rate of callus tissues, where the concentration (2mg/l) of BA + (2mg/l) 2,4-D added to culture medium gave the higher fresh weight rate of callus and differed significantly from all treatments Cytokinin (BA) in culture medium that stimulate and increase of callus cell division , expansion , increase water absorption and nutrient leading to increase in biomass of callus(Mohommed,1990) .

Table (2) Effect of BA concentrations on callus fresh and dry weight of *p.anisum*.

Concentration mg/l		Fresh weight (g)	Dry weight (mg)
2,4-D	BA		
2	1	1.53	148
	2	2.11	208
	3	1.2	118
L.S.D. (0.05)		0.077	14.15

Determination of *t*-anethole essential oil by Thin layer chromatography (TLC): The results showed the presence of six, seven and five compounds in the leaves, seeds and callus extract respectively and in calculating (R*f*) values of separated spots were (0.13, 0.31, 0.53, 0.82, 0.88, 0.96) of leaves extract and (0.11, 0.27, 0.48, 0.75, 0.79, 0.85, 0.95) of seeds extract and (0.14, 0.30, 0.50, 0.79,0.94) of callus extract respectively and when compared with the value of (R*f*) of *t*-anethole standard solution that its value is equal to (0.94) were identical with one spot in alcoholic extract of leaves, seeds and callus as in fig (3). These results correspond to the description of (Najdoska *et al*., 2010) in separation of *t*-anethole compound from essential oils using TLC technique.

Fig (3) Detection of *t*-anethole by TLC technique in leaves, seeds and callus of *P. anisum*

(A: standard, B: callus, C: seeds, D: leaves)

Effect of salinity on callus fresh (g) and dry weight (mg): Callus fresh weight was determined after 40 days in salt treatment .The results showed a gradual decrease in fresh weights with an increase in salt concentration as compared to the control treatment. Salt concentration (6 Ds/m) gave the higher fresh weight rate reached to (2.51)g compared with control treatment (4 Ds/m) and significant difference with all treatments .Also ,there was a significant decrease at concentrations of (9, 12 and 15 Ds/m) and the less fresh weight rate was recorded with concentration of (15 Ds/m) reached to (0.90) g which differed significantly with control and all other treatments. However, the results in the same table is clear that the values of dry weight commensurate with the values of fresh weight of callus grown in medium supplemented with different NaCl concentrations and the best dry weight rate of callus was in (6Ds/m) reached to (240) mg compared with control treatment (4 Ds/m) and with all other treatments and differed significantly with control and all concentrations of NaCl whereas (15ds/m) gave less dry weight and differed significantly with control treatment and all other treatments as showed in the table (3). Perhaps the reason for the significant enhancement of callus fresh weight in the low concentration (6 Ds/m) due to may stimulate bioactivities necessary for the growth to resistance the salt stress or may be due to increased nutrient uptake by cells as a means of resistance to salt stress conditions (Rains *et al*, 1986).But the reduction in callus fresh weight at high salt stress (15 Ds/m) due to negative effect of salt on cells grown at high salt concentrations (Al-hechamy, 2010). Or caused by a reduced ability to adjust osmotic potential, consequently this results in saturation of solute uptake system, or because of excessive demand for energy requirements of such systems (Aghaleh *et al*., 2011). This results agreement with the results reported by (Al-Khayri, 2014 and Sharma, 2014).

Effect of salinity on proline content: The data indicate that salt stress had a significant effect on proline content in callus cultured in MS medium with different concentration of NaCl. An increase in NaCl concentration cause significant increase in proline content which lead to (0.810)µmole/g in (15 Ds/m) treatment in compared with control treatment (4 Ds/m) which reached to(0.261)µmole/g. Whereas (6Ds/m) salt stress gave less proline content and differed significantly with control treatment and with all treatments as showed in table (3). This results agreement with the results of (Sharma and Ramawat, 2013 and Jasim *et al*., 2010). proline may act as a signaling/regulatory molecule able to activate multiple responses that are component of the adaptation process (Ashraf & Harris, 2004) and act as a scavenger of ROS, osmolyte, stabilizer of the proteins structure, thereby protecting cells from damage caused by stress (Szabados and Savoure, 2010) and as stabilizer of cellular structures and membranes (Van Rensburg *et al*., 1993).Accumulation of Prolinein response to increase salt stress has been reported in many studies. Proline accumulation useful as biochemical marker to understand how plant tolerance the abiotic stresses (El Hadrami *et al*., 2011).

Salinity effect on catalase activity: The maximum catalase activity was recorded in (9 Ds/m) about (22.67) unit/ml and differed significantly with control treatment (4 Ds/m) and with (15Ds/m), except with (6Ds/m) and (12Ds/m) treatments. Also, the lowest

catalase activity was found in callus grown in high salt NaCl level (15Ds/m) which reached to (8.13) unit/ml and differed significantly compared with all treatments except control treatment as shown in table (3). An antioxidant system of plant includes of both enzymatic and non-enzymatic systems and is important for controlling the production of ROS under stress (Mittler *et al*., 2004). Antioxidant enzymes such as catalase play an important role in scavenging H_2O_2 and converted it into water and oxygen (Yang *et al*., 2010).The first defense line of plant in responding to stress in converting of O_2^- to H_2O_2 and then convert H_2O_2 by antioxidant enzyme (catalase and ascorbate peroxidase) into oxygen and water (Lokhande & Suprasanna, 2012).This results agreement with (Sharma and Ramawat, 2014).

Quantitative determination of *t*-anethole content by (HPLC)

The results of HPLC indicate to presence of *t*-anethole compound in samples, Where was obtained absorbance peaks of callus samples cultured for 30 days under salt stress with retrieval time which closed to retrieval time of *t*-anethole standard sample (5.270) minuets with one absorbance peak Fig (4a). Through the study appeared that the concentration (6 Ds/m) salinity encouraged the plant to produce of *t*-anethole more than in control treatment which reached to (77.09) ppm DW Fig (4b) and represent the higher amount of *t*-anethole and differed significantly with control and all treatments. While the concentrations (9, 12 and 15) Ds/m gave decrease amounts of *t*-anethle compared with control treatment (4Ds/m) reached to (32.27) ppm DW and the lowest concentration of *t*-anethole was in (15 Ds/m) reached to (14.89) ppm DW and differed significantly with control and all treatments as shown in table (3).

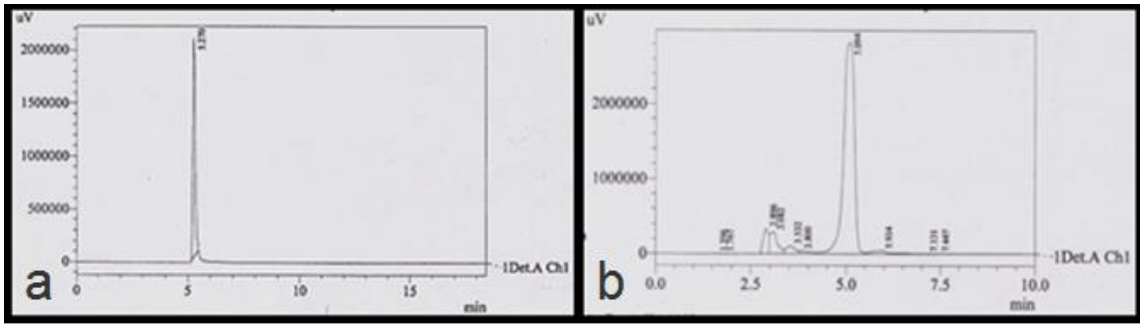

Fig (4) a: Peak of standard *t*-anethole by HPLC technique

b: peak of *t*-anethole in callus cultured in MS medium with 6DC/m salinity by HPLC technique

This results agreement with (Neffati and Marzouk, 2008) when he is study the production of menthol from peppermint plant grown under salt stress. Ashraf and Akhtar (2004) reported that the sweet fennel grown under salt stress resulted in progressive decrease in oil content. Fennel plant irrigated with saline water resulted in reduction of *t*-anethole content (El-Wahab, 2006).The reason for reduction of *t*-anethole compound under high level of salt may be due to affected of some enzyme included in biosynthesis of essential oils (Sakar, 2008) .While the increase production of *t*-anethole under low level of salt may be due to that the essential oils have a potential to remove of

free radicals which would Preserving the cells from damage, so the plant may resort to increase production of essential oil under low level of salt (Bakkali *et al*., 2008) .And some studies pointed to present positive relationship between the essential oils and chlorophyll and the defect in this relationship may be causes variation in production of essential oils (Maffei and Scannerini, 1999). Also the reason for reduction of *t*-anethole may be due to low absorption of metal elements under salt stress such as, phosphorus, calcium and nitrogen which have a role in biosynthesis of essential oils (YanCai, 2005). These results are consistent with the general explanations of Al-shahat (1999) .that the plants grown under salt stress produce low level of essential oils.

Table (3) effect of NaCl on callus fresh and dry weight, catalase activity, proline content, and *t*-anethole content of *P. anisum*

NaCl conc. Ds/m	Fresh weight g	Dry weight mg	Proline content µ mole/g	Catalase activity Unit/ml	*t*-Anethole content Ppm
0	2.11	208	0.261	9.26	32.27
6	2.51	240	0.482	12.31	77.09
9	2.06	190	0.534	22.67	31.20
12	1.27	130	0.782	16.84	21.38
15	0.90	100	0.810	8.13	14.89
L.S.D (0.05)	0.1283	8.94	0.0419	3.031	6.921

Comparative study in vitro and in vivo of *t*-anethole production from callus grown under different stresses with leaves and seeds of *P.anisum*

The maximum content of *t*-anethole under different concentration of salt stress was selected and compared with other maximum amount of *t*-anethole in leaves and seeds of *P.anisum*. Results refer to that the maximum amount of *t*-anethole under salt stress was in (6 Ds/m) reached to (77.09) ppm DW. *t*-anethole amount in leaves was about (27.70) ppm DW and in seeds was about (78.42) ppm DW. When compared the results of the amount of *t*-anethole produced from callus grown under salt stresses with amount of *t*-anethole in leaves and seeds of *P.anisum* resulting in that the maximum amount of *t*-anethole as shown in fig (5). Data reveled that salt stress significantly increases *t*-anethole contents in callus tissues cultured in different concentration of salt stress comparison with control callus in induced phase of *P.anisum* mother plants. Level of NaCl (6 Dc/m) in callus culture medium caused significantly increased in *t*-anethole content up to (2) fold with callus grown in culture medium without salt treatment and about (2.8) fold in comparison with *t*-anethole content in leaves extracts. Also, seeds extracts up to (0.11) fold increase in *t*-anethole content in comparison with *t*-anethole content in callus grown in salt stress at levels of (6 Dc/m) and (4) fold increases in *t*-anethole content were found in comparison with leaves extract. Several researches have shown that the treatment of salts tress on *P.anisum* callus tissue can enhance *t*-anethole production and potentially other antioxidant concentrations. The increase in *t*-anethole

content in response to stress in the callus varied from 10% to 30%. Evidence suggests that increasing antioxidant concentrations is a primary physiological response of the plant to salt stress. Additionally salinity stress during cultivation increased the antioxidant capacity while maintaining the secondary metabolite concentration Proline also plays roles in scavenging free radicals, stabilizing sub-cellular structures, and buffering cellular redox potential under stresses. Plant tissue culture techniques are the more advantage methods to studying and observing morphological, physiological and biochemical changes in callus cultures and organized tissue (i.e. shoot tip,leaves, mature embryo) .

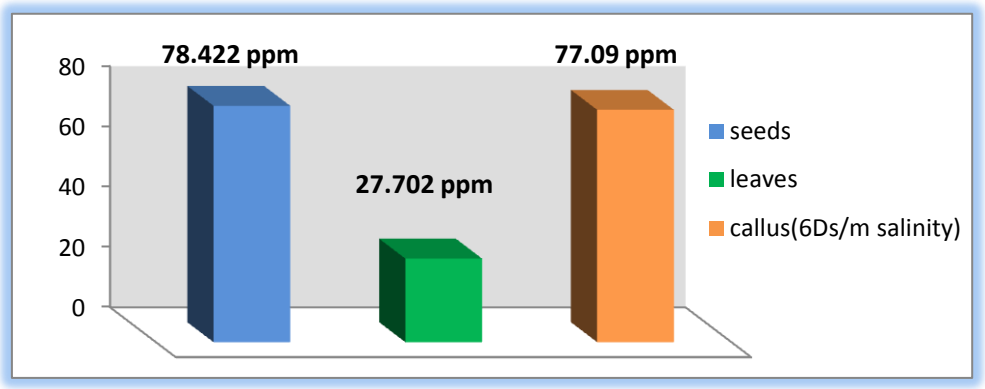

Fig (5) *T*-anethole content in seeds ,leaves and callus grown in MS medium with PEG, NaCl

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