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Full Length Research Paper

Large scale production of antitumor cucurbitacins from *Ecballium Elaterium* using bioreactor

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Bioreactor plays a vital role in the commercial production of secondary metabolites and pharmaceuticals from plant cells. Many physical factors, like the mixing intensity, shear stress and operation conditions were optimized as a first step in scaling up process. Qualitative and quantitative determination of cucurbitacins E and I in the extract were carried out using high-performance liquid chromatography (HPLC). After two weeks of equipping the reactor with marine impeller, both the growth rate and the concentration of cucurbitacins were increased. However, the increase in the aeration rate from 0.3 to 0.6 vvm induced the production of cucurbitacin E, it reduced the cucurbitacin I production. The highest recorded level of cucurbitacins was 0.3 and 0.1 g/L for cucurbitacin E and cucurbitacin I, respectively. Crude cucurbitacins extract showed potent antitumor activity in a range of 15.6 to 23.5 µg/ml against various carcinoma cell lines. In the current study, the optimizing condition for the production of cucubitacins E and I in *Ecballium Elaterium* and their biological activities as an antitumor and antimicrobial agent were evaluated.

Key words: *Ecballium Elaterium,* cucubitacins E, cucubitacins I, bioreactor, antitumor.

INTRODUCTION

Ecballium elaterium is an important wild cucurbit germplasm in the Mediterranean region known as squirting cucumber. The plant extract is known to be rich in cucurbitacins, which has numerous activities. Protease inhibitors peptides were isolated from its seeds' extract (Favel et al., 1989). Cucurbitacin E tested on peripheral human lymphocytes and showed an immunodulatory activity (Attard et al., 2005). Cucurbitacin I showed cytotoxic effects against four human cancer cell lines and significant activity against HIV replication in H9 lymphocyte cells (Wu et al., 2004). Cucurbitacin B has a potent antiproliferative effect on breast cancer (Wakimoto et al., 2008). Furthermore, *Ecballium* extract has antimicrobial effect (Oskay and Sari, 2007) using plant cell and tissue culture techniques may be useful for the production of these effective substances economically and could be developed on the semi industrial production level (Attard and Spiteri, 2001; Chen et al., 2005). Bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology.

To successfully cultivate the plant cells at large scale, several engineering parameters such as, cell aggregation, mixing, aeration, and shear sensitivity are taken into account for selection of a suitable bioreactor. The media ingredients, their concentrations and the environmental factors are optimized for maximal synthesis of a desired metabolite. Increased productivity in a bioreactor can be achieved by selection of a proper cultivation strategy (batch, fed-batch, two-stage, etc.), feeding of metabolic precursors and extraction of intracellular metabolites. Proper understanding and

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rigorous analysis of these parameters would have the way towards the successful commercialization of plant cell bioprocesses (Chattopadhyay et al., 2002). The main objectives of this work is the large scale production of the bioactive compounds, cucurbitacin E and I using bioreactor and the determination of their antimicrobial and antitumor activities on different carcinoma cell lines.

MATERIALS AND METHODS

Plant Material

Mature seeds of *Ecballium elaterium* were collected from their growing habitats in north Sinai, Al-arish, el-sheikh zowaid, Egypt collected in July 2008, and it was identified by professor Soad el-Gengeihi Department of Medicinal and Aromatic plants, National research center, Egypt. A voucher specimen (number 2012.050) was deposited at the herbarium of Faculty of Pharmacy, Cairo University, Egypt.

Batch culture system

Briefly, 25 g of the selected cotyledon soft calli which contain highest amount of cucurbitacin E and I were cultured in liquid Murashige and skoog, 1962 (MS) medium supplemented with 5 mg/benzyl adenine (BA) and 0.1 mg/L naphthalene acetic acid (NAA) and 3% sucrose. The pH of the culture medium was adjusted to 5.8. The suspension culture was inoculated in a 2.0 L stirred tank bioreactor. The cell culture conditions were performed as follows: temperature 27°C, aeration rate 0.3 vvm, agitation rate 150 rpm, non controlled pH and marine impellers were used. Samples were taken every 48 h for 3 weeks and the cell fresh weight (g) was determined in each sample according to the described method by Choi et al. (2008) (Figure 1). Furthermore, the effect of change the aeration rates from 0.3 vvm to 0.6 vvm on accumulation of cucurbitacin in *Ecballium elaterium* suspension culture was investigated.

Chemical analysis

Determination of total carbohydrate

This was done according to the method reported by Dubois et al. (1956) and Krishnaveni et al. (1984).

Qualitative and quantitative determination of cucurbitacin E and I using HPLC technique

HPLC Condition

High performance liquid chromatography (HPLC) was used for qualitative and quantitative determination of cucurbitacin E and I contents in the total crude extract of each sample. C18 column was used with a flow rate of 1 ml/min. The column was equilibrated for 10 min with the mobile phase before each run and it was eluted using gradient mobile phase: A: Water/B: Acetonitrile starting with 100% of water and minutes A 80%, B 20%, after 10 min A 60%, B 40% and after 5 min A 40%, B 60%. Cucurbitacins E and I were detected using a UV detector at λ 229, 254 nm wave length, respectively. Calibration curves of authentic samples of cucurbitacins E and I (Carl Roth GbH + Co KG. 76185 Karlsruhe, Germany) were illustrated (R = 0.999), retention time (RT) for cucrbitacin E was 7.2 min at 229 nm and for cucrbitacin I was 2.7 min at 254 nm. All the prepared samples were analysed under the same conditions (Sturm and Stupper, 2000). All samples were dissolved in HPLC grade acetonitrile: distilled water (1:1) and analysed under the same condition as aforementioned.

Qualitative and quantitative determination of cucurbitacin E and I

Calibration curve of authentic cucurbitacin E and I was carried out using five different injection volume of the authentic sample of cucurbitacin E and I at a concentration of 1 mg/ml. The crude extract of each sample after evaporation were dissolved in acetonitrile: water (1:1) and filtered through millipore filter. The amount of cucurbitacins E and I were calculated in each sample by comparing the peak area with that at the same retention time with that of the authentic samples. At first, determine the response factor (RF) of each authentic which is the concentration (C) divided by the area (A). RF = average of (C/A) at 5 different concentrations. Determination of the concentration of cucurbitacin E and I in each sample by getting its peak area at the same retention time of the corresponding authentic under the same condition. Conc. = RF X A, where: Conc. is the concentration of cucurbitacin E or I in each injected sample, A is the peak area of each sample at the same retention time corresponding to that of the authentic cucurbitacin E and I (Figure 2).

Statistical analysis

All obtained results were subjected to analysis of variance ANOVA to test the significance in the all experiments. The least significant difference (L.S.D) at P = 0.05 level was calculated using SPSS 10.0 windows package according to the statistical analysis method described by Casanova et al. (2004).

Antimicrobial activity of cucurbitacins extract

Preparation of the extract

MS medium supplemented with 5 mg/L NAA + 1 mg/L BA and elicited with 20% mannitol showed the highest amount of cucurbitacin E and I crude extract. The obtained crude extract was air dried by rotatory evaporator and then extracted using ethanol: methanol: chloroform 1:1:1 on water bath at 60°C for 2 h. Then the extract was centrifuged for 15 min at 10,000 rpm. The supernatant was collected and evaporated with a rotatory evaporator and tested with bacteria according to (Dogruoz et al., 2008).

Bacterial strains

Different strains of bacteria were used in this study; *Escherichia coli* 0157, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S.typhimurium* and *Listeria Monocytogenes*. All strains were obtained from Cairo Microbiological Resource Centre - Cairo Mircen, Faculty of Agriculture, Cairo, Egypt.

Antimicrobial activity evaluation

In this experiment, agar-well diffusion was used in the antimicrobial activity tests. Mueller Hinton agar was used as media. The bacteria suspensions were prepared equal to the turbulence of Mac Farland 0.5 standard and cultivated (100 µl) on agar medium. After that 6 mm diameter were punched in agar plate. The standard

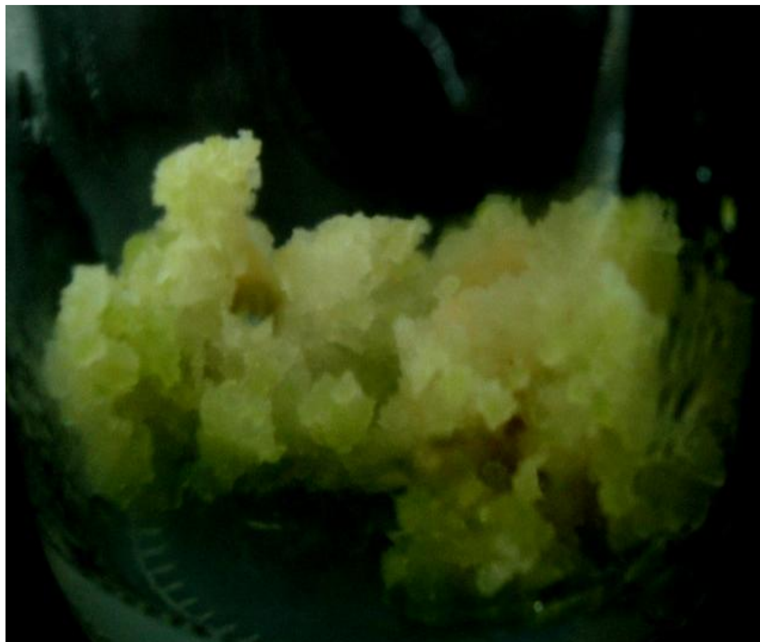
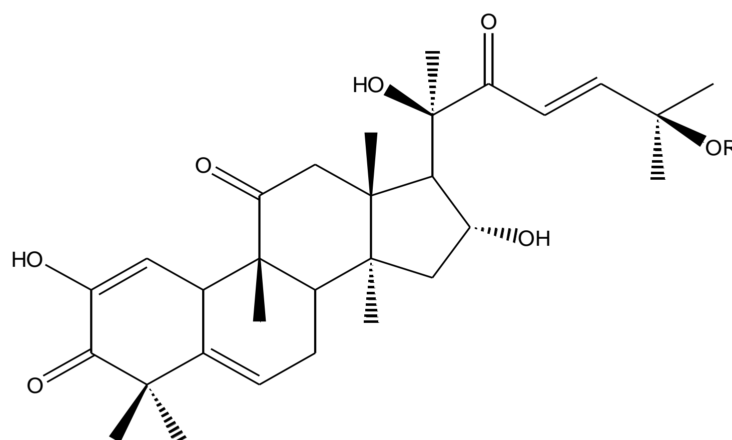


Figure 1. Callus induction from *Ecballium elaterium* leaf explants after 4 weeks of culturing on MS-medium supplemented with 5 mg/BA and 0.1 mg/l NAA.



Cucurbitacine I (R= H)

Cucurbitacine E (R= $COCH_3$)

Figure 2. Structure of cucurbitacin I and E.

antibacterial agent tobramycin (30 µg/ml) was tested in the same manner as control. All plates were incubated at 37°C, for 18 to 24 h. After incubation the different bacterial strains, the antimicrobial activity was evaluated by measuring the inhibition zone diameter observed (Barry et al., 1999; Ulusoylu et al., 2001). Each test was performed twice and the average of the results was taken. The standard antibacterial agent (tobramycin) was carried out in all tested bacteria.

Antitumor activity of cucurbitacins extract

Sample that showed a higher concentration of cucurbitacin E and cucurbitacin I using HPLC technique was taken from the bioreactor

and tested against different human tumor cell lines.

Human tumor cell lines

Human tumor cell lines: breast carcinoma cell line (MCF7), liver carcinoma cell line (HEPG2), cervical carcinoma cell line (HELA), colon carcinoma cell line (HCT116) were obtained frozen in liquid nitrogen (-180°C) from the American Type Culture Collection. The tumor cell lines were maintained in the National Cancer Institute, Cairo, Egypt, by several sub-culturing.

Sulphorhodamine-B (SRB) assay method for cytotoxic activity

This method was carried out according to that of Skehan et al. (1990). The sensitivity of the human tumor cell lines to thymoquinone was determined by the SRB assay. SRB is a bright pink aminoxanthrene dye with two sulphonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content.

Procedure

At the National Cancer Instistute in Egypt, cells were used when 90% confluence was reached in T25 flasks. Adherent cell lines were harvested with 0.025% trypsin. Viability was determined by trypan blue exclusion using the inverted microscope (Olympus 1 × 70, Tokyo, Japan). Cells were seeded in 96-well microtiter plates at a concentration of 5×10^4-10^5 cell/well in a fresh medium and left to attach to the plates for 24 h. After 24 h, cells were incubated with the appropriate concentration ranges of drugs, completed to total of 200 µl volume/well using fresh medium and incubation was continued for 24, 48 and 72 h. Control cells were treated with vehicle alone. For each drug concentration, 4 wells were used. Following 24, 48 and 72 h treatment, the cells were fixed with 50 µl cold 50% trichloroacetic acid for 1 h at 4°C. Wells were washed 5 times with distilled water and stained for 30 min at room temperature with 50 µl 0.4% SRB dissolved in 1% acetic acid. The wells were then washed 4 times with 1% acetic acid.

The plates were air-dried and the dye was solubilized with 100 µl/well of 10 mM tris base (pH 10.5) for 5 min on a shaker (Orbital shaker OS 20, Boeco, Germany) at 1600 rpm. The optical density (O.D.) of each well was measured spectrophotometrically at 564 nm with an ELIZA microplate reader (Meter tech. Σ 960, U.S.A.). The mean background absorbances was automatically subtracted and mean values of each drug concentration was calculated. According to the National Cancer Institute guideline a natural product extract with IC_{50} values < 20 µg/ml is considered active (Boik, 2001).

Calculation of the cytotoxic activity

The percentage of cell survival was calculated as follows: Survival fraction = O.D. (treated cells)/ O.D. (control cells). The IC_{50} values (the concentrations of thymoquinone required to inhibit 50% of cell growth). The result was calculated as the mean of 3 repeated experiments for each cell line.

RESULTS AND DISCUSSION

Batch fermentation using 0.3 vvm aeration rate

The correlation between the pH degree and the dry weight are presented in Figure 3a and b. The highest value of dry weight was recorded after 12 days about 0.8 g/L at pH 4.2. However, the lowest dry about weight was 0.1 g/L at pH 5.5. It was observed that the growth increased after a week from the beginning of the batch culture and reaching stationary phase for about one week. Then the growth rate was constant and decreased markedly. The pH was 5.7 in the beginning of the batch and was constantF for about 12 days then decreased markedly to reach 4.3 in the end of the batch.As shown in Figure 3c and d,the concentration of cucurbitacin E in plant cell reaches its maximum value after one week from the beginning of the batch to become 0.008 g/L and in the medium cucurbitacin E reaches its maximum value after 20 days from the beginning of the batch to become 0.3 g/L.

However, the concentration of cucurbitacin I in plant cell reaches its maximum value after one week from the beginning of the batch to become 0.04 g/L and in the medium cucurbitacin I reach its maximum value after two weeks to become 0.1 g/L (Figure 3c and d). These results are in agreement with previous reports regarding the pH changes in the medium which can change the permeability of cell membranes, accordingly, release more metabolites into the culture medium (Carbonell et al., 1993). Taya et al. (1992) and Mukundan et al. (1998) both used pH shift to release compounds normally stored in vacuoles, and attributed to cell damage.

Batch fermentation using 0.6 vvm aeration rate

The correlation between the pH degree and the dry weight are presented in Figure 4a and b. The highest value of dry weight was recorded after 12 days about 1.6 g/L at pH 3.5. However, the lowest dry weight was 0.1 g/L at pH 5.5. It was observed that the growth increased markedly from the beginning of the batch. The pH was 5.7 in the beginning of the batch then decreased markedly to reach 2.28 at the end of the batch. As shown in Figure 4c and d, the concentration of cucurbitacin E in plant cell reaches its maximum value after two weeks at the end of the batch 0.04 g/L and in the medium cucurbitacin E reaches its maximum value at the end of the batch to become 0.17 g/L. However, the concentration of cucurbitacin I in plant cell reaches its maximum value after 3 days from the beginning of the batch to become 0.005 g/L then decreased markedly and began to increase after 10 days to reach 0.004 g/L at the end of the batch. In the culture medium cucurbitacin I reach its maximum value after two weeks to become 0.077 g/L (Figure 4c and d).

Determination of total carbohydrate

The initial sugar concentration in the medium was 30 g/L,

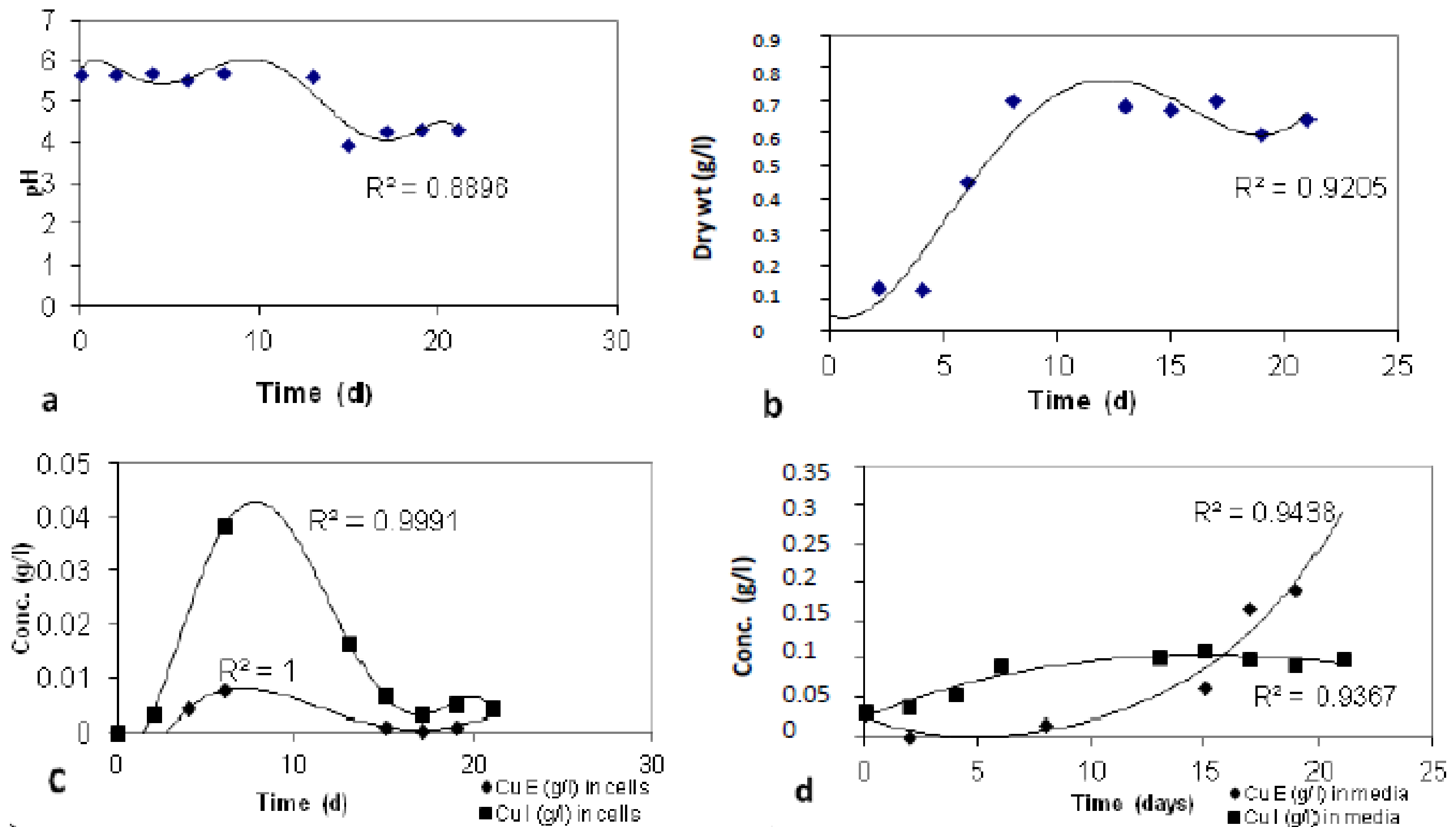


Figure 3. (a): Changes in pH degree, (b): Changes in the growth rate, (**c**): The concentration of cucurbitacin E and cucurbitacin I (g/l) in cells, (d): The concentration of cucurbitacin E and cucurbitacin I (g/L) in the liquid culture of of *E. elateriu*m by using aeration rate 0.3 vvm in batch culture of 2 L stirred tank bioreactor.

after cultivation, the concentration of sucrose fell rapidly. After 10 days of culture, the concentration of sucrose in the 2 L Stirred tank bioreactor was 20 g/L and after 12 days, the concentration of sucrose was down to 15 g/L, and after 14 days of culture, it fell to zero in the medium. The consumption of sucrose might be due to its conversion to glucose and fructose by cell wall bound invertase of plant cells (Martinez and Park, 1993) (Figure 5).

Comparison between 0.3 and 0.6 vvm aeration rates using batch fermentation

Illustrated data in Figure 6 revealed that changing the aeration rate from 0.3 to 0.6 vvm, the

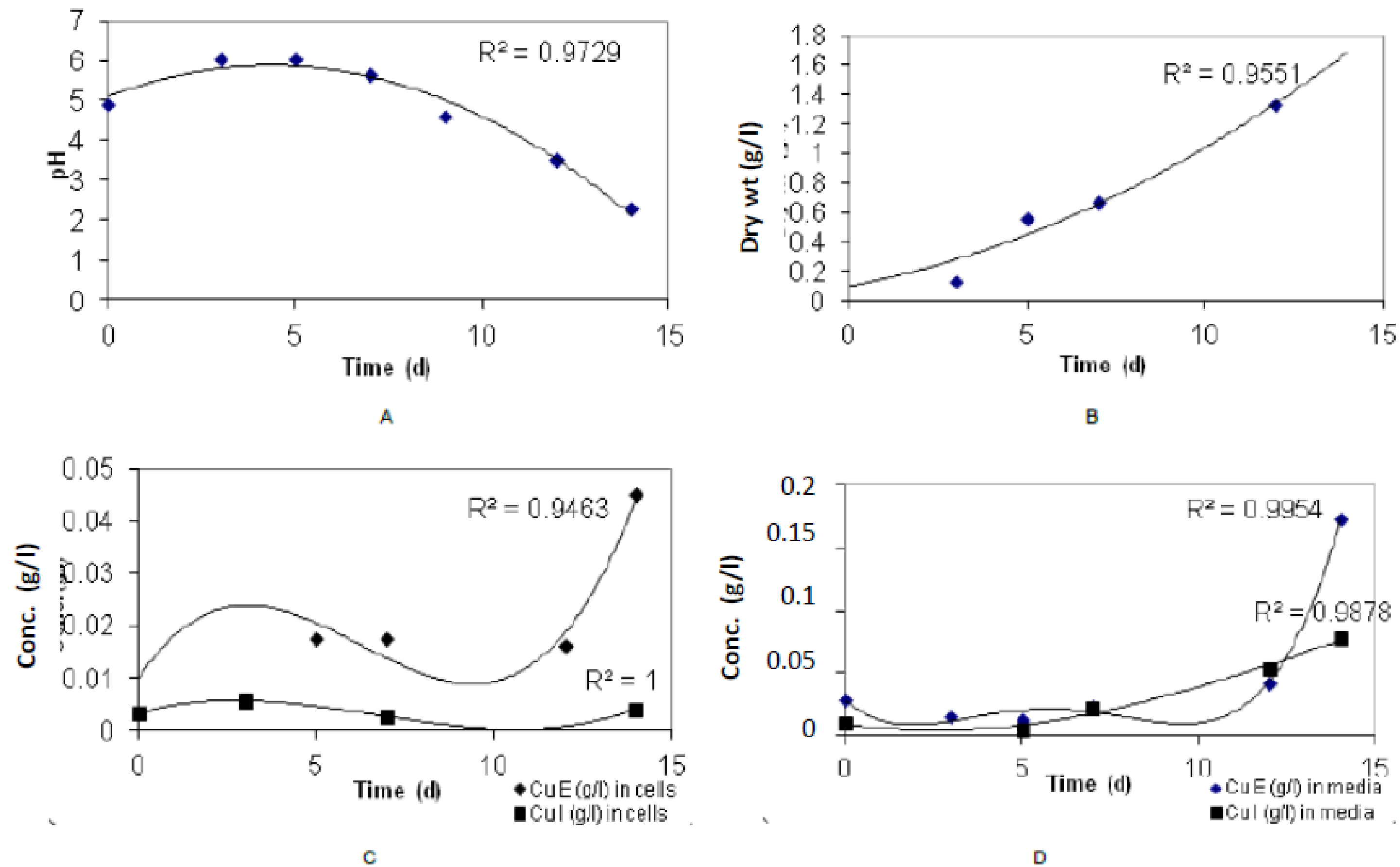


Figure 4. (a): Changes in pH degree. (b): Changes in the growth rate. (**c**): The concentration of cucurbitacin E and cucurbitacin I in cells. (d): The concentration of cucurbitacin E and cucurbitacin I in the liquid culture of *E. elaterium* by using aeration rate 0.6 vvm in batch culture of 2 L stirred tank bioreactor.

amount of cucurbitacin E in both cells increased from 0.001 to 0.045 g/L and in the liquid medium increased from 0.06 to 0.17 g/L and this is a proof that this secondary metabolite needs aeration for growthand production but cucurbitacin I did not need aeration for production. However, increasing the aeration from 0.3 to 0.6 the amount of cucurbitacin I in the cells decreased from 0.006 to 0.004 g/L and in the liquid medium decreased from 0.11 to 0.077 g/L as shown in (Figure 6). These results are in agreement with different reports about O_2 supply affects both growth and metabolite production in a number of plant cell cultures including *Perilla frutescens* (Zhong et al., 1993); and *Catharanthus roseus* (Leckie et al.,

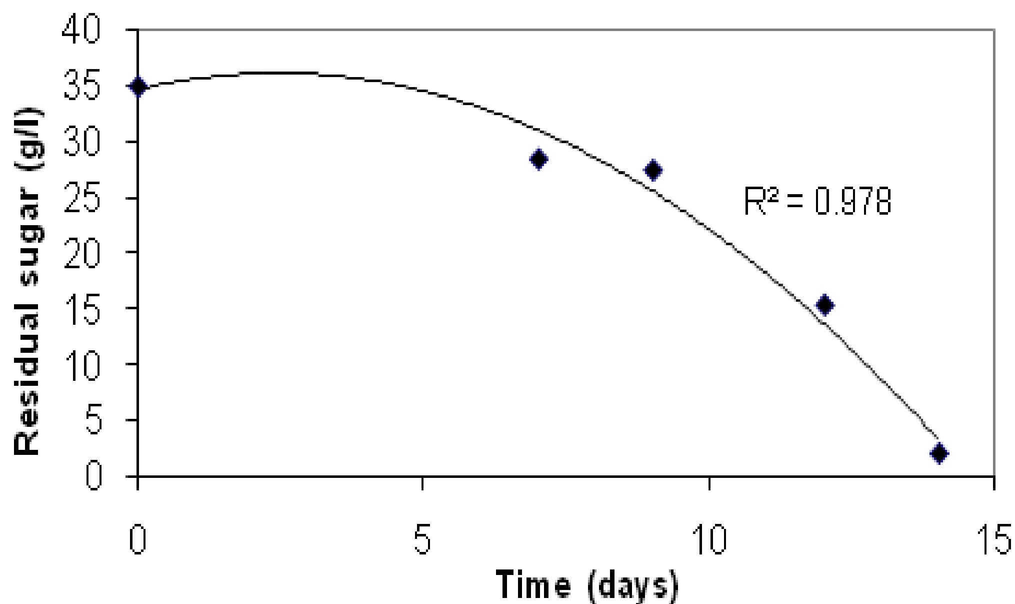


Figure 5. Sucrose consumption of *E. elaterium* in batch culture of 2 L stirred tank bioreactor and the batch conditions were: temperature 27°C, aeration rate 0.6 vvm, agitation rate 150 rpm, non controlled pH and marine impellers.

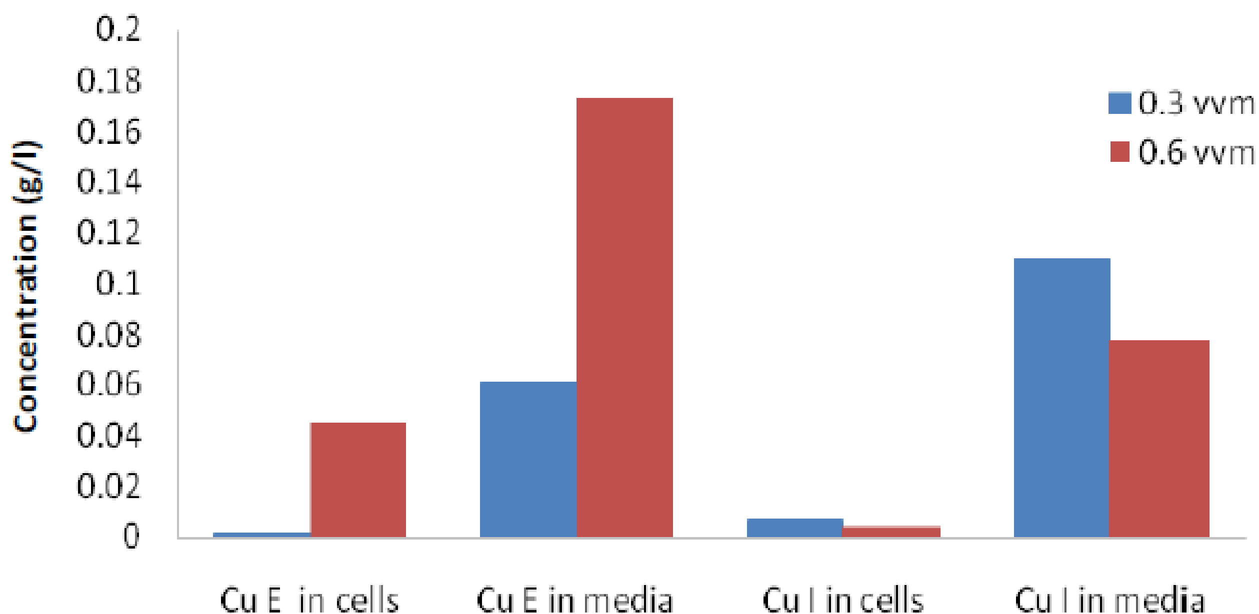


Figure 6. Comparison between different aeration rates 0.3 to 0.6 vvm and the concentration of cucurbitacin E and cucurbitacin I in cells and in the liquid media of *E. elaterium* (g/L) in batch culture of 2 L stirred tank bioreactor.

1991).

Antimicrobial activity evaluation of cucurbitacins

The obtained results of the antimicrobial activity are shown in Table 1 clearly showed that the plant extracts had antimicrobial activity in different degrees. In addition it was remarked that the inhibition zone of bacterial growth by plant extract varies from species to another according to the tested bacterial isolates. The greatest zone of inhibition was observed against *E. coli* O157 with

Table 1. Zone inhibition (*) of the tested bacterial strains (*E. coli O157, S. typhimurium, L.monocytogenes*, *Ps. aeruginosa* and *S. aureus*) aganist different concentrations of *Ecballium elaterium* extract with reference antibiotics.

Concentration (mg/ml)	*E. coli* O157 (mm)	*S. Typhimurium* (mm)	*L. monocytogenes* (mm)	*S. aureus* (mm)	*Ps.argenosus* (mm)
10	-	-	-	-	-
25	29	16	13	-	9
50	33	23	26	-	10
Tobramycin (10µg/ml)	15	14	14	16	15

*Mean zone of inhibition of three replicates in mm.

Table 2. Cytotoxic activities of cucurbitacins crude extract on MCF7, HCT116, HEPG2 and HELA carcinoma cell lines.

Carcinoma cell line	IC_{50} (µg/ml) Doxorubicin	IC_{50} (µg/ml) Cucurbitacins extract
MCF7	2.97	19.70
HCT116	3.73	23.50
HEPG2	3.73	18.20
HELA	4.19	15.60

Breast carcinoma cell line (MCF7); colon carcinoma cell line (HCT116); liver carcinoma cell line (HEPG2); cervical carcinoma cell line (HELA). Doxorubicin was used as a +ve control.

mean diameter equal 33 and 29 mm in conc. of 50 and 25 mg/ml respectively, followed by, *L.monocytogenes* with mean zone of inhibition equal 26 mm in conc. of 50 mg/ml. *S. typhimurium* showed mean zone of inhibition 23 and 16 mm in conc. of 50 and 25 mg/ml respectively.

On the other hand, extract conc. of 10 µg/ml did not show any hindrance for all tested bacterial species. Also, *S. aureus* did not show any hindrance in growth when tested against the different conc. of *Ecballium elaterium* extract. Results were compared with reference antibiotic tobramycin 10 µg/ml which showed zone of inhibition ranged from 14 to 16 mm. These results are in agreement with many reports of the antimicrobial activity of different plant extracts from cucurbitaceae family such as the ethanolic extract of *Lagenaria siceraria* that show zone of inhibition ranged from 11 to 14 mm and its aqueous extract that show zone of inhibition ranged from 7 to 9 mm against different bacteria such as *Ps. Aeruginosa, S. aureus* and *E. coli* (Badmanaban and patel, 2010).

Antitumor activity of cucurbitacins

The selected cucurbitacins extract which showed a higher concentration of cucurbitacin E and cucurbitacin I was evaluated as antitumor agents. The antitumor activities on different carcinoma cell lines; breast carcinoma cell line (MCF7), liver carcinoma cell line (HEPG2), cervical carcinoma cell line (HELA), and colon carcinoma cell line (HCT116) were summarized in Table 2. From these results, the strongest inhibition was exhibited on HELA cell line (IC_{50} = 15.6 µg/ml), on other cell lines the IC_{50}s were 18.2, 19.7 and 23.5 µg/ml for HEPG2, MCF7 and HCT116 cell lines respectively. These results were in agreement with those previously reported on the activity of cucurbitacins against different cancer cell lines (Bartalis and Halaweish 2005; Miró, 2009; Kongtun et al., 2009; Gitter et al., 1961).

REFERENCES

Attard E, Brincat MP, Cuschieri A (2005). Immunomodulatory activity of cucurbitacin E isolated from *Ecballium elaterium*. Fitoterapia 76:439-441.

Attard EG, Spiteri A (2001). *Ecballium elaterium*: an *in vitro* source of cucurbitacins. Fitoterapia 72:46-53.

Badmanaban R, Patwl CN (2010). Studies on anthelmintic and antimicrobial activity of the leaf extracts of *Lagenaria siceraria mol*. J. Glob. Pharma. Technol. 2(4):66-70.

Barry AL, Craig W, Nadler A, Reller HL, Sanders CHC, Swenson JM (1999). Methods for determining bactericidal activity of antimicrobial agents. Proposed Guideline, National Committee for Clinical Laboratory Standards NCCLS document M26-A [ISBN 1-56238-384-1] USA, 19(18).

Bartalis J, Halaweish FT (2005). Relationship between cucurbitacins reversed-phase high-performance liquid chromatography hydrophobicity index and basal cytotoxicity on HepG2 cells. J. Chromatogr. B, 818(2):159-166.

Boik J (2001). Natural compounds in cancer therapy. Oregon Medical Press Minnesota, USA.

Carbonell LA, Mendoza IE, Valenzula O,Uitz R, Meyer M, Vargas VM (1993). Effect of the medium pH on the release of secondary metabolites from roots of *Datura stramonium*, *Catharanthus roseus*,

and *Tagetes patula* cultured *in vitro*. Appl. Biochem. Biotechnol. 38:257-267.

Casanova E, Valdes AE, Zuker A, Fernandez B, Vainstein A, Trillas MI, Moysset L (2004). *rolC*- transgenic carnation plants: adventitious organogenesis and levels of endogenous auxin and cytokinins. Plant. Sci. 167:551-560.

Chattopadhyay S, Farkya S, Srivastava AK, Bisaria VS (2002). Bioprocess Considerations for Production of Secondary Metabolitesby Plant Cell Suspension Cultures. Biotechnol. Bioprocess. Eng. 7:138-149.

Chen JC, Chiu MH, Nie R, Cordell GA, Qiu SX (2005). Cucurbitacins and cucuurbitane glycosides: structures and biological activities. Nat. Prod. Rep. 22:386-399.

Choi DS, Andrade MH, Willis LB, Cho C, Schoenheit J, Boccazzi P, Sambanthamurthi R, Sinskey AJ, Rha C (2008). Effect of agitation and aeration on yield optimization of oil palm suspension culture (special issue on Malasia- MIT biotechnology partnership program). J. Oil. Palm. Res. 1:23-34.

Dogruoz M, Zeybek Z, Karagoz A (2008). Antibacterial activity of some plant extracts. IUFS J. Biol. 67(1):17-21.

Dubois M, Gilles KA, Hamilton JK, Repers PA, Smith F (1956). Calorimetric method for determination of sugars and related substances. Anal. Chem. 26:50.

Gitter S, Gallily R, Shohat B, Lavie D (1961). Studies on the antitumor effect of cucurbitacin. Cancer Res. 21:1-8.

Favel A, Mattras H, Coletti-Previero MA, Zwilling R, Robinson EA, Castro B (1989). Protease inhibitors from *Ecballium elaterium* seeds. Int. J. Pep. Proc. Res. 33(3):202-208,

Kongtun S, Jiratchariyakul W, Kummalue T, Tan-ariya P, Kunnachak S, Frahm AW (2009). Cytotoxic properties of root extract and fruit juice of *Trichosanthes cucumerina.* Planta. Med. 75(8):839-842.

Krishnaveni S, Balasubramanian T, Sadasivam S (1984). Sugar distribution in sweet stalk sorghum. Food Chem. 15(3):229-232.

Miró M (2009). Cucurbitacins and their pharmacological effects. Phytother. Res. 9:159-168.

Mukundan U, Bhide V, Singh G, Curtis WR (1998). PH-mediated release of betalains from transformed root cultures of *Beta vulgaris L*. Appl. Microbiol. Biotechnol. 50:241–245.

Oskay M, Sari D (2007). Antimicrobial screening of some Turkish medicinal plants. Pharm. Biol. (Formerly Int. J. Pharmacog.) 45:176-181.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. 82(13):1107-1112.

Sturm S, Stuppner H (2000). Analysis of cucurbitacins in medicinal plants by high pressure liquid chromatography-mass spectrometry. Phytochem. Anal. 11:121-127.

Taya M, Mine K, Kino-Oka M, Tone S, Ichi T (1992). Production and release of pigments by culture of transformed hairy root of red beet. J. Ferment. Technol. 73:31-36.

Ulusoylu M, Öndersev DV, Soyoğul Ü, Gürkan E, Tuzlacı E (2001). The Cytotoxic and the biological (antibacterial and antifungal) activities of *Centaurea iberica* and *Ferulago confusa*. J. Fac. Pharm. Gazi Univ. 18(2):75-80.

Wakimoto N, Yin D, O´Kelly J, Haritunians T, Karlan B, Said J, Xing H, Koeffler HP (2008). Cucurbitacin B has a potent antiproliferative effect on breast cancer cells *in vitro* and *in vivo*. Cancer Sci. 99:1793-1797.

Wu PL, Lin FW, Wu TS, Kuoh CS, Lee KH, Lee SJ (2004). Cytotoxic and anti-HIV principles from the rhizomes of *Begonia nantoensis*. Chem. Pharm. Bull. 52(3):345-349.