BIOCHEMICAL STUDIES ON PRODUCTION OF SOME SECONDARY METABOLITES IN *ECBALLIUM ELATERIUM* USING TISSUE CULTURE TECHNIQUES

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ABSTRACT

This investigation was undertaken to study the effect of using different *Ecballium elaterium* tissue culture techniques whether by changing concentration of basal medium components or elicitors on biomass and cucurbitacins accumulation. It was found that 5% sucrose was the best for biomass and total cucurbitacins accumulation while 7% sucrose was the best for CuE accumulation. Addition of high concentration of calcium to culture medium inhibited plant cell growth while increased cucurbitacins accumulation. There was a positive proportional between phosphate concentration and biomass depletion of phosphate from culture media leads to marked increases in cucurbitacins accumulation. Lower NH_4^+/NO_3^- ratios in the medium were favorable for cell growth while decreasing nitrate level increased the production of cucurbitacins. Yeast elicitor improved the growth and enhanced cucurbitacins accumulation. The fungal elicitor treatment depressed the biomass yield at all concentrations except the lowest (0.5 g/l) while 1 g/l of fungal elicitor was the best for Cu content % and 0.5 g/l gave the highest value for Cu yield & Cu E accumulation. There was inversely proportional between concentrations of salicylic acid or hydrogen peroxide and biomass accumulation on the other hand they showed positive proportional with cucurbitacins accumulation.

Keywords: Ecballium elaterium, plant tissue culture, cucurbitacins**.**

INTRODUCTION

The squirting cucumber *Ecballium elaterium* (L.) A. Rich belongs to the family Cucurbitaceae. The various attributes of *Ecballium elaterium* have been popular in Maltese folk medicine, its main use being as a cathartic. The Maltese utilised the fruit juice in the treatment of jaundice (Lanfranco, 1980). Elaterium can also be used to remove oedema, which is the accumulation of excessive water in the body tissues (Penza, 1969), hence its use in the treatment of dropsy. At high doses, the elaterium can cause vomiting (Lanfranco, 1975). So it has been possibly used in the treatment of poisonings where induced vomiting has been a necessity.

Although several cultivation studies have been conducted for *Ecballium elaterium* (Balbaa et al., 1979), no studies exist on in vitro culture studies on this plant except that of (Attard and Scicluna-Spiteri (2001) , Attard and Attard (2002), Attard and Scicluna-Spiteri (2003), Attard, (2004) Attard and Vujicic (2005) and a study carried out by Toker and co-workers (2003).

Recently, Attard and Scicluna-Spiteri (2001) studied the tissue culture production of *E. elaterium* and reported the effects of different plant hormones on callus biomass accumulation and cucurbitacin production, particularly, the cytotoxic cucurbitacin E.

The present work was undertaken to study the production of some secondary medicinal metabolites using tissue culture techniques from Ecballium elaterium and evaluation their nutritional and medicinal effects.

MATERIALS AND METHODS

All chemicals, solvents (ethanol, Petroleum ether (40-60), chloroform) all at Analar grade. Solvents were purified and redistilled before use. Seeds of *Ecballium elaterium* were purchased from local market.

Plant materials

Seeds of *Ecballium elaterium* were surface sterilized by dipping into Ethanol (70% v/v) for 30s , followed by soaking in 20 % (v/v) of commercial bleach (Clorox , containing 5.25 % sodium hypochlorite NaOCl) for 25 min and rinsed thoroughly with sterile

distilled water . After cracking, aseptically, the seed coat according to Attard and Scicluna – Spiteri (2003), then sterilized seeds were germinated in 250-ml jars containing 50-ml of MS medium until plantlets reached about 10-12 cm in length and were used as starting material.

Media preparation

The basal medium described by Murashige and Skoog was used (1962) containing 3 % Sucrose and solidified with 0.7 % Agar. The pH was adjusted to 5.8 before autoclaving. Most of macronutrients, micronutrients, vitamins and plant growth regulators (PGRs), prepared in stock solutions to minimize the experimental errors of weighting, were used as treatments to enhance growth and cucurbitacin (Cu) / cucurbitacin E (CuE) accumulation of *Ecballium elaterium* tissue culture. The media were sterilized by autoclaving at 121 °C at 1.2 kg/cm^2 for 25 min. (Evans et al., 2003)

Callus induction

Stem segments obtained from 15- day-old seedlings were cultured in 100-ml jars containing 25 ml of Murashige - Skoog medium (MS) supplemented with NAA / BAP (0.5 and 1 mg / l , respectively) , incubated in growth chambers at 25 ±1ºc under a 16 h photoperiod (2000 LUX) .Initiated callus was maintained by transferring approximately 2 g of callus every 4 weeks Toker et al(2003) .

Optimization of medium composition

The effect of the manipulation of the component of the culture medium was studied to obtain the best medium give high growth and Cu & CuE yield by two means

a)- Investigating the effect of using different carbon source (sucrose) concentration on both of growth and productivity by culturing approximately 2 g of initiated callus material in callus induction medium containing different concentrations of sucrose (3 , 5, 7, and 10 % w/v)

b) - Modification of the composition of MS medium

Callus material was subcultured in callus induction medium (control) and in media in which the concentrations of the salts had been modified as follows:-

1- Medium containing different concentration of $CaCl_2.2H_2O$ (zero, 440, 880, 1320 mg/l).

2 - Medium containing different concentrations of KNO_3 (zero, 1900, 3800, 5700 mg/l).
3 - Medium containing different concentrations of KH_2PO_4 (zero, 170, 340, 510 mg/l).

Effect of different biotic and abiotic elicitors on callus proliferation and Cu / Cu E production

Callus was cultured on MS – medium without KNO_3 supplemented with NAA / BAP (0.5 and 1 mg / l, respectively) with different concentrations of elicitor treatments.

Preparation of elicitors

Commercially available yeast extract (Y-4000, Sigma chemical Co) was prepared according to Peltonen et al (1997). Yeast extract was diluted in deionized water, autoclaved at 121º C for 15 min and adjusted to various elicitor concentrations (0.01, 0.1, 1, 2 and 3 g/l). Stock solution of Salicylic acid was prepared by dissolving it in distilled water, autoclaved at 121 ° C for 15 min and adjusted to various elicitor concentrations (25, 50 and 100 μM). H_2O_2 was used by adding the appropriate volume to the medium to obtain concentration (1 and 2 PPm). The mycelial homogenate elicitor was prepared from the liquid culture of the isolate of *Aspergillus Niger*, liquid cultures were initiated from 7-d potato dextrose agar (PDA) by transferring 2 cm^2 squares of medium to 150 ml (PDA) . Liquid culture flasks of Aspergillus Niger were incubated in the dark at 120 rpm at 25 ºc for 7 days. One- week old mycelia were collected by filtration and elicitor was prepared according to the method described by Zhang et al (2000). The elicitor dose was measured by the total carbohydrate content of the fungal homogenate, which was determined by the phenol/sulfuric acid method using glucose as the standard.

Dry weight measurement

According to Attard and Scicluna –Spiteri (2001) callus samples were collected after four weeks and dried at 40 ºC for 24 h to obtain the dry weight. .

Determination of Cucurbitacins

(1) For total Cu assay, dried callus material (100mg per sample) was extracted with absolute ethanol (5 ml) for 2h, after centrifugation (2000 rev/min, 3 min) the supernatant was reduced to 2 ml on a water

bath (40°C). (2) For Cu E assay, dried callus was extracted with chloroform (5 ml) for 2 h; after centrifugation, the supernatant was mixed with an equal volume of petroleum ether, the precipitate obtained was filtered and dissolved in absolute ethanol (5 ml) and then reduced to a volume of 2 ml as above. The reference standard CuE was dissolved in ethanol and serial dilutions (0.01- 1.09 mg/ml). all samples (100 µl , in triplicate), together with various concentrations of CuE standard , were mixed with 100 µl of a 2 % solution of Phosphomolybdic acid in absolute ethanol according to Yang et al (1991) at room temperature using a 96 well plate. The absorbance was measured at 492 nm after 5 min on ELISA reader (Model Expert plus uv , ASYS Hitech , AUSTRIA) the results were worked out as w/w % , calculated from dry callus weight .

Statistical analysis

Statistical analyses were carried out according to the method described by Snedecor and Cochran (1980) using Mstat- C microcomputer program for analysis of Randomized Complete Blocks design. Duncan New Multiple Range Test at 5% level was used to differentiate between means.

RESULTS AND DISCUSSION

Effect of different concentrations of sucrose on biomass and cucurbitacins (total and CuE) yield:

As shown in Table (1) altering of sucrose concentration in culture medium resulted in significant increase to show maximum values of biomass , total Cu yield , Cu content to 571.9 mg , 61.97 mg and 10.84% respectively at 5% sucrose then it significantly decreased with the increase in sucrose concentration compared with control . On the other hand, CuE yield showed significant increase to 26.45 mg (maximum value) then it significantly decreased to 14.09 mg at 10 % sucrose while the increase in sucrose concentration showed positive proportional with CuE content as it increased non significantly with the increase of sucrose content in culture medium to 5.217 % compared to control While 3% sucrose showed greatest value of CuE/Cu ratio which was 65.51%.these results are in a good agreement with those of Choi *et al.* (1994 a, b) who found that the optimal concentration of sucrose for cell growth was between 30 and 50 g/l and that 70 g/l sucrose inhibited cell growth and Xu *et al.* (1999) who

reported that sucrose has positive effects on salidroside products. The salidroside content was the highest in media containing 40 – 50 g /l sucrose, and higher concentrations of sucrose reduced salidroside formation.

Table (1): Effect of different carbon source (sucrose) concentrations on biomass and cucurbitacins (total and CuE)

Treatment	Biomass (mg)	Total Cucurbitacin yield (mg)	Cucurbitacin content (% w/w)	Cucurbitacin E yield (mg)	Cucurbitacin E content (% w/w)	Ratio of CuE /Cu
Control (3%)	419.1 b	28.07 b	6.7 c	18.38 b	4.387 a	65.51 a
(5%) sucrose	571.9 a	61.97 a	10.84 a	25.92 a	4.537 a	41.87 c
(7%) sucrose	545.7 a	56.14 a	10.29 a	26.45 a	4.873 a	47.38 bc
(10%) sucrose	273.0 c	25.03 b	9.10 b	14.09 c	5.217 a	58.39 ab
L.S.D at $_{0.05}$	59.04	6.245	0.937	3.103	1.063	15.52

Means with the same letter (s) are not significantly different ($p \leq 0.05$)

Effect of different concentrations of calcium on biomass and cucurbitacins (total and CuE) yield:

Data presented in Table (2) reveal that elimination of $CaCl_2$ from culture medium strongly suppressed biomass to 303.1 mg while it significantly increased to 419.0 mg with 440 mg/l $CaCl_2$ (control) and inhibited slightly to reach it's minimum value 351.9 mg at 1320mg/l $CaCl_2$. Values of Cu yield or content and CuE yield or content increased with the increase of $CaCl_2$ concentration to reach the highest value at 1320 mg/l of $CaCl_2$. However, at 440mg/l $CaCl_2$ (control) showed the highest value of CuE/Cu ratio (65.51%).

Table (2): Effect of different calcium concentrations ($CaCl_2$) on biomass and cucurbitacins (total and CuE)

Treatment	Biomass (mg)	Total Cucurbitacin yield (mg)	Cucurbitacin content (% w/w)	Cucurbitacin E yield (mg)	Cucurbitacin E content (% w/w)	Ratio of CuE /Cu
Zero mg/l	303.1 c	17.83c	5.88 c	10.53 c	3.493 bc	59.47 a
440 mg/l (Control)	419.0 a	28.07 b	6.7 c	18.38 ab	4.387 c	65.51a
880mg/l	359.7 b	61.19 a	16.51 b	17.19 b	4.620 b	28.30 b
1320mg/l	351.9 b	64.28a	18.29 a	21.65 a	6.163 a	33.66b
L.S.D at $_{0.05}$	25.61	1.048	1.743	3.379	1.048	11.45

Means with the same letter (s) are not significantly different ($p \leq 0.05$)

These results are in harmony with those of Nakao *et al.* (1999) who found that the growth of *polygonum hydropiper* cultured cells was strongly suppressed in the culture medium without $CaCl_2$, promoted most in that with 3 mM $CaCl_2$ (control) and inhibited slightly in medium with 6 mM $CaCl_2$ or more and they mentioned also that a high concentration of $CaCl_2$ in the medium stimulated an increase in flavanol levels in cells which can be a result from indirect effect of calcium induced phosphate deficiency .

Effect of different concentrations of phosphate (KH_2PO_4) on biomass and cucurbitacins (total and CuE) yield:

Data recorded in Table (3) show that elimination of phosphate from medium showed a significant decrease on biomass accumulation to 310.6 mg promoted to 419 mg with control (170 mg/l KH_2PO_4) then it showed a non significant increase on biomass to reach a maximum value 679.9 mg at 510 mg/l KH_2PO_4. These data indicate that there is a positive proportional between phosphate concentration in culture medium and biomass. On the other hand, depletion of phosphate from medium showed the highest value of Cu yield 53.46 mg, Cu content 17.21% and CuE content 7.443 % then it significantly decreased with the increase of KH_2PO_4 level to reach 37.05 mg, 5.443% and 3.730% for Cu yield, Cu content and CuE content respectively at 510mg/l KH_2PO_4 while the same phosphate concentration (510mg/l) showed the most significant value of CuE yield 25.23 mg while using 340mg/l of KH_2PO_4 on culture medium showed the highest value of CuE/Cu ratio which was 69.43 . These results are in agreement with those of Knoboloch and Berlin (1981) who reported that phosphate depletion of culture media leads to marked increases in phenylalanine ammonia-lyase activity followed by increases in the biosynthesis of Cinnamoyl putrescines. Ramachandra Rao and Ravishandkar (2002) mentioned also that the phosphate concentration in the medium can have a major effect on the production of secondary metabolites in plant cell cultures. Higher levels of phosphate were found to enhance the cell growth, where it had negative influence on secondary product accumulation

Effect of different concentrations of KNO_3 on biomass and cucurbitacins (total and CuE) yield:

Data shown in Table (4) demonstrate that elimination of KNO_3 from medium strongly suppressed the biomass accumulation to 325.0 mg then it increased to reach its maximum yield with 3800 mg/l KNO_3 then it non significantly decreased to 733.4 mg at 5700 mg/l KNO_3 . Elimination of KNO_3 from the medium showed a remarkable increase in Cu content %, Cu yield and CuE content which were 20.30%, 66.01 mg and 8.163 % respectively but it did not give significant difference between CuE yields While at 1900 mg /l KNO_3 (control) show significant increase in CuE/Cu ratio to 65.51% then it non

Table (3): Effect of different phosphate concentrations (KH_2PO_4) on biomass and cucurbitacins (total and CuE)

Treatment	Biomass (mg)	Total Cucurbitacin yield (mg)	Cucurbitacin content (% w/w)	Cucurbitacin E yield (mg)	Cucurbitacin E content (% w/w)	Ratio of CuE /Cu
Zero mg/l	310.6 c	53.46 a	17.21 a	23.11 ab	7.443 a	43.26 b
170 mg /l (Control)	419.0 bc	28.07 b	6.700 b	18.38 b	4.387b	65.51 a
340 mg/l	534.1 ab	32.06 b	6.100 c	22.42 ab	4.213 bc	69.43 a
510 mg/l	679.9 a	37.05b	5.443 d	25.23 a	3.730 c	68.67a
L.S.D at $_{0.05}$	168.1	9.083	0.5508	5.996	0.543	9.531

Means with the same letter (s) are not significantly different ($p \leq 0.05$)

Table (4) Effect of different nitrogen concentrations (KNO_3) on biomass and cucurbitacins (total and CuE)

Treatment	Biomass (mg)	Total Cucurbitacin yield (mg)	Cucurbitacin content (% w/w)	Cucurbitacin E yield (mg)	Cucurbitacin E content (% w/w)	Ratio of CuE /Cu
Zero	325.0 b	66.01 a	20.30 a	27.23 a	8.163 a	40.22 b
1900 mg/l (control)	419.0 b	28.07 b	6.70 b	18.38a	4.387 b	65.51 a
3800 mg/l	754.1 a	44.29 b	5.937 c	23.51a	3.093 bc	53.15 ab
5700 mg/l	733.4a	40.11 b	5.490 c	17.93a	2.443 c	44.71 b
L.S.D at $_{0.05}$	153.5	15.82	0.7148	13.57	1.437	17.02

Means with the same letter (s) are not significantly different ($p \leq 0.05$)

significantly decreased to show it's minimum value 44.71 % at 5700 mg/l and gave the most significant decrease value (40.22) at zero mg/l of KNO_3 compared with control (65.51%).These results are in accordance with Franklin and Dixon (1994) who mentioned that it is a general trend that a lower ratio of NH_4^+ to NO_3^- ratio is more favorable for plant tissue and cell growth and Pan *et al.* (2004) who reported that cell dry weight was improved in a medium with a higher NO_3^- / NH_4^+ratio. Nitrate as the sole nitrogen source (i.e. 60mM NO_3^- without NH_4^+) gave the highest cell dry weight. While more ammonium was optimal for camptothecin accumulation in cell suspension cultures of *C. acuminate.*

Effect of different concentrations of yeast extract on biomass and cucurbitacins (total and CuE) yield:

Data regarded in Table (5) illustrate that using yeast extract as an elicitor showed significant increase to 546 mg at 0.01 g/l yeast extract compared with control followed by a gradual increase by increasing yeast extract dose to reach its maximum 978.8 mg at 3g/l of yeast extract Cu content was increased to 23.72 % at 3g/l of yeast extract compared to 20.16 %of control and the same concentration 3g/l of yeast extract show the highest value in Cu yield (232 mg) while concentration of 1g/l showed the highest value of CuE content (14.24 %) and CuE yield 133.2mg but CuE/Cu ratio increased to 90.30% at 0.01g/l of yeast extract compared to 65.83% in control then it showed a significant decrease with the increase of yeast extract concentration to reach its minimum value (54.54% at 3g/l of yeast extract) these results in a good agreement with those of Chen *et al*. (2001) who found that yeast elicitor improved the growth of hairy roots (from 3.9 g/l to 7.3g/l on a dry weight basis), Sánchez-Sampedro et *al.* (2004) who reported also that only yeast extract stimulated production of silymarin in *S. marianum* cultures when compared to chitin and chitosan effect, Yan *et al.* (2006) who mentioned that yeast extract was much more effective than Ag^+ in stimulating the accumulation of rosmarinic acid and phenolics and Cho et *al.* (2008) reported that yeast extract treatment strongly elicited accumulation of sanguinarine with maximum value of 146.8 ±3.5 mg/l at 168 h after treatment.

Table (5): Effect of yeast extract concentrations on biomass and cucurbitacins (total and CuE)

Treatment	Biomass (mg)	Total Cucurbitacin yield (mg)	Cucurbitacin content (% w/w)	Cucurbitacin E yield (mg)	Cucurbitacin E content (% w/w)	Ratio of CuE /Cu
Control	325.00	65.62 e	20.16 b	42.80 c	13.26 a	65.83 cd
0.01	546.0	79.96 de	14.64 c	72.10 b	13.21 a	90.30 a
0.1	617.6	94.30 d	15.21 c	78.34 b	12.68 a	83.78 ab
1	936.1	79.96 c	19.13 b	133.2 a	14.24 a	74.37 bc
2	961.8	65.62 b	20.98 b	121.5 a	12.63 a	60.57 d
3	978.8	232.2 a	23.72 a	126.5 a	12.92 a	54.54 d
L.S.D at $_{0.05}$	64.92	20.36	1.025	18.37	1.971	11.47

Means with the same letter (s) are not significantly different ($p \leq 0.05$)

Effect of different concentrations of fungal elicitor (*Aspergilus niger*) on biomass and cucurbitacins yield:

Data presented in Table (6) manifest that using elicitor derived from the cell walls of *Aspergillus niger* in culture medium increased the biomass and total Cu yield to 779.1 mg, 223 mg respectively at 0.5 g/l of fungal elicitor then it decreased significantly to 398.3 mg , 50.52 mg respectively . on the other hand Cu content % , CuE yield and CuE content increased significantly to show it's maximum values which were 29.95 % , 184.5 mg , 17.93% respectively then it decreased gradually to show it's minimum values to 22.64 % , 90.30 mg , 12.61 % respectively while using concentration of 0.5 g/l of fungal elicitor showed the highest value of CuE/Cu ratio 76.83 . These results in accordance with those of Chong *et al.* (2005) who found that higher elicitor concentration of *Aspergillus niger* could retard cell DW, however, the extracellular anthraquinone content (mg/g DW) was significantly higher than at lower concentration and Xu and Dong (2005) who mentioned also that elicitor , derived from the cell walls of *Aspergillus niger* , induced rapid generation of reactive oxgen intermediates (ROI) , including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) , sequentially followed by phenylalanine ammonia-lyase (PAL) activation and catharanthine biosyntheses in *C. roseus* suspension cells.

Table (6): Effect of different (AN) concentrations on biomass and cucurbitacins (total and CuE)

Treatment	Biomass (mg)	Total Cucurbitacin yield (mg)	Cucurbitacin content (% w/w)	Cucurbitacin E yield (mg)	Cucurbitacin E content (% w/w)	Ratio of CuE /Cu
Control	325.0 e	42.80 b	20.17 e	65.52 e	13.26 c	65.67 b
0.5	779.1 a	223.0 a	20.70 e	161.1 b	15.89 b	76.83 a
1	616.0 b	110.4 ab	29.95 a	184.5 a	17.93 a	59.84 bc
1.5	528.3 bc	82.18 b	27.80 b	146.9 bc	15.56 b	55.95 cd
2	491.4 cd	65.76 b	26.82 c	131.9 c	13.39 c	49.96 d
2.5	398.3 de	50.52 b	22.64 d	90.30 d	12.61 c	55.68 cd
L.S.D at $_{0.05}$	97.71	134.1	0.6831	21.98	1.280	5.934

Means with the same letter (s) are not significantly different ($p \leq 0.05$)

Effects of different concentrations of salicylic acid on biomass and cucurbitacins (total and CuE) yield:

Data recorded in Table (7) demonstrate that using salicylic acid as an elicitor significantly increased biomass to 785.2 mg at 25 µM of salicylic acid compared to 325 mg in the control (without elicitor) followed by significant decrease to lowest value 250.7 mg at 100 µM of salicylic acid. Cu content % was increased gradually with salicylic acid increase to reach its maximum value 28.06 % at 100 µM of salicylic acid while Cu yield showed a significant increase to 164.4 mg at 25 µM of salicylic acid compared to 65.52 mg at control then it decreased significantly to show its minimum value 70.36 mg at 100 µM of salicylic acid. For CuE content it showed a significant increase in CuE content to 15.33 % at 100 µM compared to 13.26 % in control while 25 µM of salicylic acid demonstrated the highest value of CuE yield 112.1 mg and CuE /Cu ratio 68.40 % compared to 42.80 mg for CuE content and 65.67 % for CuE/Cu ratio in control. These results are harmony with those of Yu *et al.* (2001) who found that treatment of 50mg/l of SA led to biomass to decrease. the reduction of biomass might be due to membrane lipid peroxidation induced by the treatment as the degree of biomass reduction was actively parallel to membrane lipid peroxidation and they found also that in spite of the biomass decrease, SA treatment resulted in the higher production of taxol compared to the control .Cho *et al.* (2008) reported that SA treatment elicited accumulation of dihydrosanguinarine on *Eschscholtiza californica*. Prakash and Srivastava (2008) found also that addition of

SA induced the azadicachtin synthise at all the concentrations studied and 2.7 fold higher azadicachtin content (8.2 ±0.05 mg/g as opposed to 3.2 ±0.02 mg/g in control).

Effect of different concentrations of hydrogen peroxide on biomass and cucurbitacins (total and CuE):-

Data showed in Table (8) demonstrate that using hydrogen peroxide as an elicitor demonstrate a non significant decrease to 319 mg and 279.5 mg at 1, 2 PPm respectively as compared to 325 mg in control (without elicitor) so we can suggest that there are inversely proportional between hydrogen peroxide concentration and biomass. Both of Cu % and its yield increased significantly to show it's maximum values which were 44.08 %, 123.1 mg respectively in a positive relation to H_2O_2 concentration when compared to control 20.16 % for Cu content and 65.52 for Cu yield while at 1 PPm showed the highest value in CuE % 29.14%, CuE yield 92.69 mg and CuE/Cu ratio 86.94 % when compared with control 13.26 % for CuE content, 42.80 mg for CuE yield and 65.67 % for CuE/Cu ratio.

Table (7): Effect of different Salicylic acid concentrations on biomass and cucurbitacins (total and CuE)

Treatment	Biomass (mg)	Total Cucurbitacin yield (mg)	Cucurbitacin content (% w/w)	Cucurbitacin E yield (mg)	Cucurbitacin E content (% w/w)	Ratio of CuE /Cu
Control	325.0 c	65.52 b	20.16 c	42.80 c	13.26 b	65.67 a
25 µM	785.2 a	164.4 a	20.93 c	112.1 a	14.30 ab	68.40 a
50 µM	570.0 b	140.8 a	24.65 b	76.86 b	13.50 ab	58.82 b
100 µM	250.7 c	70.36 b	28.06 a	38.44 c	15.33 a	54.64 b
L.S.D at $_{0.05}$	125.1	31.28	1.281	14.47	1.877	9.621

Means with the same letter (s) are not significantly different ($p \leq 0.05$)

These results are in a good agreement with those of Lamb and Dixon (1997) who found that H_2O_2 functions as a substrate for oxidative cross – linking in the cell wall, as a threshold trigger for hypersensitive cell death, and as a diffusible signal for induction of cellular protecting genes in surrounding cells. Chong et al (2004) reported that H_2O_2 generation above the threshold level can cause lipid peroxidation and programmed cell death depending on the duration of exposure of the cells.

Table (8): Effect of different hydrogen peroxide concentrations on biomass and cucurbitacins (total and CuE)

Treatment	Biomass (mg)	Total Cucurbitacin yield (mg)	Cucurbitacin content (% w/w)	Cucurbitacin E yield (mg)	Cucurbitacin E content (% w/w)	Ratio of CuE /Cu
Control	325.0a	65.52 b	20.16 c	42.80	13.26 b	65.67 b
1PPm	319.9a	108.3 ab	33.66 b	92.69	29.14 a	86.94 a
2 PPm	279.5a	123.1 a	44.08 a	78.59	28.09 a	64.11b
L.S.D at $_{0.05}$	154.7	42.93	4.197	32.13	3.949	19.69

Means with the same letter (s) are not significantly different ($p \leq 0.05$)

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دراسات كيميائية حيوية على انتاج بعض المركبات الثانوية فى نبات قثاء الحمار بواسطة تقنية زراعة الانسجة.

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اجريت هذة الدراسة بهدف دراسة تاثير استخدام تقنيات زراعة الانسجة لنبات قثاء الحمار من خلال تغيير تركيز مكونات البيئة الرئيسية و كذا تركيز المستحثات المستخدمة فى بيئة الزراعة على كلا من الكتلة الخلوية الناتجة و مدى تراكم المادة الفعالة و هى الكيوكيوربيتاسينات و كذلك دراسة التاثير الطبى لمستخلص النبات فى معالجة الفئران المحقونة برابع كلوريد الكربون كمادة مسببة لالتهاب الكبد. و قد وجد ان تركيز 5% من السكروز كان افضل تركيز فى تراكم كلا من الكتلة الخلوية و الكيوكيوربيتاسينات الكلية فى حين ان تركيز 7% سكروز كان افضل تركيزات السكروز المستخدمة على تراكم CuE .

اضافة تركيزات عالية من الكالسيوم لبيئة الزراعة ادى الى تثبيط نمو الخلايا النباتية فى حين ان زيادة تركيزة فى بيئة الزراعة ادى الى زيادة تراكم الكيوكيوربيتاسينات و قد وجد ان هناك علاقة طردية بين تركيز الفوسفات و مدى تراكم الكتلة الخلوية فى حين ان استخدام بيئات ينقصها الفوسفات ادى الى زيادة انتاج المادة الفعالة . الانخفاض فى نسبة الامونيا الى النترات فى البيئة كان مفضلا لنمو الخلايا فى حين ان الانخفاض فى نسبة النترات ادى الى زيادة تراكم الكيوكيوربيتاسينات. استخدام مستخلص الخميرة ادى الى زيادة نمو الخلايا و تراكم الكيوكيوربيتاسينات . ادى استخدام مستخلص الفطر (الاسبرجيلس نيجر) لمستحث الى تثبيط تراكم الكتلة الخلوية الناتجة فى جميع التركيزات ماعدا الاقل تركيزا(0.5 جم/لتر) بينما تركيز 1جم/لتر من مستخلص الفطر كان الافضل فى نسبة الكيوكيوربيتاسينات و قد اعطى تركيز 0.5 جم/لتر زيادة فى كلا من محتوى الكيوكيوربيتاسينات و CuE و قد وجد ان هناك علاقة عكسية بين تركيز كلا من حمض الساليسليك او فوق اكسيد الهيدروجين و تراكم الكتلة الخلوية و من جهة اخرى فكلا منهما اظهر وجود علاقة طردية مع تراكم و الكيوكيوربيتاسينات .