In Vitro Effect of Cholesterol and Different Sugars on Digitonin Production in Multiplied Shoots of *Digitalis purpurea* L. Plant

Zainab J.Awad[*,1]

* Department of Pharmacognosy ,College of Pharmacy, University of Baghdad ,Baghdad, Iraq.

Abstract

Production of the steroidal saponin digitonin in multiplied shoots of *Digitalis purpurea* , (var. Excelsior Mixed) has been achieved in vitro by two experiments. In the experiment 1, shoot tips (1cm length) explants from the sterilized seedlings were excised and cultured on MS medium (Murashige and Skoog medium) supplemented with 0.5 mg/L TDZ (thidiazuron) and cholesterol at the concentrations 0.0, 0.1, 0.3, 0.5, 1.0, 1.5, 2.0 or 4.0 mg/L. After 45 day, results showed that the treatment with 0.5 mg/L TDZ and 2.0mg/L cholesterol had a positive effected on increasing the dry weight of multiplied shoots and their production of digitonin when compared with other treatments, where this treatment gave 2.98 g dry weight of multiplied shoots and digitonin at amount of 64.42 µg/g dry weight, while the other studied characteristics of multiplied shoots (content the total chlorophyll, soluble sugars and starch.) also this treatment had appositive effected and was gave the following values:- (3.51 mg/g fresh weight, 5.06% ,6.09%) respectively. After that experiment 2 was carried out . The objective of this experiment was to increase the degree of digitonin production in multiplied shoots compared with the experiment 1.Therfore in the experiment 2, we were selected supplements the best treatment of experiment 1(0.5mg /L TDZ and 2.0mg/ L cholesterol) and supplemented to the MS medium with the sugars glucose, fructose, sucrose or maltose at the concentrations 30,50,70 or 90 g/L for each sugar. After 45 days results showed that the treatment with 0.5 mg/L TDZ, 2.0 mg/L cholesterol and 50g/L maltose was the best a compared with other treatments, where this treatment gave 4.52 g dry weight of multiplied shoots and digitonin at amount of 191.87 µg/g dry weight . The content of multiplied shoots from the total chlorophyll, soluble sugars and starch also this treatment gave highest values and they are 4.97 µg/g fresh weight , 5.91% and 8.30% respectively .

Key words: *Digitalis purpurea,* Cholesterol, Sugars, Digitonin .

الخلاصة

انتاج السيتروئيد الصابوني الديجيتونين (Digitonin) في الافرع المتضاعفة لنبات الديجيتالس *Digitalis purpusea* (ضرب Excelsior Mixed) تم انجازه خارج الجسم الحي بواسطة تجربتين في التجربة الأولى، فُصلت اطراف الافرع بطول ١ سم من البادرات المُعقمة وزرعت على وسط MS (مراشيك وسكوك) مضافاً اليه TDZ بتركيز ٠.٥ ملغم/لتر والكولسترول بالتراكيز ٠.٠، ٠.١، ٠.٣، ٠.٥، ١.٠، ١.٥، ٢.٠ او ٤.٠ ملغم/لتر، بعد ٤٥ يوماً أظهرت النتائج بان المعاملة المتالفة من ٠.٥ ملغم/لتر TDZ مع ٢.٠ ملغم/لتر كولسترول كانت ذات تأثير ايجابي على زيادة الوزن الجاف للأفرع المتضاعفة ومحتواها من الديجيتونين مقارنةً مع المعاملات الاخرى، حيث بلغ الوزن الجاف للأفرع المتضاعفة ٢.٩٨غم محتوية على الديجيتونين بمقدار ٦٤.٤٢ مايكروغم/غم وزن جاف. اما الصفات الاخرى التي دُرست للافرع المتضاعفة وهي محتواها من الكلوروفيل الكلي، السكريات الذائبة والنشاء، فقد كانت هذه المعاملة أيضا ذات تأثير إيجابي على هذه الصفات حيث أعطت أفضل القيم وهي :- (٣.٥١ ملغم/غم وزن طري , ٥.٠٦%، ٦.٠٩%) وعلى التوالي. بعد ذلك أجريت التجربة الثانية والهدف منها هو إمكانية إحداث زيادة أكثر في إنتاج الديجيتونين في الأفرع المتضاعفة مقارنة مع التجربة الاولى، لذلك اضيف الى وسط MS اضافات أفضل معاملة في التجربة الأولى وهي ٠.٥ ملغم /لتر TDZ و ٢.٠ ملغم/لتر كولسترول مع السكريات سكروز، كلوكوز، فركتوز او مالتوز وبالتراكيز ٣٠، ٥٠، ٧٠ او ٩٠ غم/لتر لكل سُكر على حده. بعد ٤٥ يوماً، اظهرت النتائج بان المعاملة المتالفة من ٠.٥ ملغم/لتر TDZ، ٢.٠ ملغم/لتر كولسترول مع ٥٠ غم/لتر مالتوز كانت هي الأفضل، حيث بلغ الوزن الجاف للأفرع المتضاعفة ٤.٥٢غم محتوية على مادة الديجيتونين بمقدار ١٩١.٨٧ مايكروغم/غم وزن جاف، أما محتوى الأفرع المتضاعفة من مادة الكلورفيل، السكريات الذائبة والنشاء فقد أعطت هذه المعاملة ايضاً أفضل القيم وهي: (٤.٩٧ملغم/غم وزن طري، ٥.٩١%، ٨.٣٠%) على التوالي.

Introduction

Digitalis purpurea (Fam: Scrophulariaceae) is a biennial herb plant [(1)]. In the medicinal and pharmacy fields this plant has a great benefit because it contains the cardiac glycosides like" Digitoxin" and steroidal saponin glycosides like" Digitonin" both of these groups representing compounds of considerable importance to the pharmaceutical industry[(2)]. As yet, these compounds can not be synthesized economically by chemical or microbiological methods [(3)] .

1Corresponding author E- mail : merciful2003@ yahoo.com
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In the field of pharmacy and medicine, the steroidal saponin digitonin had a great importance because it was used to determine the ratio of cholesterol in the blood, plasma and bile acids but the great importance and interest of this compound and some steroidal saponins like the Diosgenin owing to their relation ship with some important substances as progesteron hormone, cortisone and vitamin D where the structures of these steroidal saponins have the same nucleus of these substances , then these steroidal saponins can be used as a precursors for the production of these substances . Originally these substances were isolated from expensive and limit sources like adrenal cortex and bile acids of cattle[4,5] For this important many studies using modern technology like tissue culture technique (in vitro) to increasing the production of steroidal saponins in some plants . Where *via* the usage of this technology different explants could be cultured and inducted to different stage of growths as a source for these important medical compounds and in a high concentrations without being restricted to the environmental conditions as well as cleans of the pharmaco which could be obtained [6].Therefore the objective of our study using this technique to inducted the multiplication shoots of *Digitalis purpurea* (var exlecior mixed) and increasing their production from the digitonin compound .

Material and method

First :- Tissue culture of the plant *Digitalis purpurea*; this work went through the following steps:-

1. ***Preparation of sterilized seedlings*** : That was, initiation a specific culture of seedlings free from any contamination which it will be a source for shoot tips and that was performed by:-
   - ***A. Medium preparation :*** Basic medium composition was that of MS[7] with 30g/L sucrose. The pH of this medium was adjusted to 5.5 then gelled with 8g/L Difco Bacto agar . Ten milliliters of this medium was dispensed into 25x125 ml tubes. After capped with clear autoclavable lids, tubes were autoclaved at 121^{0}C for 20 min .
   - ***B. Seeds germination :*** Seeds of the plant *Digitalis purpurea* (var exlecior mixed) were surface sterilized in 70% ethyl alcohol for 30 second. This was followed with three sterilize distilled rinses[8], after that the seeds were cultured on MS medium , and allowed to germinate by incubated in growth room at 25 ± 2 °C with artificial illumination given by fluorescent light of about 1600 lux at 16 hour / day[9]. Incubation was for30 days and then the seedlings are ready to take the explants shoot tips which excised and cultured in step 2.
2. ***Shoots multiplication and the production of steroidal saponin "Digitonin":*** These processes were inducted by two experiments:-

Experiment 1

This experiment was carried out to inducted multiplication the cultured shoot tips and their digitonin production and by the following steps:-

1. ***Preparation of the nutrient medium:-*** Basic medium MS was used supplemented with 30g/L sucrose plus 0.5 mg/L TDZ [10] (for multiply the cultured shoot tip) and Cholesterol at a concentrations of 0.0, 0.1, 0.3 , 0.5, 1.0, 1.5, 2.0 or 4.0mg/L (for induct the production of digitonin). The pH of the media were adjusted to 5.5 and gelled 8g/L Difco Bacto agar. Twenty five milliliter medium was dispensed into 25x125 ml tubes. After capped with clear autoclavable, tubes were autoclaved at 121 ^{0}C for 25 min .
2. ***Cultured the shoot tip:-*** Excised the shoot tips (1cm length) from the sterilized seedlings and one shoot tip in every tube was cultured, then allowed to multiplication by incubated in a growth room at the same condition of seeds germination [11] .

Experiment 2

This experiment was carried out after experiment 1 .The objective of this experiment was to increasing the degree of digitonin production in multiplied shoots compared with the experiment 1. Therefore we were selected supplements the best treatment of experiment 1 (0.5 mg/L TDZ and 2.0 mg/L cholesterol) and supplemented to MS medium with the sugars glucose, fructose, sucrose or maltose at a concentrations of 30,50,70 or 90 g/L for each sugar , the pH of these media and their gelled , distribution, autoclaving , cultured the shoot tip and condition the incubated in a growth room as in the experiment 1.

Second :- Analysis of the multiplied shoots .

After 45 days in cultures, multiplied shoots are ready for studying the following characteristics:-

1. Total chlorophyll content:- This characteristic was estimated according to goodwing method[12].
2. Dry weight estimation:- The multiplied shoots were taken out of the tubs and washed in water to remove agar from them, after wiped with clean cloth ,the shoots were separated and differentiated on filter paper to be dried in an oven at 40°C until they reached constant weight [13], later, shoots were grinded to hard powder and kept in paper sacks in dry condition and dark place .
3. Soluble sugars and starch content:- These characteristics were estimated according to joslyn method [14].
4. Extraction of the steroidal saponin "digitonin" according to the procedure of Brain et al[15]
5. HPLC analysis of digitonin:-This analysis was carried out by using [16] C 18 – column and the mobile phase used was acetonitril: water (25:75). The flow rate was 1.5 ml/min .The measurements were carried out , using uv detector at 205 nm. Statistical analyses in our study were made by using complete randomized design. The mean differences were tested utilizing LSD test. [17]

Results

Multiplication of cultured shoot tip and yielded the highest number of shoots in the experiment 1 and 2 could be inducted by supplemented 0.5 mg/L TDZ to MS medium . In experiment 1, supplemented different concentrations of cholesterol to MS medium with o.5 mg/L TDZ , showed positive effect on digitonin production in multiplied shoots and other studied characteristics for these shoots (total chlorophyll content, soluble sugar, starch and dry weight) as show in table (1). But a significant effect was observed with the concentration 2.0 mg/L cholesterol and 0.5 mg/L TDZ. Therefore we were selected this treatment and was used in the experiment 2 with different sugars . In this experiment , all concentrations of maltose sugar had signification effect on digitonin production, but the best concentration was 50 g/L which had more effect on the production of this compound and other studied characteristics of multiplied shoots as compared with other treatments of this experiment (Table 2 ,3 ,4 and 5).

Table 1 : Effect of different concentrations of Cholesterol. with the presence of 0.5 mg/L TDZ on the content of multiplied shoots of *Digitalis purpurea* from the total chlorophyll , soluble sugars , starch , digitonin and on their dry weight.

Concentration mg/L	Total Chlorophyll mg/g fresh weight	Soluble Sugars (%)	Starch(%)	Digitonin µg/g dry weight	Dry weight (g)
0.0	3.31	4.36	5.11	33.13	2.11
0.1	3.39	4.44	5.14	33.60	2.09
0.3	3.33	4.42	5.31	35.71	2.13
0.5	3.47	4.54	5.24	37.56	2.36
1.0	3.49	4.61	5.66	43.49	2.41
1.5	3.47	4.88	5.84	49.46	2.71
2.0	3.51	5.06	6.09	64.42	2.98
4.0	3.48	4.41	5.86	44.40	2.31
L.S.D.5%	0.05	0.21	0.17	6.19	0.23

Table 2 : Effect of different concentrations of sugars with the presence of 0.5mg/L TDZ and 2mg/L Cholesterol on total chlorophyll content of multiplied shoots of *Digitalis purpurea* .

Concentration g/L	Total Chlorophyll mg/g fresh weight.			
	Sugars			
	Sucrose	Glucose	Fructose	Maltose
30	3.51	3.41	3.18	4.69
50	3.90	3.51	3.85	4.97
70	3.96	3.56	3.84	4.31
90	3.42	3.15	3.61	3.60
L.S.D 5%	0.23			

Table 3 :- Effect of different concentration of sugars with the presence of 0.5 mg/L TDZ and 2mg/L Cholesterol on soluble sugars and starch content of multiplied shoots of *Digitalis purpurea* .

Concentration g/L	Soluble Sugars (%)			
	Sugars			
	Sucrose	Glucose	Fructose	Maltose
30	5.06	4.78	4.59	5.69
50	5.31	4.81	4.55	5.91
70	5.54	4.20	4.38	5.49
90	4.01	3.08	3.33	4.44
L.S.D 5%	0.30			
Concentration g/L	**Starch(%)**			
	Sugars			
	Sucrose	**Glucose**	**Fructose**	**Maltose**
30	6.09	5.79	5.74	7.91
50	6.81	5.87	5.82	8.30
70	6.76	5.55	5.50	7.30
90	5.65	5.01	4.49	6.12
L.S.D 5%	0.41			

Table 4 :- Effect of different concentrations of sugars with the presence of 0.5 mg/L TDZ and 2mg/L Cholesterol on digitonin content of multiplied shoots of *Digitalis purpurea* .

Concentration g/L	Digitonin µg/g dry weight.			
	Sugars			
	Sucrose	Glucose	Fructose	Maltose
30	64.42	51.01	42.13	113.11
50	98.93	68.18	83.78	191.87
70	108.55	73.28	81.91	171.22
90	61.72	67.33	51.67	165.61
L.S.D 5%	17.35			

Table 5 :- Effect of different concentrations of the sugars with the presence of 0.5 mg/L TDZ and 2mg/L Cholesterol on the dry weight of multiplied shoots of *Digitalis purpurea* .

Concentration g/L	Dry weight (g)			
	Sugars			
	Sucrose	Glucose	Fructose	Maltose
30	2.96	2.42	2.28	3.14
50	3.18	3.61	2.91	4.52
70	3.51	3.72	2.71	4.03
90	2.40	2.31	2.51	3.90
L.S.D 5%	0.25			

Discussion

In the field of tissue culture, shoots cultures (multiplied shoots) of Digitalis plant was considered one of the main sources to obtain some medicinal compounds like the cardiac and saponin glycosides[18] because the site of biosynthesis of these medicinal compounds is the shoots of this plant[19] for this reason , in the present study we were inducted multiplication the cultured shoot tip and formation the shoots cultures by supplemented the growth regulator TDZ (cytokinin – like) to MS medium at a concentration 0.5 mg/L[10], and because the strong effects of this growth regulator[20] , shoot cultures in our study were appeared as leaf masses consisted from a high number of small and dwarf shoots as shown in figure (1).

Figure 1 : Multiplication shoot tip of *Digitalis purpurea* (Var. excelsior Mixed) in the treatment 0.5 mg/L TDZ and 2.0 mg/L cholesterol after 45 days.

So it was difficult to distinguish the stem tips of these shoots then it was not possible to count their number and arrange the statistical analysis for them.Total chlorophyll content, soluble sugars and starch of the shoot cultures was studied as an indicator to degree of the changes in quantity of digitonin in these shoots, where if the content of chlorophyll was high this mean the green plastids have high degree of differentiation and that reflected positively on the ability of green plastids to provide some necessary enzymes to produce these compounds [21, 22] . As for the soluble sugars and starch , these compounds are the main source for energy and carbon element which are necessary to formation the great carbon structure of steroidal compounds [23]. Thus the rich places of chlorophyll , sugars and starch will be also rich in these medicinal compounds.In experiment 1, Presence of the cholesterol with 0.5 mg/ TDZ in MS medium had effected on increasing the internal level of digitonin inside the tissue of multiplied shoots especially in the concentration 2.0 mg/L , because this material was considered as a precursor to form the digitogenin (aglycon part of digitonin) [24] as shown in figure (2). Also presence the cholesterol in MS medium was led to increasing the content of multiplied shoots from the total chlorophyll, soluble sugars and starch. This increasing may be due to analysis this precursor and supply of some elements for these shoots like the carbon element which is necessary to formation the chlorophyll , soluble sugars and starch [25] and reflected that positively on shoots production from the digitonin. All of these increases reflected positively on increasing the dry weight of these shoots ecpically in the concentration 2.0 mg/L . Therefore we were selected this concentration with 0.5 mg/ L TDZ and used in the experiment 2 with different sugars. In the experiment 2, the treatment 50 g /L maltose with 2.0 mg/ L cholesterol and 0.5 mg/L TDZ significantly increased the digitonin , total chlorophyll, soluble sugars and starch in multiplied shoots and their dry weight as compared with other treatments of this experiment . The reason may be due, the maltose sugar is disaccharide and consist of two molecule of glucose . Therefore presence of this sugar in MS medium was led to increasing the number of glucose molecules inside the tissue of these shoots and that reflected positively on different bioprocesses of these shoots like: 1- increasing the supply of sugar part (glucose) which is necessary to building the digitonin where this compound consist from 2 molecules of glucose, 2 molecules of galctose and 1 molecule of xylose [25]. 2 – increasing the supply of energy which is necessary to growth and development of the shoots[23], 3- increasing the supply of carbon element which is necessary to building the great carbon structure of chlorophyll, different sugars, starch and digitonin [5,25].

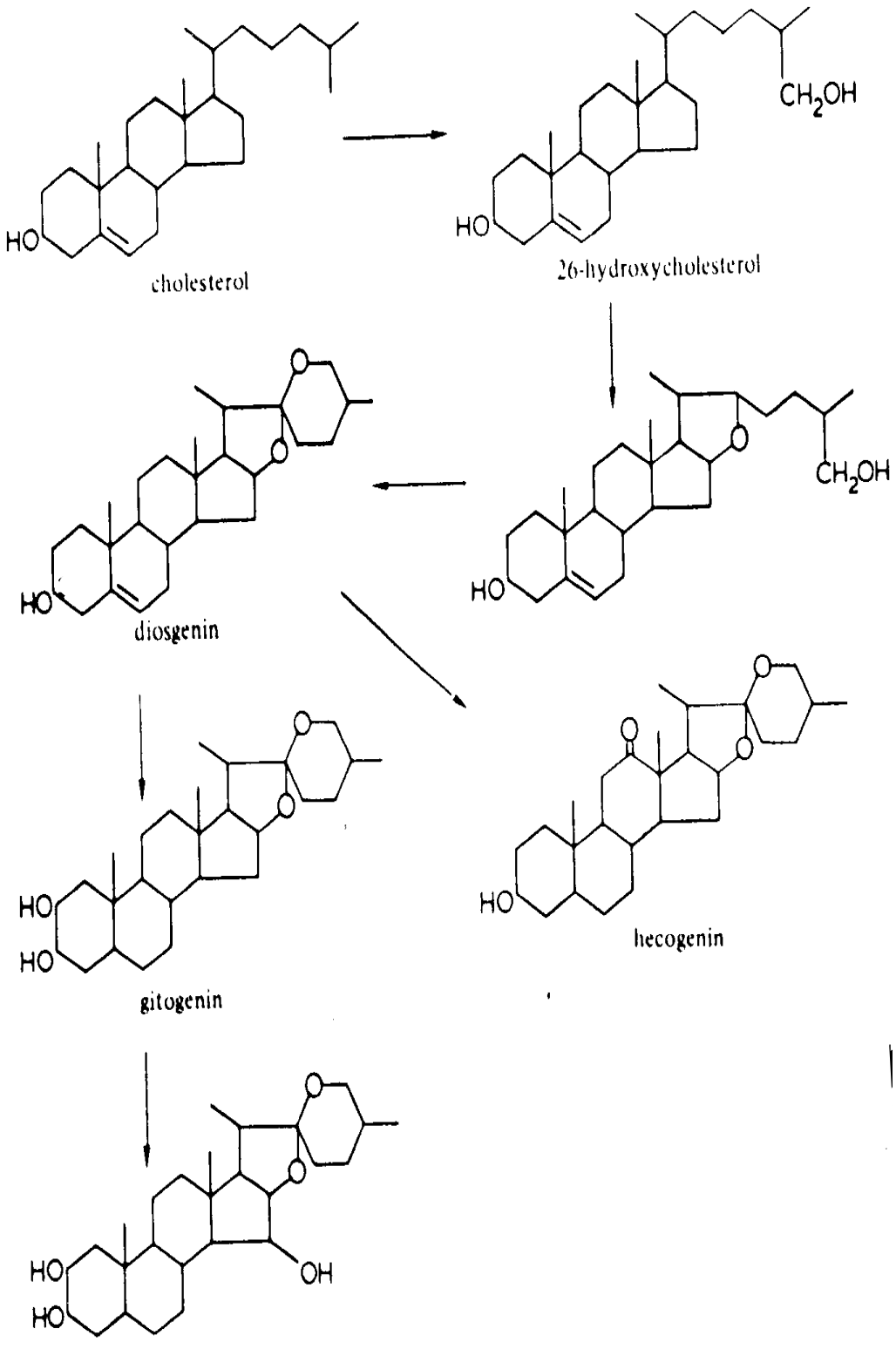

Figure 2 : The biosynthesis of steroidal sapogenin digitogenin[24]

References

1. Tyler ,V.E, Brod , L.R . and Robbers , J.E.1988. Pharmacognosy.9th edition .K.M. Varghese , com , Bombay.
2. Evans , F. J . and cowleg , P.S . 1972 .variation in cardenolides and sapogenins in *Digitalis Purpurea* during germination phytochemsitry 11.2729 – 2733 .
3. James , A.G.1999. Physiology of plants and their cells : Phytochemistry (saponins).Pergamon press INC .New York .
4. Trease , G.E. and Evans,W.C . 1983. Pharmacognosy (Saponins , cardio – active drug and other steroids) . 12th edition . Published by Bailliere Tindall a division of cassell LTd .
5. Aua Zeid , S.N.1986 . Medicinal Plants and Herbales . Dar Al Bihar . Bayrute .
6. Stafford, A. 1986. Secondary Metabolism in plant cell culture .(Eds.).Cambridge University Press . Cambridge , London , New Rochelle , melbourme , Sydney .
7. Murashige, T. and Skoog, F. 1962 .Arevised medium for rapid growth and bioassay with tobacco tissue culture . Physiol . Plant .15:473 – 497 .
8. Kubalakova , M., Spitzova , I . and Novak , F.J.1987 stability of lanatoside C content in the in vitro propagated *Digitalis lanata* clones . Biologia plant arum .29(1):7 – 9 .
9. Hay, F.R. , Robert , R.J. and coomber , S.A . 1997 . Development of desiccation tolerance and longevity in seed detached capsules of foxgloves (*Digitalis purpurea*) Annals .Botany . 79 : 419 – 427 .
10. Zeinab , J.A. 2002 . The production of cardiac glycosides from the plant of zahrat al kishtiban (*Digitalis purpurea* L .) by using tissue culture technique .Ph.D thesis Agric.College .Baghdad University .Iraq .
11. Coner, S.and Reinhard , E.1986 . Longterm cultivation of *Digitalis lanata* clones propagated in vitro : cardenolide glycosides tent of the regenerated plants . Plant Medica . 7:478 – 481 .
12. Goodwing , T.W .1976 . Chemistry and biochemistry of plant pigments , 2nded Academic press ,London , New York , sanfrencisco
13. -Brugidou ,C. ,Jacques ,M., Cosson ,L., Jarreau F.X. and ogerau , T.1988 . Growth and digoxim content in *Digitalis lanata* in controlled condition and natural environment .Plant medical 262 – 265 .
14. Joslyn , M.A. 1970 . Method in food analysis , physical , chemical and instrumental methods of analysis ,2nd .Academic press , New York and London .
15. Brain , G.L. , Brain , K.R. 1976.Influence of hormonal supplementation on steroid level during callus induction from seeds of Trigonella foenum - graecum . Phytochemistry , 15: 1655 – 1660 .
16. Barnes , J. , Anderson , L.A . and Phillipson , J.D. 2002 . Herbal medicines , 2nd ed , Pharmaceutical Press ,UK .
17. Alrawi, K.M. and khalaf – Allah, A.M. 1980 . Design and analysis of agricultural experiments , Mosul University . Agric and Forest College Iraq .
18. Abdul – Mutalib , S.M . and Mubashar , S.O. 1990 . The basic conception in plant cell , Tissue and Organ Culture. University of Baghdad , Ministry of Higher Education and Scientific Research . Iraq .
19. Stuhlemmer , U., Kreis, w., Eisenbesis , M. and Reinhard , E. 1993 . Cardiac glyrosides in party submerged shoots of *Digitalis lanata* .Planta Media ,59 : 539 – 545 .
20. Carl , A.H. and Jhon ,E.P.1993 . Thidiazuron a potent cytokinin for woody plant culture .palnt cell tissue and organ culture .33:105 – 119 .
21. Hagimori , M ., Matsumoto , T. and Obi , Y.1982 .Studies on the production of Digitalis cardenolides by plant tissue culture .II.Effect of light and plant growth substances on digitoxin formation by undifferentiated cells and shoots – forming cultures of *Digitalis Purpurea* grown in light media .Plant physiol .69 : 635 – 656 .
22. Morales , C., Cusido , R., Palzon , J., Bonfill , M.and Pinol L.1999 . Digitalis Plants and tissue culture , improved conditions for cardinolide production .Plant Biology , 1:35.
23. Alrawi, A.M. and Sulieman ,R.R.1988 .Metabolitei bioefficiencies .Ministry of high education and scientific research ,Baghdad university ,Iraq .
24. Margaret, L.V. and Vickery , B.1981 .Secondary Plant metabolism . The macmillan press LTD. London and Basingstoke .
25. Mohamed, A.A.K.and Younis , M.A.1991 .Principles of Plant Physiology. V2 . ministry of higher education and scientific research .Baghdad University . . College of Agriculture .Iraq .