Vol 4/2 (2004) 137 - 144

JOURNAL OF NATURAL REMEDIES

Cytotoxicity of Cucurbitacin E extracted from *Ecballium elaterium in vitro*

E. Attard*[1], A. Cuschieri[2]

1. Institute of Agriculture, University of Malta.
2. Department of Anatomy, Faculty of Medicine and Surgery, University of Malta.

Abstract

Objective: The objective of this present investigation was to determine the effects of Cucurbitacin E (CuE) extracted from *Ecballium elaterium* A.Rich., Fam. Cucurbitaceae, on three cancer cell lines (breast, prostate and melanoma) and a normal transformed cell line (fibroblasts), compared to other cytotoxic agents. Methods: Cytotoxicity was assessed by an array of assays including total and viable count determinations, lactate dehydrogenase enzyme release, morphological changes, effects on mitochondrial metabolism, agarose gel electrophoresis for apoptotic changes, median inhibitory concentration determination and interaction studies. Results: CuE exhibited a marked effect on prostate adenocarcinoma cells at a median inhibitory concentration (IC_{50}) of 9.35 nM and moderate effects on melanoma and breast carcinoma cells (IC_{50} = 0.87 and 1.95 μM, respectively). Parameters that showed a reduction in cell viability were prominent with the compound, as compared to the controls. Morphologically, the cancer cells exhibited nuclear and cytoplasmic (N/C) changes such as condensation of chromatin, an increase in the N/C ratio, and rounding up of the cytoplasm. Surface blebbing and morphological signs of apoptosis occurred in all cancer cell types. In the agarose-gel electrophoresis analysis, DNA ladder characteristic of apoptosis, was exhibited by the CuE treatment on both prostate and breast cancer cell lines, as for tamoxifen and mesterolone, respectively. Negligible cytotoxic effects were observed on normal fibroblasts as compared to the control. Conclusion: Owing to its chemical structure, CuE can be used as a lead drug in the development of cytotoxic agents with low toxicity on normal cells.

Keywords: *Ecballium elaterium*, cucurbitacin E, cell proliferation, cytotoxicity, apoptosis.

1. Introduction

Ecballium elaterium (L.) A. Rich. (Cucurbitaceae, the squirting cucumber), a Mediterranean medicinal plant that has been investigated for its several pharmacological properties [1-4], contains a group of compounds collectively termed cucurbitacins. One particular cucurbitacin that has been studied for its cytotoxic activity is cucurbitacin E (CuE), a

* Corresponding author
E-mail: everaldo.attard@um.edu.mt

tetracyclic triterpenoid with a high cytotoxic potential.

This has been previously tested on ascites carcinoma, Sarcoma Black and E0771 mammary adenocarcinoma (Shohat *et al.,* 1965), HeLa and KB human cell lines (Konopa *et al.,* 1974), Epstein-Barr transformed B-lymphocyte JY cells (Musza *et al.*, 1994), human adenocarcinoma (PC-3) cells (Duncan *et al.,* 1994), ovarian (OV-95-CC3) and stomach (ST-95-AT2) human carcinomas (Attard *et al.*, 1996), *in vitro*. This work was aimed at investigating new cancer cell lines (ZR-75-1 and COLO 679), a normal transformed cell line (L929) and a previously tested cell line (PC-3).

2. Materials and methods

ZR-75-1 (breast carcinoma), COLO 679 (melanoma), PC-3 (prostate adenocarcinoma) and L929 (murine connective tissue), were purchased from the European Tissue Culture Collection (Porton Down, Salisbury, U.K.). The media used for the cell lines were as indicated in table 1.

The compounds tested on the cell lines were Cucurbitacin E (CuE; 0.2, 2 and 20 µM) extracted from *Ecballium elaterium,* and melatonin (20 µM), tamoxifen (20 µM and 2mM), ethinyloestradiol (20µM), dexamethasone (2 mM) and mesterolone (20 µM), all purchased from Sigma (Germany), according to the cell line (table 1).

These were dissolved in the respective supplemented culture medium to the stated final concentrations. To aid dissolution, all compounds required the use of DMSO (Sigma, Germany) at a concentration not exceeding 0.25 %, a dose that does not influence cell viability [5].

Cell monolayers were detached from culture flasks using the standard trypsinisation procedure. Prewarmed cell culture medium was added to adjust the cells to a concentration range of 10^5-10^6 cells/ml, after pooling the cell suspensions from the separate flasks.

The cultures were assayed at time intervals as indicated for each individual cell line (table 1) for up to 72 h, exposure to the specific substances, according to the following procedures performed in triplicate: (a) total cell counts; (b) the trypan blue dye exclusion test for viable cells [6]; (c) cytotoxicity assay using the LDH cytotoxicity kit (Boehringer-Mannheim, Germany) in 96-well plates and spectrophotometric measurement of optical density at 492/650 nm in an ELISA reader (Statfax 2100, Awareness, U.S.A.); (d) morphological observations on Papanicolau stained preparations; (e) cell proliferation assays using the WST-1 tetrazolium kit (Boehringer-Mannheim, Germany) for mitochondrial activity in round-bottomed microtiter test plates, and spectrophotometric measurement of optical density at 450/650 nm in an ELISA reader (Statfax 2100, Awareness, U.S.A.); (f) apoptotic activity was estimated in 48-hr cultures of PC-3 and ZR-75-1 cell lines by the DNA fragmentation test using the DNA ladder kit (Boehringer-Mannheim, Germany) and agarose gel electrophoresis; (g) median inhibitory concentration using 6-well plates with CuE concentrations of 0.1 to 20 µM, and cell viability analysed after 72 h; and (h) interaction studies between CuE and mesterolone for PC-3 cells, between CuE, dexamethasone and tamoxifen for COLO 679 cells, and between CuE, tamoxifen and ethinyloestradiol for ZR-75-1 cells.

Numerical data were analyzed using the BMDP/DYNAMIC (v 7.0) (Cork, Ireland) statistical package for one-way analysis of variance (ANOVA), the Bonferroni post-hoc test for comparison of means with the control, one-way analysis of co-variance (ANCOVA) and two-tailed adjusted means T-test.

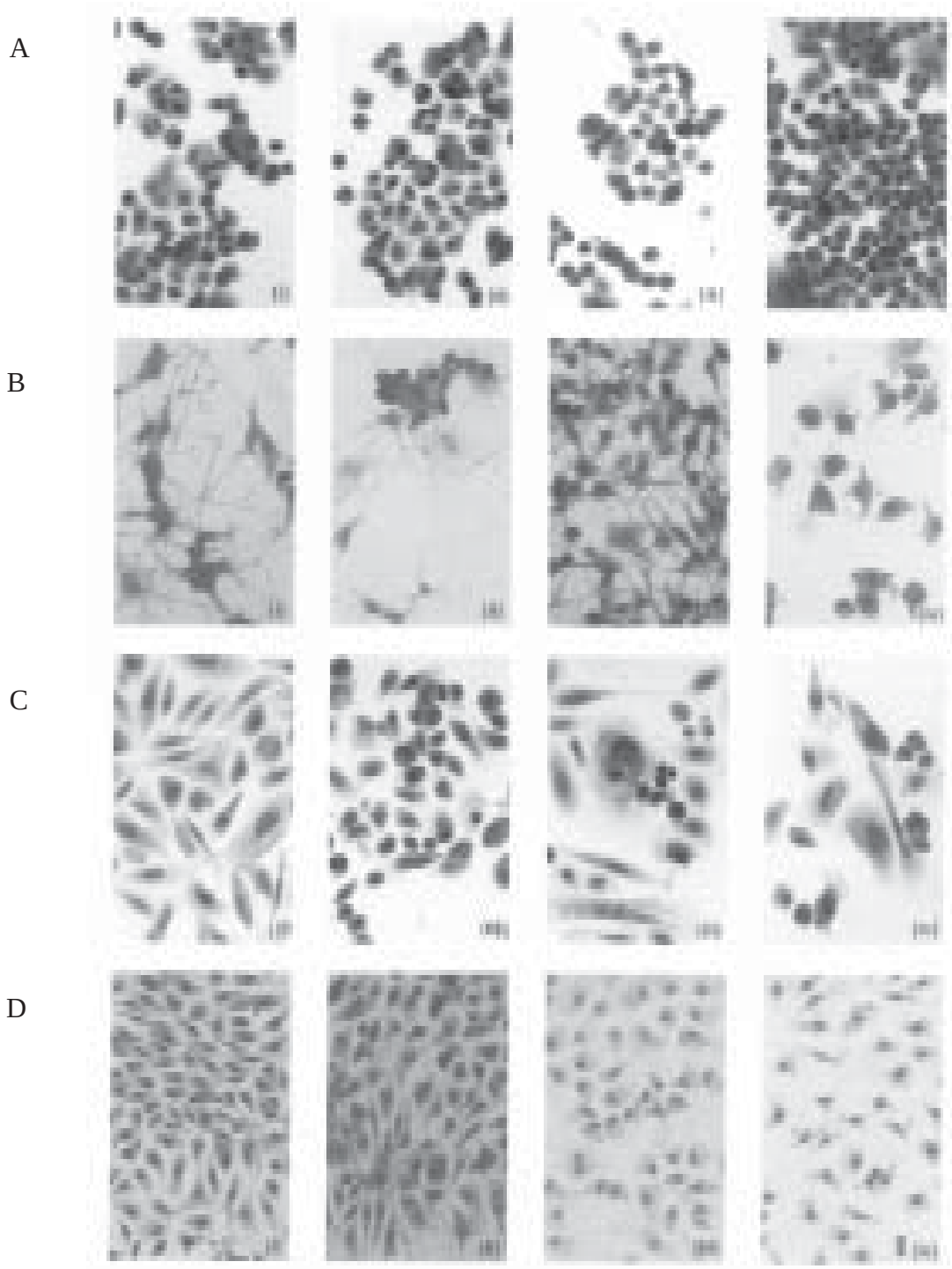

Figure 1. The effects of compounds on the morphology of (A) ZR-75-1, (B) COLO-679, (C) PC-3 and (D) L929 cells after 48 h.
A [(i) Control, (ii) CuE, (iii) Ethinyloestradiol, (iv)Tamoxifen]
B [(i) Control, (ii) CuE, (iii) Dexamethasone, (iv)Tamoxifen]
C [(i) Control, (ii) CuE, (iii) Mesterolone, (iv)Tamoxifen]
D [(i) Control, (ii) CuE, (iii) Dexamethasone, (iv) Tamoxifen]

Table 1.
The parameters for the four cell lines tested.

Parameter	ZR-75-1	COLO 679	PC-3	L929
ECACC No.	87012601	87061210	90112714	85011425
Cell Type	Epitheloid	Fibroblastoid	Epitheloid	Fibroblastoid
Culture Media	RPMI 1640 10% FCS 100 mg/l Na pyruvate	RPMI 1640 10% FCS 2mM glutamine	Ham's F12 7% FCS 1% NEAA 2mM glutamine	DMEM 10% FCS 2mM glutamine
Time Intervals	6, 24, 48, 72 h	1, 6, 24, 48 h	6, 24, 48, 72 h	1, 12, 24, 48, 72 h
Investigations	TC, VC, CA, CM, CP, Ap, IC_{50}, CIS	TC, VC, CA, CM, CP, IC_{50}, CIS	TC, VC, CA, CM, CP, Ap, IC_{50}, CIS	TC, VC, CA, CM, CP, IC_{50}
Compounds used	CuE, Mel, Tam, Eth	CuE, Tam, Dex	CuE, Mel, Tam, Mes	CuE, Dex, Tam

Legend: TC (total counts), VC (viable counts), CA (cytotoxicity assay), CM (cell morphology), CP (cell proliferation), Ap (apoptotic assay), IC_{50} (median inhibitory concentration) CIS (compound interaction studies); CuE (cucurbitacin E), Mel (melatonin), Tam (tamoxifen), Eth (ethinyloestradiol), Dex (dexamethasone), Mes (mesterolone).

3. Results and discussion

Initial studies had demonstrated that CuE had a toxic effect on cancer cells *in vitro*, inhibiting cell proliferation and producing morphological changes indicative of apoptosis. [7] The present study, originally intended to assess cytotoxicity on other cancer cell lines and a normal cell line showed that Cucurbitacin E had an inhibitory effect on the cancer cell lines with a lower inhibitory activity on the fibroblastic cell line. Cell viability was reduced with CuE treatment in a concentration-dependent manner.

It manifested a similar decline in cell viability to tamoxifen on ZR-75-1 (20 µM), PC-3 (20 µM) and COLO 679 (2 mM) cells, and melatonin (20 µM) on PC-3 cells (table 2). The effects of CuE on L929 cell viability were concentration-dependent although not significantly different from the control. A slightly significant decline was manifested with the 20 µM CuE concentration after 72 h exposure.

CuE exhibited the lowest IC_{50} on PC-3 cells (9.35 nM or 5.21 ng/ml), followed by COLO 679 cells (0.87 µM or 0.483 µg/ml), ZR-75-1 cells (1.95 µM or 1.08 µg/ml) and finally L929 cells (93.8 µM or 52.22 µg/ml). According to Ratsimamanga-Urverg and co-workers [8], compounds with IC_{50} of 30 µg/ml or higher are considered as being weakly toxic. The effects of the compound on PC-3 cells goes in accordance with Duncan and co-workers [9]. These results show that CuE is very effective against the COLO 679 and ZR-75-1 cells and weakly toxic to the L929 cells.

From the results obtained, cell membrane integrity assay and the cellular metabolism assay complemented the morphological manifestations of cells with CuE treatment. The compound manifested signs of apoptotic cell death on the cancer cell lines. Although all cell types had different morphology, with treatment certain manifestations were common.

In fact, cellular blebbing, cells with high nuclear to cytoplasmic ratio, nuclear fragmentation and

Table 2.
The effects of compounds on percentage cell viability of (A) ZR-75-1, (B) PC-3, (C) COLO-679 and (D) L929 cells against time.

	(A) ZR-75-1 Percentage Viability (%) [Mean ± S.E.]						(B) PC-3 Percentage Viability (%) [Mean ± S.E.]					
	ZR-75-1 Control†	**CuE (20µM)**	**CuE (2µM)**	**CuE (0.2µM)**	**Tamoxifen (20µM)**	**Ethinyl-oestradiol (20µM)†**	**PC-3 Control**	**CuE (20µM)†**	**CuE (2µM)**	**CuE (0.2µM)**	**Tamoxifen (20µM)**	**Me-sterolone (20µM)**
6h	100±	80.562±	85.513±	89.101±	69.788±	94.993±	99.29±	96.52±	97.01±	98.52±	97.62±	98.32±
	0.0000	1.4329***	2.2215**	0.8051**	1.2365***	1.4450***	0.7140	2.3261	1.5511	0.9895	1.2162	1.1235***
24h	96.275±	74.573±	78.992±	85.535±	66.389±	97.181±	99.38±	54.21±	60.71±	67.50±	78.54±	59.97±
	0.7280	0.5001***	0.9688***	1.5930***	0.9262***	0.7974	0.6250***	2.8773***	2.1946***	2.7493***	2.3651***	1.6927***
48h	95.988±	52.228±	76.228±	83.736±	59.718±	98.114±	98.97±	49.83±	55.86±	59.83±	70.73±	55.53±
	0.4092***	0.5831***	1.0872***	1.3251***	0.5202***	0.4954***	0.7135***	1.4578***	2.8396***	2.4757***	2.8531***	2.9350***
72h	98.750±	44.596±	47.153±	58.393±	48.731±	96.417±	97.60±	20.89±	37.58±	40.04±	55.86±	31.34±
	0.4535***	0.5034***	0.5048***	0.3255***	0.3708***	0.2360***	0.8698***	1.5798***	1.2060***	0.6807***	1.3243***	2.0243***

	(C) COLO679 Percentage Viability (%) [Mean ± S.E.]						(D) L929 Percentage Viability (%) [Mean ± S.E.]				
	COLO679 Control†	**CuE (20µM)**	**CuE (2µM)**	**CuE (0.2µM)**	**Tamoxifen (20mM)**	**Dexa-methasone (20mM)**	**L929 Control**	**CuE (20µM)**	**CuE (2µM)**	**CuE (0.2µM)**	**Dexa-methasone (2mM) †**
1h	100±	40.508±	83.751±	89.252±	69.667±	48.599±	97.048±	93.996±	94.837±	95.910±	80.388±
	0.0000***	0.9165***	0.5411***	0.4929***	2.2607***	2.7308***	0.9877	1.0105	0.9209	0.9234	0.8404***
6h	100±	28.356±	65.565±	86.833±	61.716±	28.547±					
	0.0000***	1.2169***	0.6655***	1.0870***	2.2253***	1.1502***					
12h							95.713±	93.238±	94.169±	95.036±	74.548±
							0.9602	1.1871	0.1794	0.8421	1.0116***
24h	98.075±	23.923±	58.997±	65.44±	27.333±	14.733±	96.159±	94.696±	93.680±	93.875±	66.639±
	0.4970***	0.5593***	0.9719***	0.8532***	1.6329***	0.7809***	0.8823	0.7616	1.5939	1.2747	0.9173***
48h	98.65±	23.76±	27.931±	46.128±	25.052±	2.00±	95.694±	91.582±	94.282±	95.351±	73.131±
	0.2961***	2.2650***	1.8442***	0.7968***	0.9059***	1.3333***	1.1286	1.1344	0.8960	0.7238	1.2178***
72h							92.536±	86.966±	91.589±	91.712±	61.577±
							0.2944	1.2529**	0.5587	1.1356	1.4999***

Each point is the mean ± S.E.M. (ANCOVA post hoc t-test: †p<0.0025, v=23, control *vs.* all for ZR-75-1, COLO-679 and PC-3, and dexamethasone *vs.* all for L929 cells; ANOVA and Bonferroni post-hoc test: **p<0.01, ***p<0.001, v=72 against control for ZR-75-1, COLO-679 and PC-3, and dexamethasone for L929 cells).

the formation of apoptotic bodies were common to the three cancer cell lines (figure 1). The release of LDH appears to be occurring from the late apoptotic bodies. Metabolic dysfunction paralleled the loss in membrane integrity occurring in those cells that have undergone apoptosis.

At high concentrations, CuE depressed cellular metabolism since like other terpenoids it probably affects the isoprenylation process [10]. The apoptotic manifestations were confirmed by agarose gel electrophoresis (figure 2). Exposure of cancer cells to CuE resulted in internucleosomal cleavage of genomic DNA, yielding a laddered pattern of oligonucleosomal fragments characteristic of apoptosis when resolved by agarose gel electrophoresis at a 20 µM CuE concentration (figure 2). These manifestations were not observed on T-lymphocytes treated with the same concentration of CuE (unpublished).

To determine whether the compound interferes with the activity of other cytotoxic compounds, combination studies were carried out (table 3). On ZR-75-1 cells, CuE did not interfere with the activity of tamoxifen or ethinyloestradiol, while on COLO 679, CuE manifested synergistic activity with dexamethasone. In PC-3 cells, the CuE-mesterolone combination did not give an optimum additive effect, due to the fact that both compounds are probably involved in the isoprenylation inhibitory mechanism.

Structure-Activity Relationships:

The moieties with cytotoxic activity in CuE are mainly the α, β-unsaturated system in the side chain and the diosphenol system which is a precursor α, β-unsaturated carbonyl system.

Table 3.
The cytotoxic effects of compounds and their combinations on (A) ZR-75-1, (B) PC-3 and (C) COLO-679 cells against time.

Time			**(A) ZR-75-1 % Cytotoxicity**				
(h)	**Control**	**CuE (20µM)**	**Tamoxifen (20µM)**	**Ethinyl-oestradiol (20µM)**	**CuE/Tam**	**CuE/Eth**	**Tam/Eth**
6	0.000	18.106	35.383	0.858	52.517***	33.41**	4.577*
24	0.000	29.11	47.398	1.938	60.679***	35.445**	7.468*
48	0.000	43.788	51.091	1.959	64.410***	48.237**	17.935*
			(B) PC-3 % Cytotoxicity				
	Control	**CuE (20µM)**	**Mesterolone (20µM)**	**CuE / Mesterolone**			
6	0.000	5.031	2.446	2.620†			
24	0.000	23.387	5.232	21.538†			
48	0.000	45.878	34.828	37.044†			
			(C) COLO 679 % Cytotoxocity				
	Control	**CuE (10µM)**	**CuE + Dxameth**	**Dxameth (1mM)**	**CuE + Tamox.**	**Tamox. (1mM)**	
1	0	32.602	65.647***	18.779	55.048*	18.902	
24	1.426	42.298	87.092***	44.238	61.104*	28.569	
48	1.228	40.685	74.194***	45.002	75.094*	45.002	

ANCOVA post hoc t-test: †p>0.1, *p<0.05, **p<0.01, ***p<0.001, v=13.

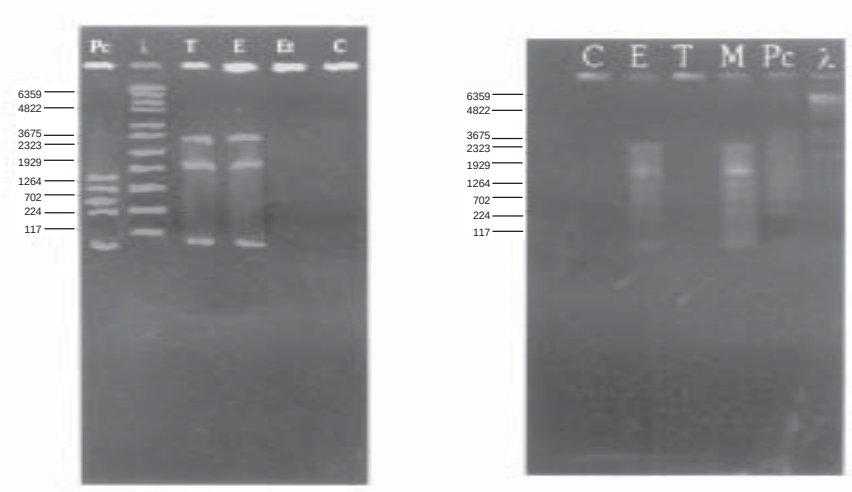

Figure 2. DNA gel electrophoresis for ZR-75-1 and PC-3 cells treated with the various compounds.
Pc=positive control, λ=λ-phage, T=tamoxifen, Et=ethinyloestradiol, E=CuE, C=control, M=mesterolone.

These moieties were termed apoptotic inducers in the cytochalasin series [11]. Terpenoids including paclitaxel are potential inhibitors of isoprenylation inside cells [10]. The fact that CuE does not inhibit ICAM-1 [12] and p53 is lacking or is defective in PC-3 cells, the apoptotic pathways related with these proteins are not suggested.

Although the CuE has direct genotoxic effects on DNA (unpublished), the effect occurs at a concentration that is much higher than the IC_{50} of CuE on the cell lines selected for the investigation. The p21-related apoptotic mechanism is strongly favoured by the fact that, like paclitaxel, CuE is an isoprenoid (terpenoid) that is capable of inhibiting protein isoprenylation in PC-3 cells by inhibiting the incorporation of prenoid units in p21ras and p21rap-1 proteins.

This inhibition led to the initiation of a cascade reaction that resulted in apoptosis [10]. This is initialised by the loss of compartmentalisation of DNase I resulting in DNA fragmentation. The endoplasmic reticulum and the nuclear membrane are affected before this could happen [13].

Isoprenylation is necessary for the maintenance of those structures especially lamins A and B which form the nuclear lamina and low molecular weight G proteins including $p21^{rho}$ that regulate cytoskeletal functions [14,15]. Inhibition of isoprenylation by isoprenoids (e.g. paclitaxel), results in impaired association of lamins to nuclear membrane and induction of actin depolymerisation and hence an alteration in cell morphology [16]. The actin-disrupting activity of CuE on PC-3 cells as suggested by Duncan and co-workers [9] is a sequel of the apoptotic events taking place inside the affected cells.

As regards the normal cell line, the same moieties were also investigated for their effects. The α, β-unsaturated system is not toxic to fibroblasts [17]. CuE lacks the 17β-hydroxyl

group as for certain corticosteroids and the epoxy group at the 5-6 position as for withanolides. But the 16α-hydroxyl group renders CuE slightly cytotoxic at doses higher than 20 μM.

The compound has minor cytotoxic effects on fibroblasts at a concentration at which it is effective against prostate and breast cancer cells, *in vitro*. This implies that CuE is a safe compound and hence could be used in phase I clinical trials especially on prostate and breast carcinoma animal models.

It is evident from the studies carried out that the compound should not pose any necrosis (i.e. necrotic cell death) hence reducing the possibility of inflammation at the treatment area. Besides, CuE like other cucurbitacins have been proved to possess anti-inflammatory activity [4].

References

1. Basaran A, Basaran N, Baser KH. (1993) *Fitoterapia* LXIV: 310 - 313.

2. Elayan HH, Gharaibeh MN, Zmeili SM, Salhab AS. (1989) *Int. J. Crude Drug Res.* 27: 227-234.

3. Favel A, Mattras H, Coletti-Previero MA, Zwilling R, Robinson EA, Castro B. (1989) *Int. J. Pept. Protein Res.* 33: 202-208.

4. Yesilada E, Tanaka S, Sezik E, Tabata M. (1988) *J. Nat. Prod.* 51: 504-508.

5. Mirossay A, Mirossay L, Tothova J, Miskovsky P, Onderkova H, Mojzis J. (1999) *Phytomedicine* 6: 311-318.

6. Freshney RI (1988) *Culture of Animal Cells: A Manual of Basic Technique,* Alan R. Liss, Inc.: New York, U.S.A.; 132-134.

7. Attard E, Scicluna-Spiteri A, Grixti M, Cuschieri A. (1996) *Xjenza* 1: 29–34.

8. Ratsimamanga-Urverg S, Rasoanaivo P, Rakoto-Ratsimamanga A, Le Bras J, Ramiliarisoa O, Savel J, Coulaud JP. (1991) *J. Ethnopharmacol.* 33: 231-236.

9. Duncan KL, Senderowicz AM, Malspeis L, Grever MR, Sausville EA (1994) *Proc. Am. Assoc. Cancer Res.* 35: 409.

10. Danesi R, Figg WD, Reed E, Myers CE. (1995) *Mol. Pharmacol.* 47:1106-1111.

11. Nagasawa H, Nagura F, Mohamad SB, Uto Y, Zhu J-W, Tsukuda T, Hashimoto T, Asakawa Y, Hori H. (1999/2000) *Phytomedicine* 6: 403-409.

12. Musza LL, Spreight P, McElhiney S, Barrow CJ, Gillum AM, Cooper R, Killar LM. (1994) *J. Nat. Prod.* 57: 1498-1502.

13. Peitsch MC, Bolzar B, Stephan H, Crompton T, MacDonald HK, Mannherz HG, Tschopp J. (1993) *EMBO J.* 12: 371-337.

14. Adamson P, Marshall CJ, Hall A, Tilbrook PA. (1992) *J. Biol. Chem.* 267: 20033–20038 .

15. Ridley AJ, Hall A. (1992) *Cell* 70: 389-399.

16. Fenton HO, Kung HF, Longo DL, Smith MK. (1992) *J. Cell. Biol.* 117: 347–356 .

17. Koganov MM, Dueva OV, Tsorin BL. (1999) *J. Nat. Prod.* 62: 481-483.