The effect of gamma ray and sugars on increasing the concentration of cardiac glycoside compounds of *Digitalis lanata In vitro*

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Received: Apr. 22, 2022

Accepted: May 30, 2022

Published: June 25, 2022

Abstract

The Explants that was used in the experiment to stimulate the production of glycoside compounds is the tips of the vegetative branches with a length of 2 cm obtained from the stage of vegetative multiplication growing from seeds treated with gamma radiation with a dose of (0, 30) gray, the tips of the branches were planted on MS nutrient medium prepared with different concentrations Of sucrose maltose (30, 60, 90, 120) g.l-1 in independent experiments to know the effect of irradiation, sucrose and maltose in stimulating the production of cardiac glycoside compounds, chlorophyll and carbohydrates. The data showed the superiority of maltose at a concentration of 90 g.l-1 significantly over the rest of the treatments, as it achieved the highest rate of production The active compounds Digitoxin, Digoxin, and Gitoxin reached (284.42, 372.65, 327.63) µg.g-1 dry weight, respectively. They also led to an increase in the amount of chlorophyll that reached 4.11 mg.g-1, while the concentration of 120 g.l-1 maltose achieved the highest rate in carbohydrate production which reached 4.51 mg.g-1. The irradiation treatment was superior in increasing the production of the active compounds, reaching (215.78, 286.28, 250.26) µg.g-1 dry weight, respectively. It also led to an increase in the amount of chlorophyll and carbohydrates, which amounted to (3.81 and 4.53) mg.g-1respectively.

Keywords: cardiac glycosides, digitalis, gamma ray, sucrose, maltose , In vitro

Introduction

The trend that took place in the past few decades is the return of interest in herbs and wild and cultivated plants and a return to what was known in the past as folk medicine or herbal medicine. There are several reasons for this trend, the most important of which is the consideration of plants as a laboratory for the production of natural pharmaceutical materials at a time when manufactured drugs cause side effects that affect the health of the patient, as well as a result of scientific and industrial progress, which provided effective methods and methods for the rapid production of effective compounds from the plant, especially through tissue culture of the parts

plants that contain these compounds and to find effective methods and methods for increasing medicinal compounds, and the ease of handling medicinal plants, their tissues and their products [1]. Moreover, scientific experiments have proven that the laboratory-manufactured active substance does not perform the physiological effect of the same active substance extracted from medicinal plants [2]. Medicinal plants are grown in the field to obtain the effective compounds, and this requires an area of land, service operations and a relatively long time until the plant's maturity is completed, in addition to the risks of field cultivation represented by unguaranteed environmental and climatic conditions that negatively affect the growth and yield of these plants. And then the quantity and type of active substances produced, and this prompted some researchers to produce some medically effective compounds with multiple uses through tissue culture hence the importance of tissue culture, which is one of the vital technologies that have played and still play an important role in human service, Especially in the field of propagating several types of plants because of the advantages of this method, perhaps the most important of which is the possibility of plants free from viral diseases and similar to the donor plant in a relatively short time and at any time of the year, as well as the use of this technology in research and applied fields, including breeding and improvement the plant, the production of medicinal drugs and medicines, and the study of the basic aspects of plant growth and development and secondary metabolism [3; 4]. *Digitalis lanata* L. is herbaceous annual or biennial flowering plant that grows naturally in eastern or western Europe, western and central Asia and northwestern Africa. It is grown as a garden ornamental plant for the beauty of its leaves in the first season, and for the beauty of its flowers in the second season or for medicinal use because it contains glycosides called Cardiac glycosides [5]. The group of glycosides constitutes an important part of the active substances in medicinal plants, and may cover most of the different types of known physiological effects،
Glycosides are the second largest group in terms of importance and prevalence after alkaloids, and they are defined as those compounds resulting from secondary metabolism in plants and consist of two parts linked to each other, one of which does not contain sugar and is called (aglycone), and the second part is a sugar, which is called (glycone), And the part that does not contain sugar molecules is a protein, which is the active part, and it represents the active substance and activates the work of enzymes. There are two main types of glycosides, namely cyanide and steroid glycosides, the second type contains 8 types of glycosides, the most important of which are cardiac glycosides, which are found in 11 plant families and more than 34 genera, the most important plant families that contain cardiac glycosides are Plantaginaceae, which contains *D. lanata*, which contains cardiac glycosides [6]. Cardiac glycoside compounds extracted from the digitalis mystic plant have been widely used as a drug for the treatment of various heart diseases, due to the high efficacy of these substances and because the failure of the heart muscle sometimes cannot be controlled by other drugs [7], as well as the indirect effects on the heart system, such as regulating the heartbeat and increasing the strength and speed of contraction of the heart muscle,

which has a positive effect [8]. In light of the foregoing, the current study was carried out in order to study the effect of stimulating doses of gamma rays and the building blocker on the level of plant growth and the production of medically active substances.

Materials and Methods

Preparation of Plant materials

The explants that was cultivated in the experiment to stimulate the production of glycosides is the ends of shoots with a length of 2 cm obtained from the stage of vegetative multiplication growing from seeds that were treated with gamma rays with a dose of (0, 30) gray, the tips of the branches were planted on the surface of the MS medium prepared with sucrose and maltose at concentrations (30,60,90,120) g.l-1 independent experiments with ten repetitions, the cultures were incubated in the growth room at a temperature of 25°C +_2 and a light intensity of 1000 lux for 16 hours/day. The study indicators, which included measuring the average concentration of glycoside compounds, chlorophyll and carbohydrates, were taken from the shoots of the seedlings after a month of planting.

Preparation of alcoholic extracts of shoots produced by stimulating treatments

The process of extraction and quantitative and qualitative estimation of the glycoside compounds (Digitoxin, Digoxin, Gitoxin) was carried out by analyzing the samples by HPLC as follows:-

The shoots obtained from the stimulation stage were dried at laboratory temperature and extracted by the ultrasonic frequencies effect according to what was mentioned by [9], 1 gm of dry matter was taken from each sample and crushed in ceramic mortar, then 40 ml of methanol at concentration of 70% was added to it, then it was placed in the ultrasonic frequency device in a water bath at a frequency of 100 Hz and at room temperature for 20 minutes, after that the samples were filtered With 0.13 mm filter paper, the process was repeated again, then the samples were ready for reading in the HPLC device.

Chlorophyll measurement

The chlorophyll pigment was estimated using the method [10] and my agencies 1gm of vegetative growths grown in tissue culture was taken and placed in a ceramic mortar and 9 ml of 85% acetone was added to it, then the plant tissues were crushed until a colorless residue was obtained. A ml was withdrawn from the solution and the appropriate dilution was made for it. The optical absorption of the solution was read at wavelengths 663 and 645 nm using a spectrophotometer, according to the total content of chlorophyll and according to the following equation:

$$\textbf{Total chlorophyll mg/l} = 20.2D(645) + 8.02D(663)$$

Carbohydrate measurement

The method of [11] called the method of phenol sulfuric acid was followed. Where 0.1 g of the crushed samples was taken and placed in a dry test tube, 10 ml of ethyl alcohol was added to it, 70% concentration. 1 ml of 5% phenol reagent was added to it with 5 ml of 99% sulfuric acid. The mixture was mixed well and incubated in a water bath at a temperature of (25-30) C for 20 minutes. The tubes were left to cool. The concentration of carbohydrates was estimated by measuring the intensity of the color by means of a spectrophotometer at the wavelength 488 nm, with three replicates for each treatment and concentration, and then compared with the standard curve of carbohydrates.

Statistical analysis

The experiments included in the study were carried out using Completely Randomized Design (CRD) and factorial experiments, and the results were analyzed using the statistical program (SAS, 2004) and the averages were compared according to the Least Significant Difference (LSD) test at a probability level of 0.05 [12].

Results and Discussion

The effect of irradiation and sucrose and the interaction between them on the rate of cardiac glycoside production

Table (1) and figures (1 and 2) show that sucrose concentrations had a significant effect on stimulating cardiac glycoside compounds from growing vegetative branches on MS food media, as the medium prepared with 120 g.l-1 sucrose significantly outperformed the rest of the other concentrations and achieved the highest rate of Digoxin. , Digitoxin and Gitoxin amounted to (277.70, 329.57, 300.87) µg.g-1 dry weight, respectively, while the lowest rate of cardiac glycoside compounds at the concentration of 30 g.l-1 sucrose reached (67.62, 106.66, 88.14) µg.g-1 dry weight, respectively. The irradiation treatment was also significantly superior and achieved the highest rate in the concentration of cardiac glycosides, which amounted to (204.16, 261.09, 239.49) µg.g-1 dry weight, respectively, compared to the non-irradiated treatment, which achieved the lowest rate of the same compounds amounted to (142.81, 179.98, 152.00) µg.g-1 dry weight on relay. As for the effect of the dual interaction, the results of the same table showed the superiority of the irradiation treatment and the interaction with the concentration of 120 g.l-1 sucrose significantly in the rate of production of cardiac glycoside compounds amounted to (312.13, 369.02, 340.11) µg.g-1 dry weight, respectively, while the lowest rate was recorded in the treatment other than Irradiated and overlapping with concentration of 30g/L sucrose reached (42.12, 58.18, 48.09) µg.g-1 dry weight, respectively.

Table (1): The effect of irradiation and sucrose and the interaction between them on the rate of concentration of cardiac glycosides compounds from the vegetative branches of digitalis after four weeks of cultivation on MS medium

Sucrose con $g.l^{-1}$	cardiac glycosides con $\mu g.g^{-1}$		
	Digoxin	Digitoxin	Gitoxin
30	67.62	106.66	88.14
60	142.40	187.81	163.63
90	206.22	256.62	230.85
120	277.70	329.57	300.87
L.S.D.(0.05)	1.23	1.23	1.23
irradiation			
radiant (30)gray	204.16	261.09	239.49
not irradiated	142.81	179.98	152.00
L.S.D.(0.05)	0.87	0.87	0.86
overlap			
30- radiant	93.11	155.14	128.18
60- radiant	169.02	225.16	205.07
90- radiant	242.37	295.05	278.60
120- radiant	312.13	369.02	340.11
30- not irradiated	42.12	58.18	48.09
60- not irradiated	115.78	150.46	122.19
90- not irradiated	170.07	218.18	183.10
120- not irradiated	243.27	290.11	255.62
L.S.D.(0.05)	1.73	1.74	1.73

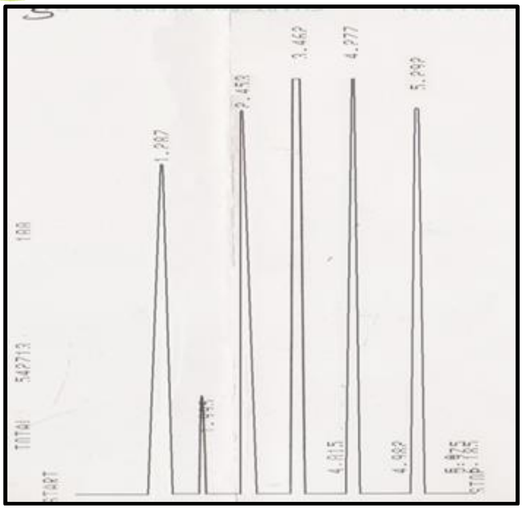

Figure (1): Effect of irradiation 30 gray on and sucrose 120 g.l-1 glycoside production

Seq	Compound	Retention time	Area
1	Digoxin	2.455	699665.40
2	Digitoxin	3.458	970264.28
3	Gitoxin	4.277	811616.41

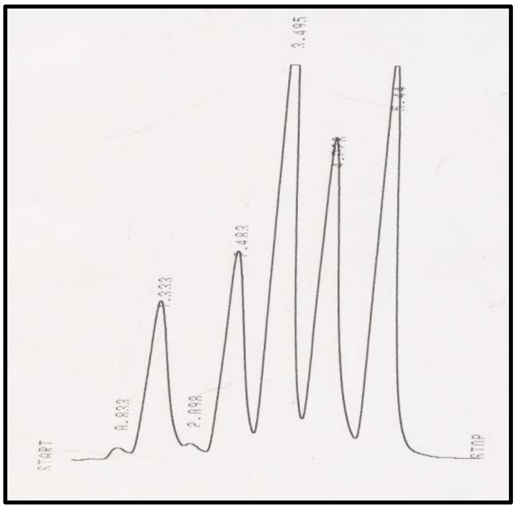

Figure (2): Effect of sucrose at a con 120 g.l-1 glycoside production

Seq	Compound	Retention time	Area
1	Digoxin	2.455	692088.85
2	Digitoxin	3.350	1079432.82
3	Gitoxin	4.267	844445.94

The effect of irradiation and sucrose and the interaction between them on the average concentrations of chlorophyll and carbohydrates

It is noticed from table [2] that the different concentrations of sucrose added to the MS medium had a significant effect on the rate of production of chlorophyll and carbohydrates in the vegetative branches of the digitalis mystic plant. The lowest rate when the neutral treatment was 2.10 mg.g-1, and the concentration 120 g.l-1 recorded the highest rate of carbohydrates amounting to 4.26 mg.g-1compared to the neutral treatment, which gave the lowest rate of 2.91 mg.g-1, the irradiation also affected the rate of production of chlorophyll and carbohydrates, as the irradiation treatment was significantly superior and gave the highest rate of (3.65, 4.23) mg.g-1 respectively, compared to the non-irradiated treatment which gave the lowest rate of (3.04, 3.50) mg.g-1 respectively. With regard to the effect of the bilateral interaction, the irradiation treatment excelled at a concentration of 90 g.l-1 sucrose, and recorded the highest rate of chlorophyll amounted to 4.25 mg.g-1, and the same treatment excelled with the interaction with a concentration of 120 g.l-1 sucrose and gave the highest rate of carbohydrates amounting to 4.63 mg.g-1, while it was recorded The lowest rate of chlorophyll and carbohydrates when un irradiated and at a concentration of 30 g.l-1 sucrose reached (1.68, 2.54) mg.g-1, respectively.

Table (2): The effect of irradiation and sucrose and the interaction between them on the average concentration of chlorophyll and carbohydrates from the vegetative branches of the digitalis plant after four weeks of cultivation on MS medium

Sugar con $g.l^{-1}$	Chlorophyll con $mg.g^{-1}$	Carbohydrate con $mg.g^{-1}$
30	2.10	2.91
60	3.59	4.20
90	3.93	4.08
120	3.78	4.26
L.S.D.(0.05)	0.03	0.02
irradiation		
radiant (30)gray	3.65	4.23
not irradiated	3.04	3.50
L.S.D.(0.05)	0.02	0.01
overlap		
30- radiant	2.51	3.28
60- radiant	3.70	4.59
90- radiant	4.25	4.40
120- radiant	4.15	4.63
30- not irradiated	1.68	2.54
60- not irradiated	3.48	3.81
90- not irradiated	3.60	3.75
120- not irradiated	3.40	3.89
L.S.D.(0.05)	0.03	0.2

Effect of irradiation and maltose and the interaction between them on the rate of cardiac glycoside production

The results of Table (3) and Figure (3 and 4) indicate that there are significant differences between the rates of cardiac glycoside concentrations: Digoxin, Digitoxin, and Gitoxin that were extracted from the vegetative branches of the Digital Woolen plant. The food prepared with 90 g.l-1 maltose was significant and gave the highest rate of cardiac glycoside compounds amounted to (284.42, 372.65, 327.63) µg.g-1 dry weight, respectively, compared to the food medium prepared with 30 mg.l-1 maltose, which achieved the lowest rate of cardiac glycoside compounds amounted to (87.67, 134.66, 98.15) µg.g-1dry weight respectively. the irradiation treatment was significantly superior in achieving the best rates of cardiac glycosides, reaching (215.78, 286.28, 250.26) µg.g1dry weight, respectively, compared to the non-irradiated treatment, which achieved rates of (164.12, 191.21, 176.15) µg.g-1dry weight respectively. As for the effect of the interaction, the data of the table and the same figures indicated that the irradiation treatment and the interaction with the concentration of 90 g.l-1 of maltose were significantly superior to the cardiac glycoside compounds (308.75, 410.54, 360.11.(

µg.g-1 dry weight, respectively, while the non-irradiated treatment and interaction with the concentration of 30 g.l-1 maltose achieved the lowest mean of concentration of cardiac glycosides compounds (58.03, 105.13, 58.10) µg.g-1dry weight, respectively.

Table (3): The effect of irradiation and maltose and the interaction between them on the rate of concentration of cardiac glycosides compounds from the vegetative branches of Woolly digitalis after four weeks of cultivation on MS medium

Maltose con $g.l^{-1}$	cardiac glycosides con $\mu g.g^{-1}$		
	Digoxin	Digitoxin	Gitoxin
30	87.67	134.66	98.15
60	232.55	292.06	255.16
90	284.42	372.65	327.63
120	150.17	205.62	171.87
L.S.D.(0.05)	1.22	1.22	1.22
irradiation			
radiant (30)gray	215.78	286.28	250.25
not irradiated	164.12	191.21	176.15
L.S.D.(0.05)	0.85	0.85	0.85
overlap			
30- radiant	117.31	164.19	138.20
60- radiant	258.97	328.08	290.15
90- radiant	308.75	410.54	360.11
120- radiant	178.09	242.32	212.56
30- not irradiated	58.03	105.13	58.10
60- not irradiated	206.13	156.04	220.17
90- not irradiated	270.09	334.76	295.15
120- not irradiated	122.24	168.92	131.18
L.S.D.(0.05)	1.73	1.74	1.73

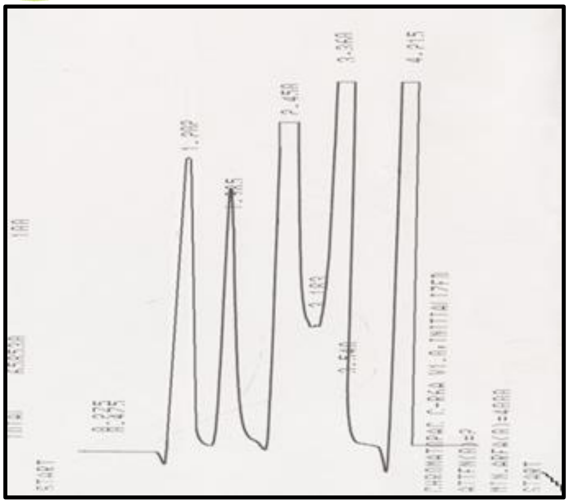

Figure (1): Effect of irradiation 30 gray maltose 90 g.l-1 glycoside production

Seq	Compound	Retention time	Area
1	Digoxin	2.450	580502.83
2	Digitoxin	3.366	862620.74
3	Gitoxin	4.215	680392.07

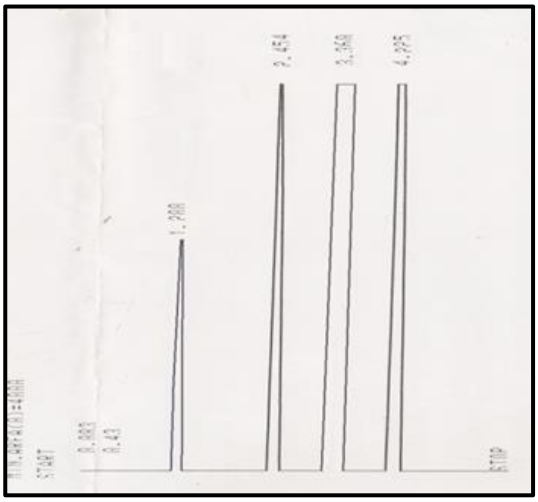

Figure (2): Effect of maltose 90 g.l-1 on glycoside production

Seq	Compound	Retention time	Area
1	Digoxin	2.454	462057.57
2	Digitoxin	3.360	673205.97
3	Gitoxin	4.225	516291.31

Effect of irradiation and maltose and the interaction between them on the average concentrations of chlorophyll and carbohydrates

Table (4) shows the effect of different concentrations of maltose on the rate of chlorophyll and carbohydrate synthesis in the vegetative branches of the digitalis woolly plant, where a significant increase in the average amount of chlorophyll at a concentration of 90 g.l^{-1} maltose was 4.11 mg.g^{-1} compared to the comparison treatment, which gave an average of 2.24 mg.g^{-1}, and the concentration of 120 g.l^{-1} of maltose recorded the highest rate of carbohydrates which reached 4.51 mg.g^{-1} compared with the control treatment which gave the rate of 3.12 mg.g^{-1}.It is also noted from the table data that the irradiation treatment was significantly superior in the rate of production of chlorophyll and carbohydrates, which amounted to (3.81, 4.53) mg.g^{-1} respectively compared to the non-irradiated treatment, which gave a rate of (3.21, 3.65) mg.g^{-1} respectively. As for the effect of the dual interaction, the irradiation treatment at a concentration of 90 g.l^{-1} of maltose achieved the highest rate of chlorophyll that reached 4.46 mg.g^{-1}, while the lowest rate of chlorophyll when treating the non-irradiated and the concentration of 30 g.l^{-1} of maltose was 1.88 mg.g^{-1}, and the treatment also affected The interaction in the rate of carbohydrate formation, the highest rate was achieved when the irradiation treatment and the concentration of 120 g.l^{-1} maltose reached 5.06 mg.g^{-1}, while the non-irradiated treatment and at the concentration of 30 g.l^{-1} maltose gave the lowest rate of 2.86 mg.g^{-1}.

Table (4): The effect of irradiation and maltose and the interaction between them on the average concentration of chlorophyll and carbohydrates from the vegetative branches of the woolly digitalis plant after four weeks of cultivation on MS medium

Maltose con $g.l^{-1}$	Chlorophyll con $mg.g^{-1}$	Carbohydrate con $mg.g^{-1}$
30	2.24	3.12
60	3.97	4.40
90	4.11	4.33
120	3.72	4.51
L.S.D.(0.05)	0.02	0.02
irradiation		
radiant (30)gray	3.81	4.53
not irradiated	3.21	3.65
L.S.D.(0.05)	0.17	0.01
overlap		
30- radiant	2.60	3.39
60- radiant	4.32	4.90
90- radiant	4.46	4.78
120- radiant	3.88	5.06
30- not irradiated	1.88	2.86
60- not irradiated	3.63	3.90
90- not irradiated	3.75	3.87
120- not irradiated	3.56	3.95
L.S.D.(0.05)	0.03	0.02

What was observed, in general, is that the concentrations of both types of sugars had a significant effect on the characteristics shown in the previous tables and figures, so that there was a significant increase in these characteristics and within certain concentrations, and this is a natural and general matter due to the fact that the increase in the concentration of sugar means an increase in the energy available to complete the various vital processes It is carried out by the cultivated plant parts and increases the various metabolic products as well as it maintains the osmotic pressure of the food medium [13]. These results are in agreement with the findings of [14] that increasing the concentration of sucrose in the medium from 3-12% led to an increase in the manufacture of the Digoxin compound in the cultures of the vegetative branches of Digitalis davisiana, and with what [15] found, there was an increase in the concentration of Digitoxin and Gitoxin when increasing the concentration of sucrose added to the medium of Digitalis purpurea from 3-12%. The data of the same tables also showed that the increase in maltose concentrations to high levels led to a decrease in the rates of the studied traits. As it was shown that the type of sugar added to the food medium had a significant effect on the studied characteristics and shown in the tables. It is clear that maltose sugar had the greatest effect on the quantities of these compounds.

The reason for this may be due to its being a double sugar that disintegrates during the process of sterilizing the food medium containing it into two units of Glucose sugar, and as it is known that glucose sugar enters into the formation of primary cardiac glycosides such as Lanatoside C, Lanatoside B, and Lanatoside A, which in turn are converted during the drying process of plant parts to Digoxin, Gitoxin, Digitoxin respectively [16]. For this reason, it was observed that the presence of Maltose sugar in the food medium had the greatest effect on the formation of cardiac glycoside compounds. This result is consistent with the findings of [17] that the quality and concentration of sugar used had a significant effect on the amount of cardiac glycoside compounds. It also agrees with the findings of [18] in her study that aimed to increase the Digitonin in the vegetative branches of Digitalis purpurea In vitro by adding different types and concentrations of sugars. As for the other traits that were studied and whose results are shown in the tables, it was also noted that the presence of maltose sugar in the food medium had the greatest effect on these traits, and the reason may be due to it being a source of glucose sugar as well as a source of energy with low concentrations of it compared to other sugars, and this was confirmed by [19 and 20] that maltose is a great source of energy.

It was concluded from this study that there is a positive effect of gamma ray and sugars on increasing the concentration of cardiac glycoside compounds of Digitalis lanata In vitro

References

1) Bisset, N.G. (2007). Herbal Drug and Phytopharmaceuticals. Boca Raton, EL, CRC Prees, UK.

2) Qin, S.; Jiang, P. and Tseng, C. (2005). Transforming kelp into a marine bioreactor. Trends in Biotechnology, 23(5): 264-268.

3) Kasumi, M.; Takastu, Y. and Manabe, T. (2005). Callus formation and plant regeneration from root explant of gladiolus (Gladiolus x grandiflora hort.). Journal-Horticultural Research (Japan), 4(1):7-10.

4) Gupta, S. and Ibarakl, Y. (2006). Plant Tissue Culture Engineering, Vol.6. Springer, Germany.

5) Karimi, M. and Kazemitabar, S.K. (2013). Study on the production of cardiac glycosides in direct regeneration shoots of foxglove (*Digitalis nervosa)*. International Journal of Agronomy and plant production, 4(2): 204- 211.

6) Dabrowski, W.M. and Sikorski, Z.E. (2005). Toxins in food. CRC PRESS., Washington. D.C. USA.

7) Desta, B. (1982). HPLC Analysis of Digoxin and Digitoxin: Development of Methods for Dosage from Assay and Separation of Potential Impurities and Metabolites. Doctoral Thesis. The University of British Colombia, USA.

8) Reddy, A.B. (2010). *Digitalis therapy* in patients with congestive heart failure. International Journal of Pharmaceutical Science Review and Research, 3(2): 90- 95.
9) Pellati, F.; Brunib, R.; Bellardi, M.G. and Benvenutia, S. (2009). Optimization and validation of a high- performance liquid chromatography method for the analysis of cardiac glycoside in *Digitalis lanata*. Journal of chromatography A, 1216-3269.
10) Goodwin, T.W. (1976). Chemistry and Biochemistry of Plant Pigment. 2nd edition Academic Press. London, UK.
11) Herbert, D.P.; Philips, J. and Stange, R.E. (1971). Method in microbiology. Narri,J.R. and D.W.Robbin (eds.). Academic Press. London, UK.
12) Al-Sahuki, M. and Wahib, K. A. (1990). Applications in designing and analyzing experiments. Ministry of Higher Education and Scientific Research - Iraq.
13) George, E.F. (2008). The components of plant tissue culture media, organic adition, osmotic and PH effects. Plant propagation by tissue culture 3rd edition, Academic Press. London, UK.
14) Gurel, E.; Yucesan, B.; Aglic, E.; Verma, S. K. and Sokmen, M. (2011). Regeneration and cardiotonic glycoside production in *Digitalis davisiana* Heywood (*Alanya foxglove*) . Journal of Plant Cell, Tissue and Organs Culture, 104: 217-225.
15) Jitendra, G.P.; Ahire, M.L.; Panda, S. and Bhatt, V.P. (2013). *In vitro* propagation and production of cardiotonic glycosides in shoot cultures of *Digitalis purpurea* by elicitation and precursor feeding. Applied Microbiology and Biotechnology, 97: 2379- 2393.
16) Theurer, C.; Treumann, H. J.; Faust, T. and Kreis, W. (1994). Glycosylation in cardenolide biosynthesis. Plant Cell, Tissue and Organ Culture, 38:327-335.
17) Akula, R. and Ravishankar G.A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. Plant Signaling and Behavior, 6 (11): 1720- 1731.
18) Awad, Z.J. (2010). *In vitro* effect of cholesterol and different sugars on digitonin roduction in multiplied shoot of *Digitalis purpurea L.* Plant. Iraqi Journal of Pharmaceutical Sciences, 19(1):22-35.
19) Huang, W.L. and Liu, L.F. (2002). Carbohydrate metabolisms in rice during callus induction and shoot regeneration induced by osmotic stress. Botanical Bulletin of Academia Sinica, 43: 107-113.
20) Fatima, Z.; Abdul Mujib; S. F.; Arshi, A. and Umar, S. (2009). Callus induction biomass growth and plant regeneration in *Digitalis lanata* Ehrh.: influence of plant growth regulators and carbohydrates. Turkish Journal of Botany, 33:393-405.