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Elicitor and Precursor Feeding as a Biotechnological Tool for the Production of Bioactive Cardiac Glycosides in Cell Suspension Cultures of *Digitalis lanata*

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In Loving Memory of Late Professor Doctor ""Mohamed Refaat Hussein Mahran""

Abstract

Digitalis lanata is commonly used as a drug for congestive heart diseases. The goal of this work is to develop bioactive components from *D. lanata* cell suspension culture. To achieve this purpose, we used progesterone as a precursor, KCl, and mannitol as elicitors, as a significant biotechnological tool to produce secondary metabolites from cell culture system. The results demonstrated that the medium containing 1.0 mg/l NAA + 1.0 mg/l BAP was more suitable for forming friable calli with acceptable texture for cell suspension culture. The medium B, which contained 1.0 mg/l NAA + 0.5 mg/l BAP, produced higher callus fresh weight and viable cell number/ml than medium A, and the greatest cell growth occurred between days 12 and 15. The linearity and recovery of digitoxin and digoxin in HPLC were within acceptable limits. The addition of 40 mM of KCl resulted in the highest digitoxin value of 5.632 mg/g DW compared to the control of 0.038 mg/g DW. In the case of digoxin, the cell suspension control without any addition showed a maximum value of 671 g/g DW.

Keywords: Digitalis lanata; Elicitors; Precursor feeding; Digitoxin; Digoxin

1. Introduction

Plants are worthy source of diverse secondary metabolites utilized in different fields including pharmaceuticals, agrochemicals and food additives [1]. *Digitalis lanata*, family Plantaginaceae is native to Europe, a herbaceous and perennial, upright, up to 1.2 m high.

The leaves are sessile, simple and narrow-lanceolate [2]. Chemical composition of *Digitalis lanata* consists of different components like cardiac glycosides, volatile oils, fatty acids, starch, gum and sugars. They axially used as cardiovascular and cardioprotective effects, antidiabetic, anticancer, immunological [3].

Cardiac glycosides (CGs) are the most abundant and preeminent components which comprise digitoxin, digoxin (Fig. 1), digitoxigenin, lanatoside A, gitoxigenin and others [4]. Cardiac glycosides, which are among the 200 most often prescribed medications in the USA as of 2018 [5]. The most well-known is likely the production of digoxin (Dg) and digitoxin (Dgt), the two most significant members of this category, by plants belonging to the Digitalis genus (Scrophulariaceae). Both chemicals are recognized medications for the treatment of cardiac arrhythmias and heart failure [6].

Cardenolides from *Digitalis* plant do not play a vital role in the cardiovascular drug market in the present time only, but use to treat other diseases, such as cancer, diabetic and viral infections [7-9]. Digoxin a cardenolide, is still produced from dried leaves of *Digitalis lanata*, however, the chemical synthesis is not economically applicable [10].

The amount of secondary metabolites produced by plant species is influenced by biotic and abiotic circumstances which associated with cultivation [11]. Several studies have described the use of elicitors in cell suspension cultures to boost the synthesis of metabolites of medicinal relevance [12]. When plants are placed *in vitro*, they naturally produce bioactive chemicals, but their content is relatively low due to the brief stationary phase that *in vitro* cultured plants present, as well as enzyme inhibition [13]. Elicitors are commonly employed to boost secondary metabolite production in plant cell cultures because they operate as defense response regulators, activating various secondary biosynthetic pathways and their associated genes [14, 15]. In the previous studies, precursors and elicitors have been used to increase cardiac glycosides, for instance, progesterone as precursor has been added to the axillary buds cultures of *Digitalis lanata* [16]. Different elicitors and

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precursors have been used in the preceding study of to increase cardiac glycosides in the shoot culture of *Digitalis purpurea* [17]. Various biotic and abiotic elicitors (methyl jasmonate, yeast extract, chitosan, salicylic acid, and KCl) as well as precursor feeding (cholesterol, progesterone, and squalene) were used to increase cardiac glycosides in *in vitro Calotropis gigantea* plantlet cultures [18]. Additionally, methyl jasmonate, salicylic acid, KCl and squalene were used to increase stigmasterol in the plantlet culture of *Bacopa floribunda* [19].

In most prior research, cardiac glycoside production in shoot culture was attempted. The synthesis of these chemicals via callus culture has received very little attention. Hence, the purpose of this work was to create an effective protocol for the production of cardiac glycosides (digoxin and digoxin) in *Digitalis lanata* via cell suspension culture. Precursors and elicitor feeding were applied in this technique to raise these secondary metabolites.

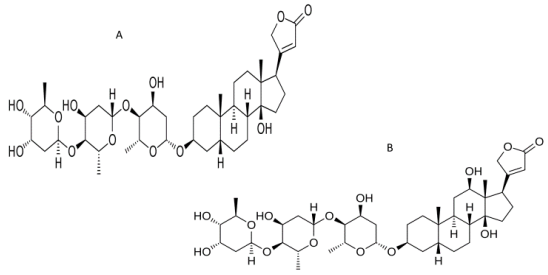

Fig. (1) Chemical structure of digitoxin (A) and digoxin (B).

2. Experimental

2.1. Preparation of explants

Seeds of *Digitalis lanata* L. were supplied by the Medicinal and Aromatic Plants Farm, Faculty of Pharmacy, Cairo University. The seeds were immersed in 70% ethanol for 2–3 min and then rinsed in sterile distilled water. The seeds were then sterilized for 20 min in 20% commercial Clorox (5% NaOCl) containing 0.5% Tween 20, followed by rinsing three times with sterile distilled water. Under aseptic conditions, the seeds were cultured on MS-medium [20] containing 3% (w/v) sucrose and solidified with 0.2% (w/v) Gelzan (Gelrite). The culture medium was adjusted to a pH 5.8. The cultures were incubated in a culture room at 26±2°C and kept under a 16-h photoperiod of fluorescent, 45 µmol cool white light tubes and 8-h dark.

2.2. In vitro callus culture initiation

Leaf explants (0.5 cm) were obtained from 21-day-old seedlings and cultivated on solidified MS medium with various combinations of 2,4-dichlorophenoxy acetic acid (2,4-D) + kinetin (kin), 2,4-D + benzyl aminopurine (BAP), and naphthalene acetic acid (NAA) + BAP. Callus initiation was performed on the following cultural media:

1) MS + 1.0 mg/l 2,4-D + 1.0 mg/l kin
2) MS + 1.0 mg/l 2,4-D + 2.0 mg/l kin
3) MS + 1.0 mg/l 2,4-D + 1.0 mg/l BAP
4) MS + 1.0 mg/l 2,4-D + 2.0 mg/l BAP
5) MS + 1.0 mg/l NAA + 1.0 mg/l BAP
6) MS + 1.0 mg/l NAA + 2.0 mg/l BAP

After 30 days of culturing, the callus initiation frequency was recorded and calculated based on
the following equation:

Callus initiation frequency%= (Number of initiated calli) /(Number of inoculated explants) × 100

Characterization of produced calli (texture and color of calli) were also observed and recorded.

2.3. Cell suspension culture and growth kinetics

The friable calli (0.2 g) produced after three subcultures on the most convenient medium were transferred into fresh liquid media containing 0.5 mg/l NAA + 1.0 mg/l BAP (A) and 1.0 mg/l NAA + 0.5 mg/l BAP (B). Every three days intervals for 21 days, cell fresh weights, viable cell number, and % viability were calculated. The viable cell number was determined using the trypan blue exclusion method [21]. In specifics, aliquots containing 150 µL of cell suspension culture were gently mixed with an identical volume of 0.4% (w/v) trypan blue and incubated in the dark for 10 min. A 10-µL sample was observed under a Leica ATC 2000 light microscope, and the number of viable (unstained) and dead (stained) cells was subsequently determined. The viable cell number and viability (%) were calculated using the following equations:

Viable cell number ⁄ ml = (number of counted live cells ⁄ number of squares) × dilution factor × 10^4

viability (%) = (number of counted live cells ⁄ total counted cells) × 100

2.4. The addition of precursors and elicitors to the cell suspension culture

After the cell subcultured on the most convenient fresh liquid medium, precursor and elicitors have been added to the cell suspension culture of *Digitali*s on the fifteen-day-old of culture, which are progesterone (200 and 300 mg/l) as a precursor, mannitol (50 and 100 mM), and KCl (40 and 80 mM) as elicitors. All additives were filter sterilized through a 0.45 µm Millipore filter (Minisart, Sartorius, Germany). All suspension

cultures were incubated by putting them on orbital shaker (120 rpm/min) at 26°C±2 under a 16-h photoperiod of fluorescent, 45 µmol cool white light tubes, and 8-h dark. After three days, the cells were harvested and subjected to cardiac glycoside extraction and determination.

2.5. HPLC of cardiac glycosides

2.5.1. Preparation of extracts

Harvested cells from all treatments and the control were lyophilized using a Labconco freeze dryer, Console, 12 l, −50°C, Stoppering Tray Dryer, FreeZone, 240 V, Catalog No. 7754030, Serial No. 100931482 D, USA. The drying was performed at −50°C, 0.1 mbar, and 48 h. The dried cells were macerated in 80% methanol for 12 h then filtered and concentrated on a rotary evaporator under vacuum. The extracts were then kept at –20°C until use.

2.5.2. Instrument and chromatographic conditions

HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using a Zorbax Eclipse Plus C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and acetonitrile (B) at a flow rate of 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (90% A); 0–5 min (30% A); 5-5.5 min (30% A); 5.5-8 min (90% A); 8-12 min (90% A). The multi-wavelength detector was monitored at 220 nm. The injection volume was 10 µl for each of the sample solutions. The column temperature was maintained at 30 °C.

2.5.3. Standard solutions

The standard markers of cardiac glycosides (digitoxin and digoxin) were purchased from Sigma- Aldrich. Individual stock solutions of digitoxin and digoxin were prepared at different concentrations in methanol (1, 5, 10, 25, 50, 100, and 200 µg/ml). These stock solutions were stored at –20°C. The different amounts of concentrations of these stock solutions were used for the preparation of calibration curve linear in the range of 1-200 µg/ml.

2.5.4. HPLC assay validation

The HPLC method was validated according to ICH guidelines [22] in terms of linearity, accuracy, precision of the digitoxin and digoxin solutions.

2.5.5. Linearity

The linearity of an analytical procedure represents its capacity to generate test results that are approximately equal to the analyte concentration in the sample. Standard methanol solutions of digitoxin and digoxin were prepared to determine the method linearity at concentrations of 1, 5, 10, 25, 50, 100, and 200 µg/mL. Each concentration was examined three times. Calibration curves were produced by plotting the peak areas *vs.* the standard concentrations.

2.5.6. Accuracy

The accuracy was determined as percent recovery of the added amount of known analyte in the sample with respect to the actual value. Five concentrations (5, 10, 25, 50, and 100 µg/mL) of standard mixtures of digitoxin and digoxin were injected into HPLC. Spiked samples were prepared in triplicate and tested three times. The percent recovery was calculated as follows: recovery (%) = (amount detected/amount spiked) × 100.

2.5.7. Precision

Precision was determined by developing the digitoxin and digoxin calibration curves in triplicates. The area under the curve was calculated by the HPLC software and relative standard deviation (RSD) was calculated using the equation below:

RSD= standard deviation/mean

Calibration curves were repeated on different days (n=6 or 9) using freshly prepared mobile phase and the RSD calculated. %RSD not more than 2 was taken as the limit.

2.6. Statistical analysis

Twenty-four explants were used per each treatment. Means and standard errors (SE) were obtained from analysis for each treatment by the use of computer program Microsoft Excel 2010. Data were presented as means ± SE. The significant difference between the means was determined using one-way analysis of variance (ANOVA) and Least Significant Difference (LSD) test at $p < 0.05$, using Statistix (version 8.0).

3. Results and discussion

3.1. Callus culture initiation

The most functional agent in *in vitro* plant cell cultures is the balance between indigenous hormones and externally added growth regulators. The formation of the callus tissue is mainly dependent on the combinations of growth regulators [23, 24]. In the current experiment, six different combinations of growth regulators have been used to induce calli from the leaf explant of *Digitalis lanata*. The data shown in Table 1 reflect that, in all combinations used, callus tissues have been induced successfully. Maximum callus initiation frequency (%) clarified with the medium which contained

NAA and BAP combination at 1.0 mg/l each of both them recorded 80.4%, followed by the medium that contained 1.0 mg/l NAA + 2.0 mg/l BAP recorded 60.2 %. All combinations containing 2,4-D either incorporated with BAP or kin showed a lower callus initiation frequency (%) ranging from 41.1% to 59.8%. The induced calli tissues and their color varied in each combination accordingly. In all used combinations, the formed calli was compact; however, the color differed with the 2,4-D and kin combination, which appeared yellow, while other combinations showed green tissues (Fig.2). After three subcultures, the developed calli on the medium containing 1.0 mg/l NAA + 1.0 mg/l BAP evolved and became somewhat friable, making this medium (1.0 mg/l NAA + 1.0 mg/l BAP) is more ideal for producing calli for cell suspension culture (Fig.3). The texture of the developed callus does not alter during sub culturing on the other treatments.

Different explants have been tried previously to induce calli from *D. lanata*. Results clarified that leaves explant were more responsive and recorded a maximum value compared to hypocotyl and root explants [25]. In the other observation, shoot tip explant induced calli with 1.0 mg/l NAA greater than 0.25 mg/l [26].

Tissue dedifferentiation occurs during callus formation from explants, resulting in structural and metabolic changes. Calluses can be compact or friable, with various cell types and high cell division frequency. A high degree of friability indicates a high meristematic system. [27].

Table (1) Effect of different growth regulators on callus initiation frequency % from leaf explants and their characterization after one month of cultivation.

Growth regulators (mg/l)				Callus initiation frequency (%)	Characterization
2,4-D	Kin	NAA	BAP		
1.0	1.0			53.2±5.3	Compact yellow
1.0	2.0			41.1±1.8	Compact yellow
1.0			1.0	59.8±1.6	Compact green
1.0			2.0	47.6±2.4	Compact green
		1.0	1.0	80.4±3.1	Compact green
		1.0	2.0	60.2±1.3	Compact green

Values=Average ± SE

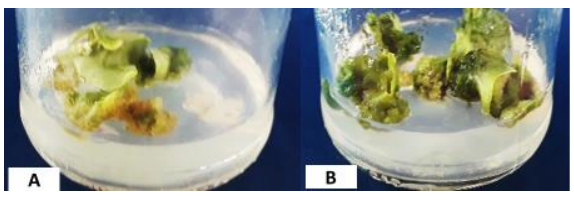

Fig. (2) Callus tissues on the media containing 2,4-D + kin combination (A) and other media combination containing NAA (B).

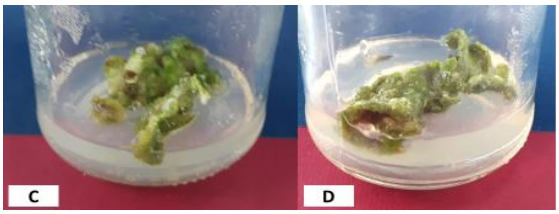

Fig. (3) Callus induction of *Digitalis lanata* from leaf explant on the medium containing 1.0 mg/l NAA + 1.0 mg/l BAP (A) compact callus compact callus, and the friable calli after three sub-culture (B) on the same medium.

3.2. Growth kinetics of cell suspension cultures

Growth kinetics of cell suspension culture are a limiting factor for cell culture management and predicting the optimal time for secondary metabolites accumulation [28].

Cell suspension culture was initiated by transferring 0.5 g of friable callus tissue to Erlenmeyer flasks containing 50 ml of liquid media with two supplementations: (A) 0.5 mg/l NAA + 1.0 mg/l BAP, and (B) 1.0 mg/l NAA + 0.5 mg/l BAP. Cell suspension growth kinetics were evaluated by determining cell fresh weight (g/l), viable cell number/ml, and percentage of viability.

The growth parameters were determined at a three-day interval and plotted against time. The values are the means of three observations. As shown in Fig. 4, callus fresh weight grew gradually from day 0 to day 12 in both media A and B, recording maximum values of 38.33 g/l and 46.67 g/l, respectively at day 12, then declining until reaching its lowest value on day 21. While the number of viable cells gradually rose from day 0 to day 15 to achieve the maximum values at day 15, which were 10000 and 20000 cells/ml in both media A and B, respectively (Fig. 5). It is also evident from Fig. 6 that the viability increased gradually until day 12, when it recorded 71% and 67% for the media A and B, respectively, and then decreased to the minimum values on day 21, when it recorded 43% and 40%, respectively. Based on these findings, it can be inferred that medium B, which contained 1.0 mg/l NAA + 0.5 mg/l BAP, produced higher callus fresh weight and viable cell number/ml than medium A, also the greatest cell growth occurred between days 12 and 15.

The large-scale manufacturing of naturally occurring products that often occur in very small amounts has been made possible through plant cell culture technology for natural products that generally occur in very low amounts [29, 30]. Suspension cultures of plant cells offer an affordable substitute for conventional growing techniques [31], and it has been proposed that they are more dependable than collecting plants in the wild [32]. Recently, liquid cultures of *Digitalis*

lanata were established using different combinations 2,4-D + BA; NAA + BA; and IAA + BA, to study the effect of these combinations on the formation of active compounds within the cell suspension cultures [33]. Some secondary metabolites were produced via cell suspension culture technique; for example, vinblastine is produced from *Catharanthus roseus* cell culture [34]. Lipid production has been scaled up from *Jatropha curcas* cell culture [35]. The production of berberine by plant cell cultures of *Coptis japonica* and the production of shikonin by cell suspension cultures of *Lithospermum erythrorhizon* [36]. Cell suspension culture is a fast, widespread, and effective method for producing secondary metabolites, with numerous commercialization efforts [37, 38]. Cell suspension culture is a fast, widespread, and effective method for producing secondary metabolites, with numerous commercialization efforts. [37].

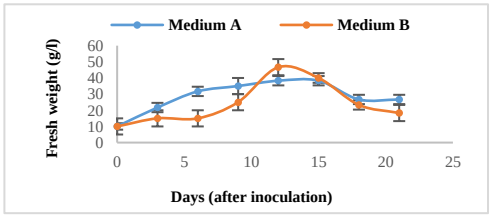

Fig. (4) Callus fresh weight (g/l) of cell suspension culture cultivated on both media containing 0.5 mg/l NAA + 1.0 mg/l BAP (A) and 1.0 mg/l NAA + 0.5 mg/l BAP (B).

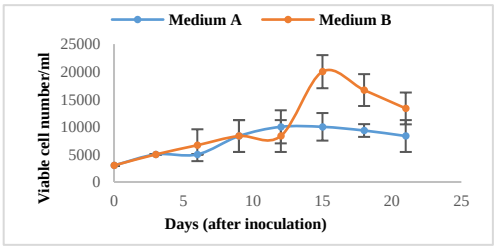

Fig. (5) Viable cell number/ml of cell suspension culture cultivated on both media containing 0.5 mg/l NAA + 1.0 mg/l BAP (A) and 1.0 mg/l NAA + 0.5 mg/l BAP (B).

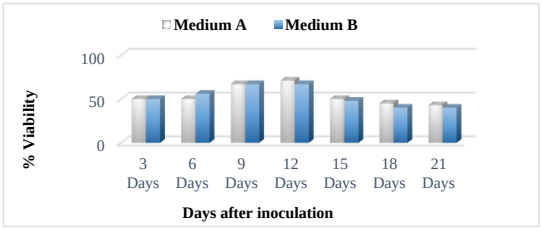

Fig. (6) Viability (%) of cell suspension culture cultivated on both media containing 0.5 mg/l NAA + 1.0 mg/l BAP (A) and 1.0 mg/l NAA + 0.5 mg/l BAP (B).

3.3. HPLC determination of cardiac glycoside

The main active components in *Digitalis lanata* were determined using the HPLC method. Fig. 7 shows HPLC chromatograms of the standards digitoxin and digoxin. ICH guidelines [22] was used to validate the method in terms of linearity, accuracy, and Precision.

3.3.1. HPLC validation

Calibration curves (Fig. 8 and 9) for digitoxin and digoxin were created by graphing the peak area from the chromatograms against the concentration throughout a concentration range of 1-200 µg/ml. The regression analysis of digitoxin yielded the line equation as y=12.28873x + 17.14378, with R2 = 0.99982. For digoxin analysis, the line equation is y= 12.21582x + 15.64294, with R2 =0.99984.

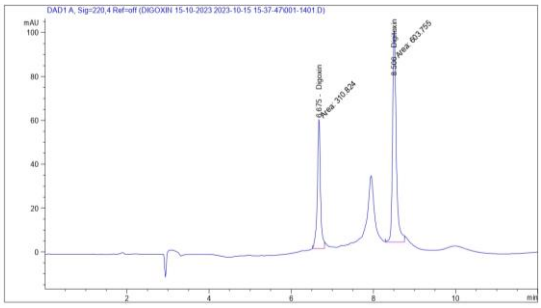

Fig. (7) HPLC chromatogram of standards digitoxin and digoxin at 50 and 25 µg/ml.

The regression coefficient parameters are shown in Table 2 within the suggested range, the high correlation coefficient indicates a linear relationship between peak area and digitoxin and digoxin concentrations. As a result, the concentration of digitoxin and digoxin in any sample may be estimated using this method using this curve.

Table (2) Regression coefficient parameters of digitoxin and digoxin concentrations to the analytical response (area under the peak).

Parameters	Values	
	Digitoxin	Digoxin
Linearity range, µg/mL	1-200	1-200
Slope of regression equation	12.28873	12.21582
Intercept	17.14378	15.64294
Correlation coefficient	0.99982	0.99984

Furthermore, the method's accuracy, as demonstrated by the recovery values of standard digitoxin and digoxin of 99.49±1.55 and 97.99±0.25, respectively, within an acceptable range, and also the acceptable ranges of %RSD at 0.64 and 0.084 for digitoxin and digoxin, respectively, demonstrates its applicability for the quantification of these components in *Digitalis lanata* samples (Tables 3 and 4).

Table (3) Highlighting of accuracy (%recovery) and RSD for the digitoxin HPLC method.

Digitoxin conc. Actual (µg/ml)	Peak area (mAU)	RSD	Digitoxin conc. calculated (µg/mL)	Recovery%
5	70.32±1.71	0.02	4.33±0.14	86.54±2.78
10	138.02±1.37	0.01	9.84±0.11	98.36±1.11
25	346.44±10.63	0.03	26.80±0.87	107.19±3.46
50	660.05±0.20	0.003	52.32±0.02	104.63±0.03
100	1254.87±2.01	0.002	100.72±0.16	100.72±0.16

Mean % recovery= 99.49±1.55
RSD%= 0.64

Table (4) Highlighting of accuracy (%recovery) and RSD for the digoxin HPLC method.

Digoxin conc. Actual (µg/ml)	Peak area (mAU)	RSD	Digoxin conc. calculated (µg/mL)	Recovery%
5	68.07±0.09	0.001	4.29±0.01	85.84±0.14
10	135.20±1.07	0.008	9.79±0.06	97.87±0.62
25	330.80±0.18	0.0006	25.80±0.02	103.20±0.06
50	639.51±0.57	0.0009	51.07±0.05	102.14±0.09
100	1248.42±0.13	0.0001	100.92±0.01	100.92±0.01

Mean % recovery= 97.99±0.25
RSD%= 0.085

3.3.2. HPLC of digitoxin and digoxin in cell suspension after addition of precursors and elicitors

After three days of adding precursor and elicitors, the cells were harvested and subjected to HPLC analysis of both bioactive compounds (digitoxin and digoxin). The data in Fig. 8 showed the differences in digitoxin content as mg/g DW as a consequence of different treatments, where there had been an increase in digitoxin contents as a result of adding both elicitors (mannitol and KCl). However, adding progesterone as a precursor led to a decrease in digitoxin content compared with the control. The most influential and prevalent treatment was 40 mM of KCl, which recorded 5.632 mg/g DW followed by 80 mM of KCl recorded 5.541 mg/g DW. There was also an increase in digitoxin content as a result of adding 50 and 100 mM of mannitol, which recorded 0.072 and 0.135 mg/g DW, respectively, compared with the control (0.038 mg/g DW). There was a significant difference between the control and the KCl and mannitol treatments, but not between the control and the progesterone addition. Regarding the digoxin results presented in Fig. 9, the digoxin content ranges from 230 to 671 µg/g DW. The cell suspension control without any addition recorded a maximum value of 671 µg/g DW, followed by adding 100 mM, and 50 mM of mannitol, which recorded 544.3 and 465.2 µg/g DW, respectively. The rest of the used treatments had a significant decrease in the content of digoxin compared with the control.

Precursor feeding, biotic and abiotic elicitors were previously utilized to increase cardiac glycosides in *Digitalis purpurea* shoot culture. Cardenolides were produced efficiently using KCl, Helminthosporium sp. mycelial, and progesterone. In the presence of progesterone (200 to 300 mg/l), digitoxin and digoxin accumulation were increased 9.1 and 11.9 folds, respectively [17], our results coincide with this study in the case of KCl elicitor, but not in the case of progesterone treatment, despite the fact that both concentrations of progesterone (200 and 300 mg/l) decreased cardiac glycosides content (digitoxin and digoxin). However, according certain *D. lanata* cell lines are capable of biotransformation of steroidal compounds, including cardenolides, in cell suspension cultures [39]. Elicitors at proper concentrations may operate as signalling molecules that are detected by a receptor on the plasma membrane, so initiating the signal transduction network involving gene expression regulation necessary for target compound formation [40, 41].

Elicitors have been shown to be effective in inducing secondary metabolite synthesis in cell and organ cultures of various species, such as berberine in *Thalictrum rugosum* [42], thiophenes in *Tagetes patula* [43], taxol in *Taxus* sp. [44], tanshinone in *Salvia miltior* [45], and hypericin and pseudohypericin in *Hypericum hirsutum* and *Hypericum maculatum* [46].

4. Conclusion

The production of natural products from natural sources is the current focus of research. Furthermore, offering these natural substances on a continual basis and at a lower cost is very important. This work is focused on the synthesis of bioactive cardiac glycosides (digitoxin and digoxin) using cell suspension culture of *Digitalis lanata*, while most prior studies relied on shoot cultures. The results showed that the medium containing 1.0 mg/l NAA + 1.0 mg/l BAP resulted in the development of friable calli with good texture for cell suspension culture performance. Among the elicitors and precursors, the KCl treatment was more successful in the synthesis of digitoxin. The production of digoxin was higher in the control than in the other treatments. It is probable that these additives altered the biotransformation of digitoxin to digoxin; nevertheless, more research on the production of cardiac glycosides by cell suspension culture is required. Overall, this approach is effective for producing adequate quantities of cardiac glycosides.

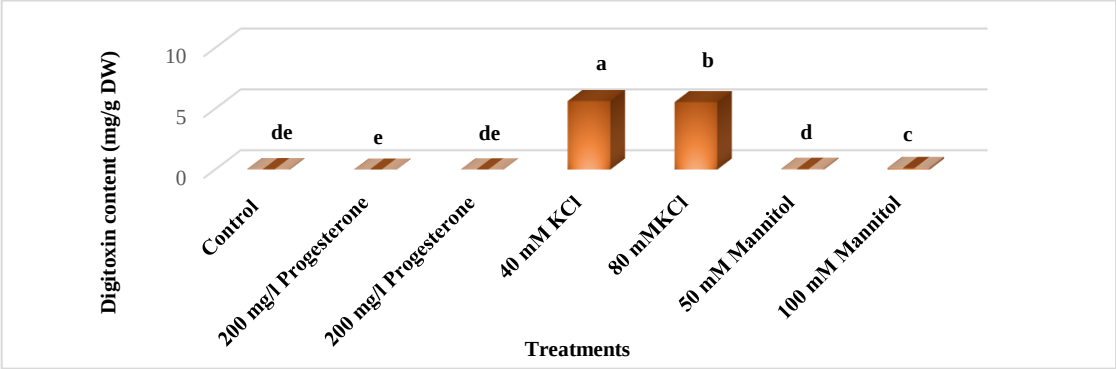

Fig. (8) Digitoxin content as mg/g DW in cell suspension treated with progesterone, KCl, and mannitol after three days. Small letters express LSD significant differences ($LSD_{0.050}$=0.0611)

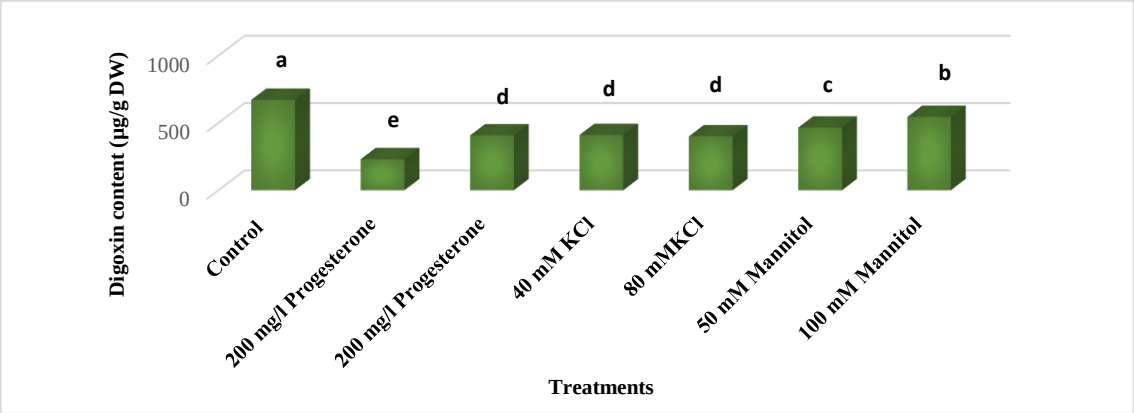

Fig. (9) Digoxin content as µg/g DW in cell suspension treated with progesterone, KCl, and mannitol after three days. Small letters express LSD significant differences ($LSD_{0.050}$=10.398)

5. Conflicts of interest

There is no conflict of interest

6. Formatting of funding source

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8. References

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