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Influence of polyethylene glycol and tyrosine on morphine alkaloids production from callus of *Papaver somniferum in vitro*

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Abstract

The research was carried out in the plant tissue culture laboratory / College of Agriculture/ Kerbala University and HPLC analyzes was completed at the White Fields Company for Investments and Environmental and Engineering Studies during 2016 and 2017. This study included the effect of PEG and tyrosine on the soft and dry weight of the induced callus from the shoot tip of poppy plantlet and the accumulation of morphine alkaloids including morphine, codeine, papaverine and noscapine in the callus. The results showed that the PEG-free medium had the highest fresh and dry weight of callus with values of 483.7 and 41.9 mg/g respectively, while the concentration of 30 g / L PEG achieved the lowest values of 148.9 and 13.4 mg/g respectively. The medium with a concentration of 40 mg / l tyrosine achieved the highest fresh and dry weight of callus of 340.6 and 30.3 mg/g respectively compared to the comparison treatment which achieved the lowest rate of 275.3 and 24.2 mg/g respectively. The results also showed that the medium supplemented with 20g/l PEG and 60 mg/l tyrosine achieved the highest weight of morphine and papaverine alkaloid production with 435.5 and 378.7 µg/g respectively, and the highest weight of codeine alkaloid was achieved in the medium supplemented with 20g/l PEG and 40 mg/l with a value of 390.0 µg/g, while achieved the medium supplemented with 30g/l PEG and 40 mg/l achieved the highest weight of noscapine with 380.0 µg/g, whereas the lowest rates of morphine, codeine, noscapine and papaverine alkaloid were obtained in the medium free of PEG and tyrosine with values of 133.5, 80.0, 75.5 and 60.0 µg/g respectively.

Key world: Morphine alkaloid, *Papaver somniferum*, PEG, tyrosine, callus, in vitro.

INTRODUCTION

The medicinal plants, including *papaver somniferum* L., which belongs to the papaveraceae family, which comprises about 26 genus and 300 species, have great medical and economic importance because they contain many alkaloids(1). Poppy cultivation has spread in Thailand, Laos, Burma, Pakistan, Afghanistan, Iran, Central Asian States, Mexico, India, Colombia. It was initially cultivated for use in non-medical purposes such as seed oil extraction ,and when the components of the plant alkaloids were separated and when their physiological and harmful effects resulting from them were known, the governments have taken control of their cultivation and laws have been issued prohibiting their cultivation or cultivation with the knowledge of the governmental authorities. Its cultivation has been limited to its alkaloids, notably morphine, codeine, noscapine and papaverine, which were found in the plant as free or as salts for some organic salts (2).

The morphine alkaloids and its salts has been used as sedatives and narcotic and is also has a hypnotic strong effect, affecting the central nervous system of the body and the effect is initially tonic and after some time give the reverse effect and shows the effect of eye narrowing, It also leads to addiction (3 and 4). Despite the similar effect of morphine alkaloids, but codeine and papaverine are less toxic and addictive and less dangerous than morphine (5).

The interest in tissue culture of plant cells and tissues has increased as a substitute for traditional agriculture. It helps in the rapid production of these substances throughout the year without limiting the planting season, reducing the areas needed for cultivation, as well as the high purity of the substances produced when compared to those manufactured (6). Many researchers have been able to increase the amount of secondary compounds used in the medical field, such as morphine alkaloids in the callus of poppy plant (7) and atropine alkaloids in the belladonna plants (8 and 9). Many sources have suggested that adding the appropriate initiator to the medium of cultivation stimulates the production of secondary metabolism, (10) noted that increasing the concentration of tyrosine added to the medium of poppy-dependent plant cultures to 30 mg / L increased the production of morphine alkaloids. This result reflected that the amino acids penetrate through the walls and membranes of the cells and help in the formation of alkaloids. Some researchers have suggested that the addition of PEG as an adjuvant stimulant stimulates the genetic expression and synthesis of enzymes that control the production of certain compounds.

PEG is an inert, non-heterogeneous substance, a long-chain polymer that has no role in the regulation of cells because it cannot pass through cellular walls and works to trap water molecules and prevent them from entering the cell (11). Due to the importance of morphine alkaloids and in order to ensure control of their production for pharmaceutical purposes but non-commercially, this experiment aimed at studying the effect of PEG and tyrosine in stimulating the increase of morphine alkaloids production in the callus of poppy plant, and then qualitative and quantitative detection of alkaloids using HPLC technology.

MATERIALS AND METHODS

100 mg of induced callus from the shoot tip of one month poppy seedling were taken and planted on MS medium (12), which was provided with a concentration of 2 mg / L 2,4-D (2,4-Dichlorophenoxy acetic acid) and 0.5 mg /L kinetin. PEG (polychlorinated glycol) at concentrations of (0, 10, 20, 30 g / L and tyrosine at concentrations of (0, 20, 40, 60) mg/ L were added to the medium with 10 replicates per each concentration. The plants were incubated in the dark at 25 ° C ± 2 ° C for four weeks.

Extraction

The extraction was carried out as mentioned by (13).A known weight of the callus was taken and then dried until the weight was constant. 1 g of dried sample were taken and 75 ml of 99% ethyl alcohol were added , then the samples were mixed using a vibrating shaker for 24 hours, The solution was filtered and placed in the oven at 40 ° C for 24 hours to get powder which was stored at 4 C^0 for use in the subsequent analyses.

Quantitative and qualitative estimation of alkaloids compounds by using High performance liquid chromatography technique (HPLC)

Alkaloids were separated from the extract of poppy callus according to (14). 20 ul of supernatant were taken and injected into the HPLC under the following conditions, Column of Euro sphere C18 5mm MSPL1 with dimensions of (12 CM x 4.6 MM), and a mobile phase consisting of (Ethanol: 0.3% ammonium carbonate in water) at 27:25 , Flow rate of 2 ml/min, and the readings were taken at a wavelength of 285 nanometers.

Table (1) Effect of PEG and tyrosine concentrations and their interaction in the wet weight (mg) of callus induced from shoot tip of poppy seedling.

Average	Conc.of tyrosine mg/l				Conc.of PEG g /l
	60	40	20	0	
483.7	527.5	493.8	465.2	448.4	0
391.8	375.3	460.9	413.8	317.1	10
231.0	240.8	254.2	238.7	190.4	20
148.9	122.8	153.3	174.2	145.1	30
18.3	20.5				LSD 0.05
	316.6	340.6	322.9	275.3	Average
	18.3				LSD 0.05

Table (2) Effect of PEG and tyrosine concentrations and their interaction in the dry weight (mg) of callus induced from shoot tip of poppy seedling.

Average	Conc.of tyrosine mg/l				g/l PEG Conc.of
	60	40	20	0	
41.9	50.2	43.0	38.0	36.5	0
31.9	28.5	37.2	32.1	30.0	10
21.1	20.3	24.7	21.8	17.4	20
13.4	9.0	16.1	15.6	12.8	30
1.6	2.4				LSD 0.05
	27.0	30.3	26.9	24.2	Average
	1.6				LSD 0.05

The concentration of alkaloids in the callus extracts was calculated according to the following formula:

Concentration of the unknown (g / µg)= Dilution times × number x concentration measurement × (sample package area) / (measurement package area).

Statistical analysis

All experiments were performed using the Randomly Randomized Design (CRD) and global experiments. The results were analyzed using the statistical program (15). The means were compared using the Least Significant Difference (LSD) and the probability level was 0.05.

RESULTS AND DISCUSSION

Effect of PEG and tyrosine concentrations and their interaction on wet and dry weight of callus.

As shown in tables (1 and 2), there was a significant decrease in the wet and dry weight of the callus by increased concentrations of PEG added to the medium. The PEG free medium resulted in the highest wet and dry weight of callus (483.7 and 41.9 mg) respectively, whereas the concentration of 30 g / l of PEG gave the lowest values of (148.9 and 13.4 mg) respectively. The same tables indicate significant differences in the wet and dry weight of callus by increasing the concentration of tyrosine added to the medium, and the medium with a concentration of 40 mg / L tyrosine had achieved the highest rate of wet and dry weight of callus with (340.6 and 30.3) mg respectively, while the comparison treatment achieved the lowest rate of (275.3 and 24.2) mg respectively. As for the effect of the interference, the medium with 60 mg / L tyrosine concentration and free of PEG was superior and gave the highest wet and dry weight of (527.5 and 50.2 mg) respectively, while the medium provided with 60 mg / l tyrosine and with a concentration of 30 g / L PEG gave the lowest wet and dry weight of (122.8 and 9.0) mg respectively.

The results of the tables (1 and 2) showed that the increase in PEG concentrations added to the medium resulted in a decrease in the wet and dry weight of the callus. This may be due to changes in the water relations of the cells due to increased stress on the cells, which requires the reorganization of the cells to the osmotic pressure effort in a way that allows them to adapt to the new conditions that are exposed to the cells which causes a decrease in the readiness of water and therefore the soluble nutrients compared to the medium in which the cells usually grow and this negatively affected the growth and development of Callus cells (16 and 17), or it may be because the cytoplasm content of plant cells exposed to such conditions becomes more condense and smaller when water is deficient in these cells (18). This is consistent with the findings of (19, 20 and 21) who found a decrease in the mean of callus weight by increasing the concentration of PEG added to the medium.

Effect of Tyrosine and PEG Concentrations and their Interaction in the Production Rate of Morphine Alkaloids in Callus of Poppy Plant

Table (3) results were obtained by using HPLC, and are also shown in Figs. (1-16). There was an increase in the concentration of morphine alkaloids in the poppy plant callus by increasing the concentration of tyrosine added to the medium, and the concentration of 60 mg / l gave the highest rate of morphine and papaverine alkaloids with (325.6, 257.9) µg / g, respectively, while the concentration of 40 mg / L gave the highest rate of codeine alkaloid of 288.5 µg / g and noscapine alkaloid of 277.9 µg / g, which were not significantly different from those obtained from the concentration of 60 mg / l, which achieved a rate of 276.3 µg / g of the same alkaloids, and the lowest rate of morphine, codeine, noscapine, and papaverine alkaloids were achieved from the comparison treatment with values of 255.0, 192.9, 172.7 and 132.2 µg / g respectively.

As indicated in the same table there was an increase in the production rate of morphine alkaloids by increasing the concentration of PEG added to the medium, as the concentration 30 g / L achieved the highest rate in the production of alkaloid morphine, codeine, noscapine with values of (386.6, 332.2, 325.0) µg / g, respectively, and the concentration 20 g / l achieved the highest rate of papaverine production with 281.1 µg / g, while the comparison treatment showed the lowest concentration of alkaloid concentrations of morphine, codeine, noscapine, papaverine with (171.6, 129.5, 119.1, 96.6 µg / g) respectively.

As for the effect of the interference between the concentrations of tyrosine and PEG on the rate of production of morphine alkaloids, it was observed from the data in the same table that the superiority

was for the medium that was provided with a concentration of 20 g / L PEG and 60 mg / L tyrosine compared to other media. It achieved the highest rate of morphine alkaloid production reaching 435.0 µg / g. An exception was that of the 30 g / L PEG and 40 mg / l tyrosine concentration, which achieved an average of 430.3 µg / g for the same alkaloids. The medium that was provided with the concentration of 20 g / l PEG and 40 mg / l tyrosine achieved the highest rate of codeine alkaloid of 390.0 µg / g which differed significantly from other concentrations, with the exception of the medium provided with a concentration of 30 g / L PEG and 40 mg / L tyrosine, which achieved an average of 380.6 µg / g. The same medium also achieved the highest noscapine alkaloid concentration of 380.0 µg / g which differed significantly from the rest of the other concentrations except the medium with a concentration of 20 g / L PEG and 60 mg / l tyrosine, which achieved a rate was 370.0 µg / g, while the highest rate of papaverine alkaloid was achieved in the medium with 20 g / L PEG and 60 mg / l tyrosine of 378.7 µg / g.

The response to morphogenetic alkaloids production decreased by increasing PEG and tyrosine added to the medium. The increased concentrations of morphine alkaloids may be due to the increase in the concentration of amino acids added to the medium as they are the structural initiator of many benzylisoguinoline alkaloids such as morphine, codeine, papaverine and noscapine (22 and 23). An important function of amino acids is to provide the carbon structure and nitrogenous components of alkaloids (24). Moreover, the increased concentrations of the amino acids will increase the dissolved nitrogen compounds within the cells, increasing their osmotic stress, forcing them to withdraw water from nearby cells which may lead to cell destruction and consequently a negative effect on the content of secondary compounds (25), or the increased stress could cause a decrease in the ability of cells to absorb the nutrients needed to produce the primary metabolites and thus a decrease of production of secondary products (26). These results were consistent with the results of 10 and 7 when tyrosine added to poppy plant significantly increased the production of morphine alkaloids compared to the comparison treatment.

The results showed that there was a gradual increase in the concentration of morphine alkaloids by increasing the concentration of PEG added to the medium. The exposure of the callus tissue to stress conditions stimulates the cells to produce secondary metabolites in larger quantities than the mother plant (27). The reduced responsiveness to the formation of morphine alkaloids by increased PEG and amino acid in the media may also lead to increased stress which has negatively affected cells and thus reduced the effectiveness of enzymes responsible for secondary products synthesis [28]. These results are consistent with (28 and 29) in regard to the production of secondary metabolites *in vitro*, and it is consistent with (29) in regard to the addition of PEG to the medium which resulted in an increase in the production of the glycosides in the callus of the *Stevia rebaudiana in vitro*.

Table (3) Effect of tyrosine (mg / l) and PEG (g / L) and their interaction in the production of morphine alkaloids (µg / g) in the callus of poppy plant.

Conc. of morphine alkaloids (µ/g)				Conc. of tyrosine (mg/l)
papaverine	**noscapine**	**codeine**	**morphine**	
132.2	172.7	192.9	255.0	0
183.2	211.2	261.1	271.9	20
223.0	277.9	288.5	314.7	40
257.9	276.3	272.2	325.6	60
6.4	6.7	6.6	6.4	LSD(0.05)
Conc. of PEG(g/l)				
96.6	119.1	129.5	171.6	0
172.4	204.9	223.6	258.8	10
281.1	289.0	329.3	350.3	20
246.1	325.0	332.2	386.6	30
6.1	6.2	6.2	6.2	LSD(0.05)
Interaction				
60.0	75.5	80.0	133.5	0-0
83.7	105.2	114.3	150.5	0-20
105.5	135.7	148.2	187.0	0-40
137.3	160.0	175.5	215.4	0-60
115.5	140.0	115.1	250.0	0-10
137.2	188.7	266.2	225.4	10-20
184.7	260.5	235.5	296.6	10-40
252.2	230.3	278.0	263.0	10-60
143.2	210.9	258.6	305.5	0-20
277.0	240.0	318.5	315.0	20-20
325.5	335.2	390.0	345.0	20-40
378.7	370.0	350.0	435.5	20-60
210.0	264.2	317.7	330.8	0-30
235.0	310.7	345.2	396.7	20-30
276.0	380.0	380.6	430.3	30-40
263.4	345.0	285.3	388.6	30-60
10.7	10.7	10.8	10.8	LSD(0.05)

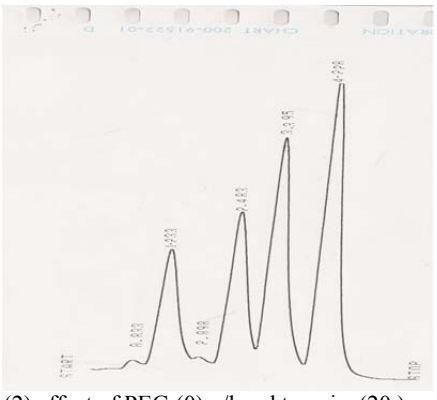

Fig (2) effect of PEG (0) g/l and tyrosine(20)mg/l

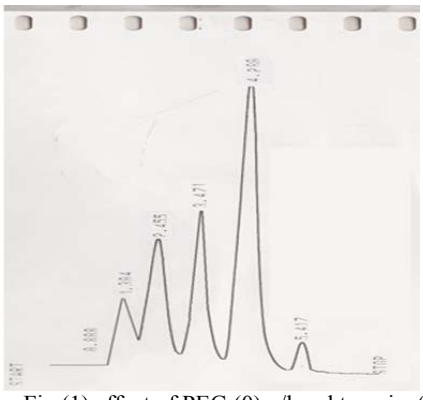

Fig (1) effect of PEG (0) g/l and tyrosine(0)mg/l

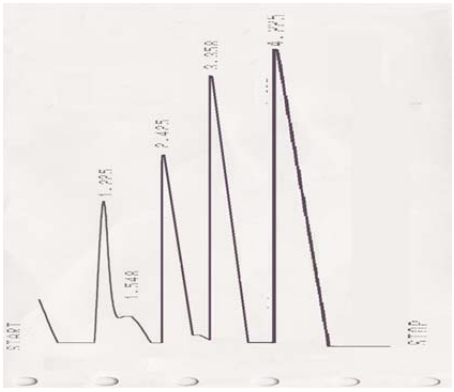

Fig (4) effect of PEG (0) g/l and tyrosine(60)mg/l

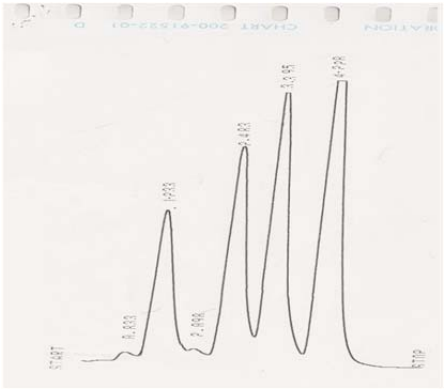

Fig (3) effect of PEG (0) g/l and tyrosine(40)mg/l

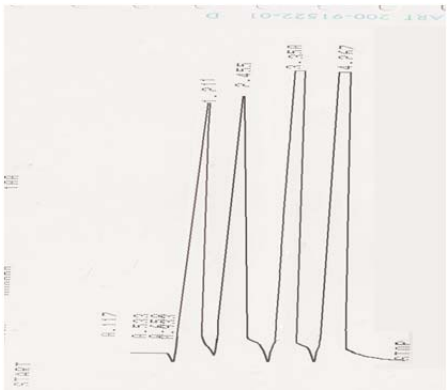

Fig (6) effect of PEG (30) g/l and tyrosine(0)mg/l

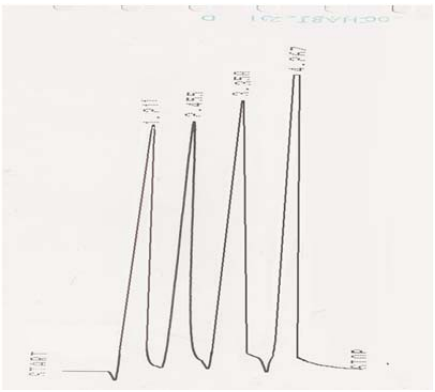

Fig (5) effect of PEG (20) g/l and tyrosine(0)mg/l

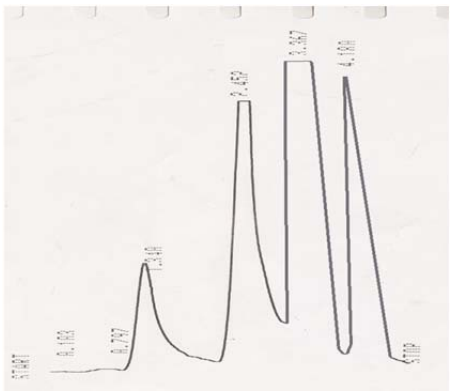

Fig (8) effect of PEG (10) g/l and tyrosine(20)mg/l

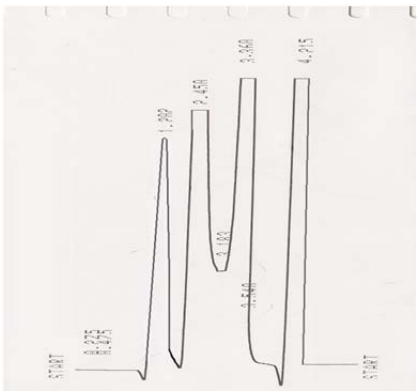

Fig (7) effect of PEG (30) g/l and tyrosine(20)mg/l

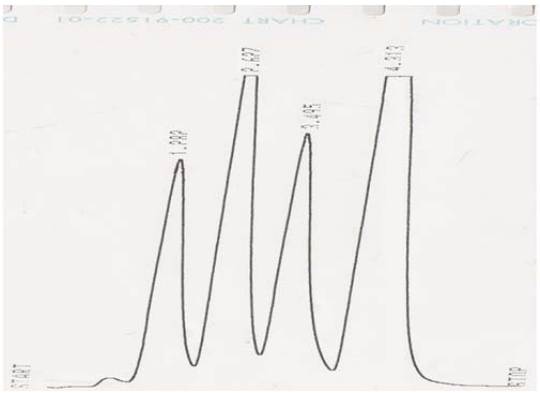

Fig (10) effect of PEG (10) g/l and tyrosine(40)mg/l

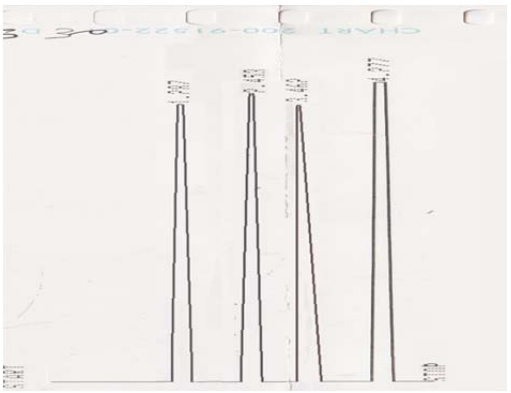

Fig (9) effect of PEG (10) g/l and tyrosine(0)mg/l

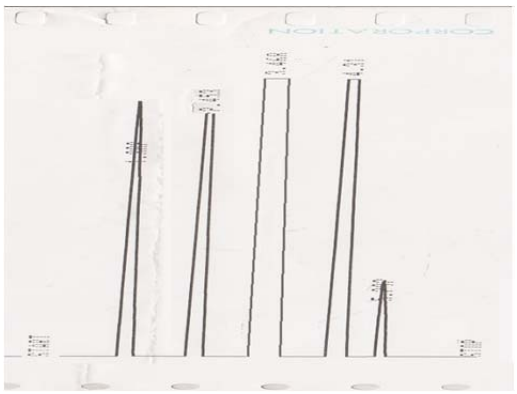

Fig (12) effect of PEG (20) g/l and tyrosine(20)mg/l

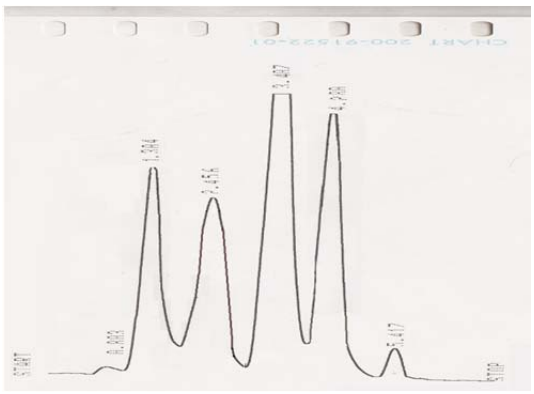

Fig (11) effect of PEG (10) g/l and tyrosine(60)mg/l

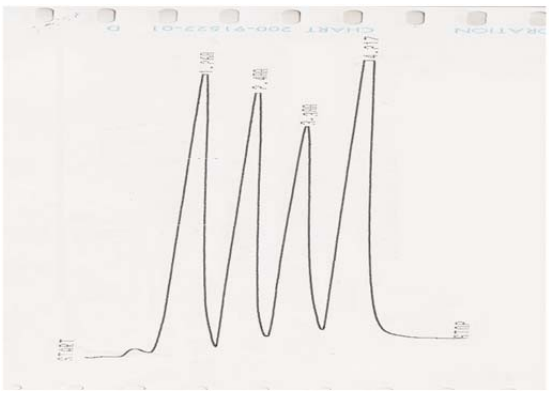

Fig (14) effect of PEG (20) g/l and tyrosine(60)mg/l

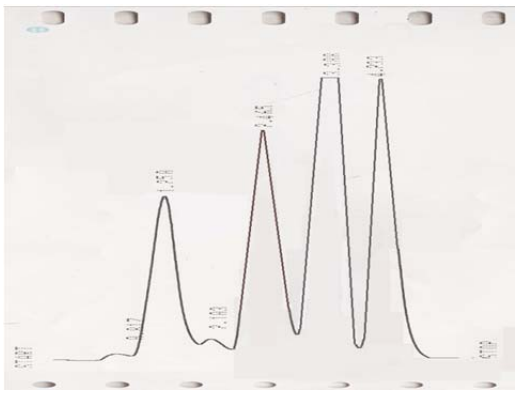

Fig (13) effect of PEG (20) g/l and tyrosine(40)mg/l

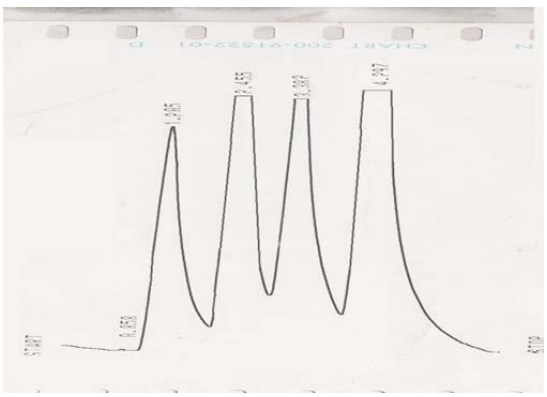

Fig (16) effect of PEG (30) g/l and tyrosine(60)mg/l

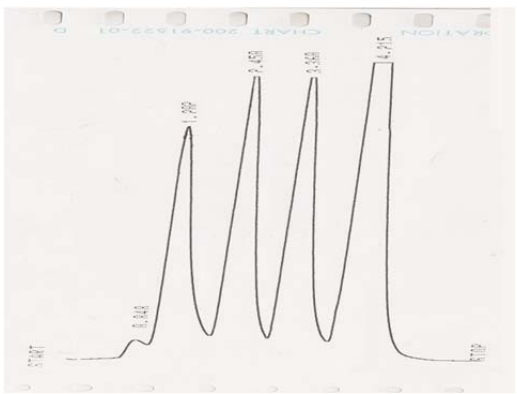

Fig (15) effect of PEG (30) g/l and tyrosine(40)mg/l

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