Research Article

The Cytotoxic Activity of Cucurbitacin E and Busulphan on Ovarian and Stomach Cancer Cells *In Vitro:* A Comparative Study

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Summary. *A comparative study of the cytotoxicity of cucurbitacin E, a natural product, and busulphan on human ovarian and stomach cell lines was carried out. The cells were exposed to different concentrations of these two compounds and cell viability was determined from day 0 to day 11. It was observed that cucurbitacin E had a marked effect on the ovarian cancer cell line while busulphan showed a similar effect when exposed to the stomach cancer cell line. These drug-cell combinations showed a pronounced cell kill exponential curve, leading to the conclusion that cucurbitacin E exerts its pinocytic activity on the ovarian cancer cells while busulphan exerts its alkylating effect on the stomach cancer cells.*

Keywords: *Ecballium elaterium, Cucurbitaceae*; cucurbitacin E; busulphan; ovarian cancer cell line; stomach cancer cell line

Cucurbitacin E and other cucurbitacins are highly oxygenated triterpenes which are found solely in plants grouped under the *Cucurbitaceae* family, including *Citrullus colocynthis* L., (the squirting cucumber), *Ecballium elaterium* L. is a local medicinal plant which has been used in folk medicine as a cathartic (Cini, 1991) and as an emetic (Lanfranco, 1975). It has also been used in dropsy (Penza, 1969) and in the treatment of jaundice (Cini, 1991).

Experiments on the juice of the plant have shown that it is effective in the treatment of constipation, oedema, sinusitis, and the prevention of liver disease (Yesilada *et al.*, 1988). However, it has been found that the juice has a low therapeutic index (Farnsworth, 1992), but that it contains cucurbitacins, including cucurbitacins B and E, which have antitumour activity. Despite this, when individual cucurbitacins were tested on various normal cells, the cell viability was not affected (Gallily *et al.*, 1962).

Cytotoxicity (Gitler *et al.*, 1961) and metabolic studies (Shohat *et al.*, 1962) were performed on Sarcoma 180, Ehrlich ascites carcinoma and Sarcoma Black using cucurbitacins D, E and I in mice. There was a higher cytotoxic effect shown by these compounds on Sarcoma 180 than on the other two cell lines. Metabolic studies showed that in Ehrlich ascites carcinoma cells, the oxygen uptake of cells was more sensitive to the action of cucurbitacins than the anaerobic glycolysis. It was observed that the inhibition of the oxidative metabolism of the cancer cells by the cucurbitacins was related to that observed by hydrocortisone. This may be due to the fact that the cucurbitacins have a steroid-like structure which may influence the permeability of the membranes of the cells and mitochondria. Combination therapy with cucurbitacins and X-rays on transplanted tumours in mice (Shohat *et al.*, 1965) was less effective on Ehrlich tumour than Sarcoma Black.

Cucurbitacins B and E showed an effect on cultured human nasopharyngeal carcinoma, and Sarcoma 37 implanted intramuscularly into right hind legs of CAF, mice when these compounds were injected intraperitoneally.

Cucurbitacin E (Figure 1) can exert its cytotoxic effect either on the cell membrane (Gallily *et al.*, 1962) or on the DNA in the nucleus of the cancer cells (Kupchan *et al.*, 1973). The cucurbitacin side chain is important for the observed cytotoxic activity (Kupchan *et al.*, 1970).

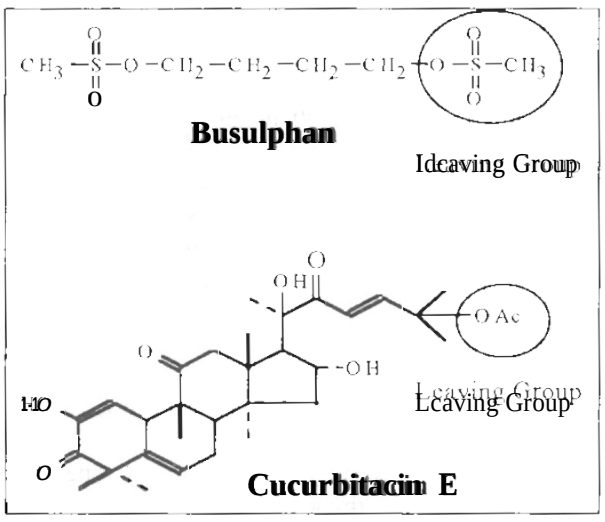

Figure 1: Structures of Busulphan and Cucurbitacin E

Busulphan (Figure I) is an alkylating agent, which exerts its action by joining two guanine residues on two strands of the DNA, leading to cross-linking. This in turn prevents the uncoiling and replication of the DNA molecule, thus halting the multiplication of the tumour cells (Rogers et al., 1976).

Several studies have been carried out on the cell lines already mentioned using different cucurbitacins, but the effect of these compounds on ovarian and stomach cancer cell lines has not been studied. The present study was therefore undertaken to examine any possible effects of cucurbitacin E on ovarian and stomach cancer cell lines and also to compare the effect of cucurbitacin E with that of a widely used cytotoxic agent, busulphan.

Experimental Procedures

Cucurbitacin E was prepared by solvent extraction of the fruit of *Ecballium elaterium* (Lavie *et al.*, 1958), collected from Marsascala (Malta). 52% (w/w) of the pure compound was obtained. Its purity was confirmed using five analytical methods: UV and IR spectrophotometry, Melting Point Determination, TLC and HPLC against a known standard. A specimen is deposited at the Institute of Agriculture, University of Malta. From a stock solution of 1.88×10^{-4} M, 10 dilutions were prepared.

Busulphan (Myleran® Wellcome, West Sussex, U.K.) 500mg tablets were ground in a mortar and then dissolved in RPMI 1640 medium to make a final stock solution of 4×10^{-3} M. 1 in 10 dilutions were then prepared.

Single stomach (SNU-1) and ovarian (OVCAR-3) cell lines were obtained from the Department of Anatomy, University of Malta. These cell lines were cultured and subcultured to propagate the cell lines. The cells were grown in RPMI 1640 medium in sterile Nunclon® culture flasks and incubated at 37°C in 6% CO_2. Subculturing was performed every seven days (Freshney, 1988).

A cell suspension was obtained by detaching the monolayer from the flask using trypsin and resuspending the cells in RPMI medium. Complete cell detachment was visualized under a x100 magnification microscope (Diavert Leitz-Wetzlar). 20ml of RPMI medium was added to each flask (x2) and the cell suspension was mixed. A small sample was withdrawn and cells were counted in an Improved Neubauer haemocytometer using the method described in the Sigma Cell Culture Catalogue (1994).

The drugs were added on day 0. Twenty-eight tubes were used in all. 1ml of ovarian cell suspension was added to each of fourteen tubes while 1ml of stomach cell suspension was added to another fourteen tubes. The concentrations of cucurbitacin E used were: 1.8×10^{-4}M, 1.8×10^{-6}M and 1.8×10^{-8}M. The concentrations of busulphan used were: 4×10^{-4}M, 4×10^{-6}M and 4×10^{-8}M. 2ml of the three solutions with different concentrations of cucurbitacin E were added to six tubes containing ovarian cancer cells, and to six tubes containing stomach cancer cells. The same procedure was repeated for busulphan. The rest (i.e. four tubes) acted as the control tubes in which 2 ml of RPMI medium were added to the cancer cell suspension.

From days 7 to 11, the number of viable and non-viable cells was counted using a haemocytometer and 0.4% trypan blue for the staining of non-viable cells (Freshney, 1988). The experiment was followed from day 7 to 11, as it was observed in our laboratory that there was no significant lethal effect on the cancer cells from day 0 to day 7. The five-day period, day 7 to 11, was sufficient to provide information on the cytotoxic activity of both cucurbitacin E and busulphan on the two cancer cell lines. A preliminary study had shown that the decrease in percentage cell viability was not significant after day 11.

The percentage cell viability was calculated using the number of viable and non-viable cells obtained. The four results were used to obtain an average percentage cell viability. The counting of non-viable cells was necessary to determine the LC_{50}, which is the concentration of cytotoxic compound required to kill 50% of the cells in suspension. The decrease in cell viability should depend on the cytotoxic activity of the compounds and not on the limited environmental factors, which include nutrient availability in the medium and the conditions inside the incubator. The cell counts for the tubes treated with the cytotoxic compounds were adjusted by taking the average cell count in the control tubes to be 100%.

Results

Tumour Cell Growth Inhibition. Figures 2 to 5 show the percentage log cell viability against number of days, for ovarian and stomach cancer cells, both treated with cucurbitacin E and busulphan.

As can be observed from Figure 2, at 1.8×10^{-6}M, cucurbitacin E showed a lower terminal percentage log cell viability than at 1.8×10^{-7}M, although cell viability, for the latter, was markedly reduced. Figure 3 shows that the inhibition of tumour growth is higher with increasing busulphan concentration. At the two lower concentrations (4×10^{-4}M and 4×10^{-6}M) of busulphan used, a rapid fall in cell viability was evident after day 10 while at 4×10^{-8}M a rapid decrease was observed after day 9. Figure 4 shows that the effect of cucurbitacin E on stomach cancer cells varied with the three different concentrations used. At 1.8×10^{-4}M cucurbitacin E, there was a rapid decrease in cell viability between day 8 and 9 but a slow steady fall thereafter. At 1.8×10^{-6}M cucurbitacin E, there was a linear decrease in cell viability. At 1.8×10^{-8}M cucurbitacin E, there was a slow decreases in cell viability after day 10. Stomach cancer cells treated with

4x10⁻⁶M busulphan (Figure 5) showed a stepwise decrease in viability with time. A considerable decrease in cell viability after day 8 was observed at the three concentrations of busulphan used, but no further effect at 4x10⁻⁶M and 4x10⁻⁵M was observed after day 10. At 4x10⁻⁴M busulphan a higher cytotoxic effect was observed than at the lower concentration of 4x10⁻⁶M.

The four different cell-drug combinations were compared using the Probit analysis. The differences in the trends for these combinations were found to be statistically significant ($P<0.05$, $F=4$).

Cucurbitacin E showed a higher cytotoxic effect on the ovarian cancer cells than busulphan. It is worth noting

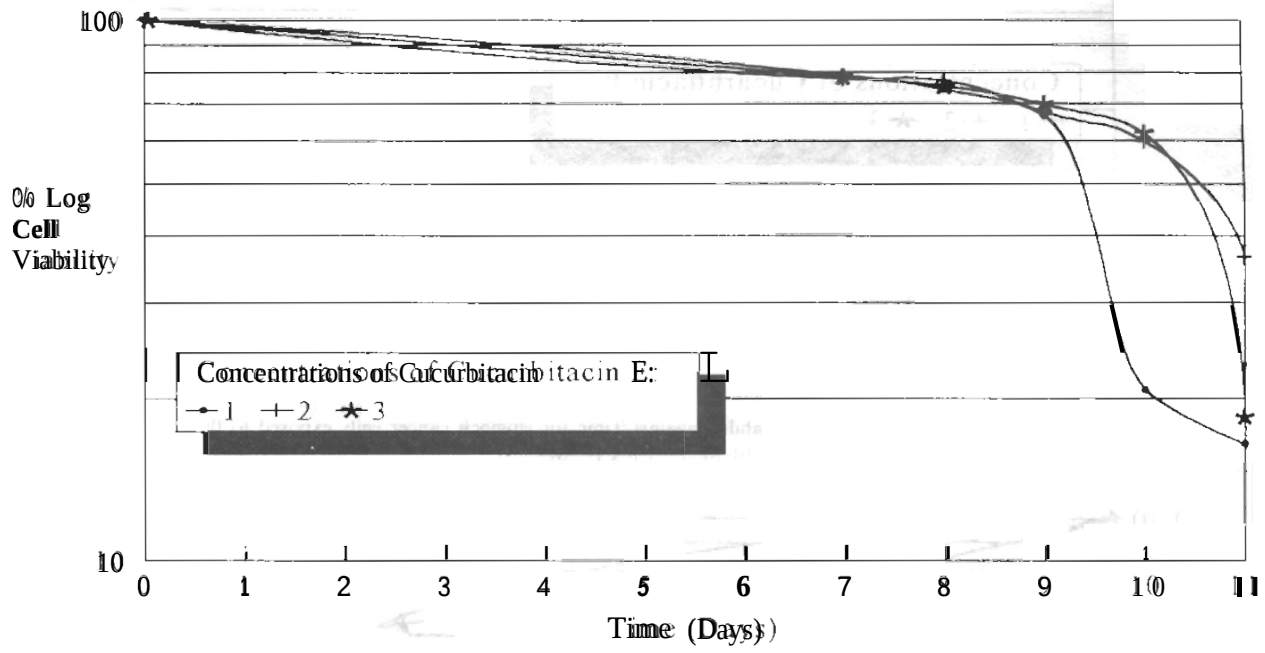

Figure 2: The Percentage Log of the Adjusted Cell Viability against Time for ovarian cancer cells exposed to three concentrations of Cucurbitacin E (Concentrations: 1 = 8x10⁻⁷M, 2 = 8x10⁻⁶M, 3 = 8x10⁻⁵M).

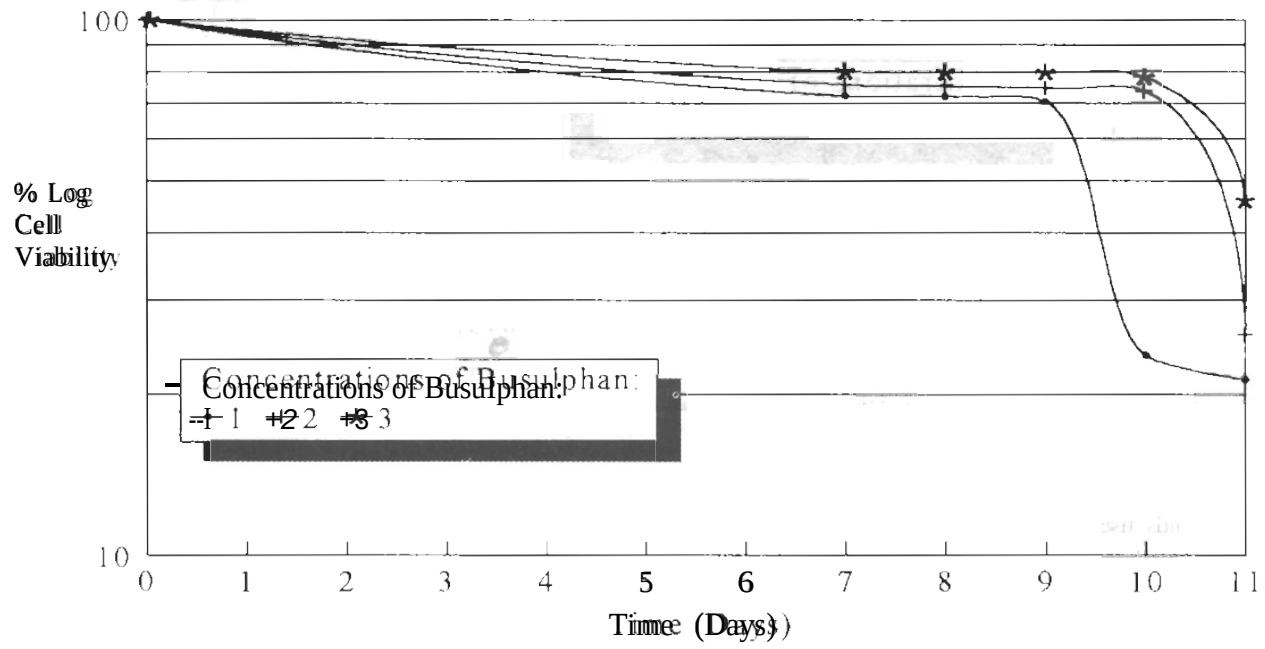

Figure 3. The Percentage Log of the Adjusted Cell Viability against Time for ovarian cancer cells exposed to three concentrations of Busulphan (Concentrations: 1 = 4 x 10⁻⁴M, 2 = 4 x 10⁻⁵M, 3 = 4 x 10⁻⁶M).

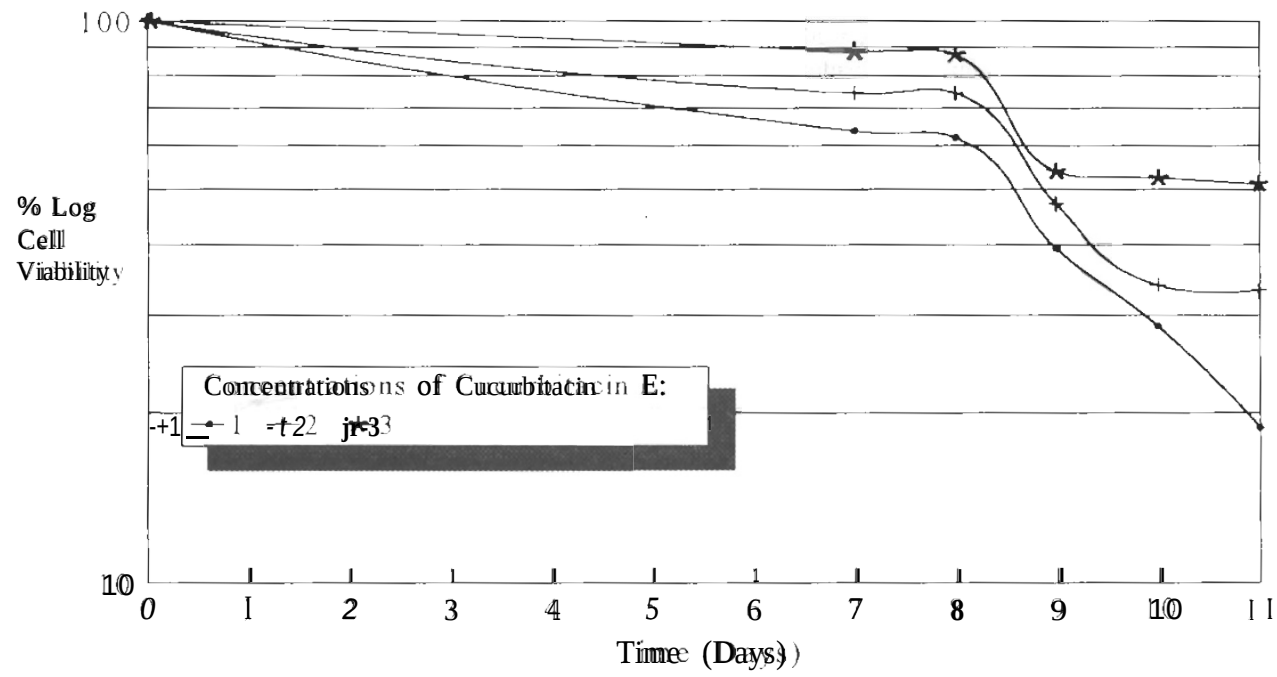

Figure 4: The Percentage Log of the Adjusted Cell Viability against Time for stomach cancer cells exposed to three concentrations of Cucurbitacin E (Concentrations: 1.8 x 10^-1 M, 1.8 x 10^-2 M, 1.8 x 10^-3 M).

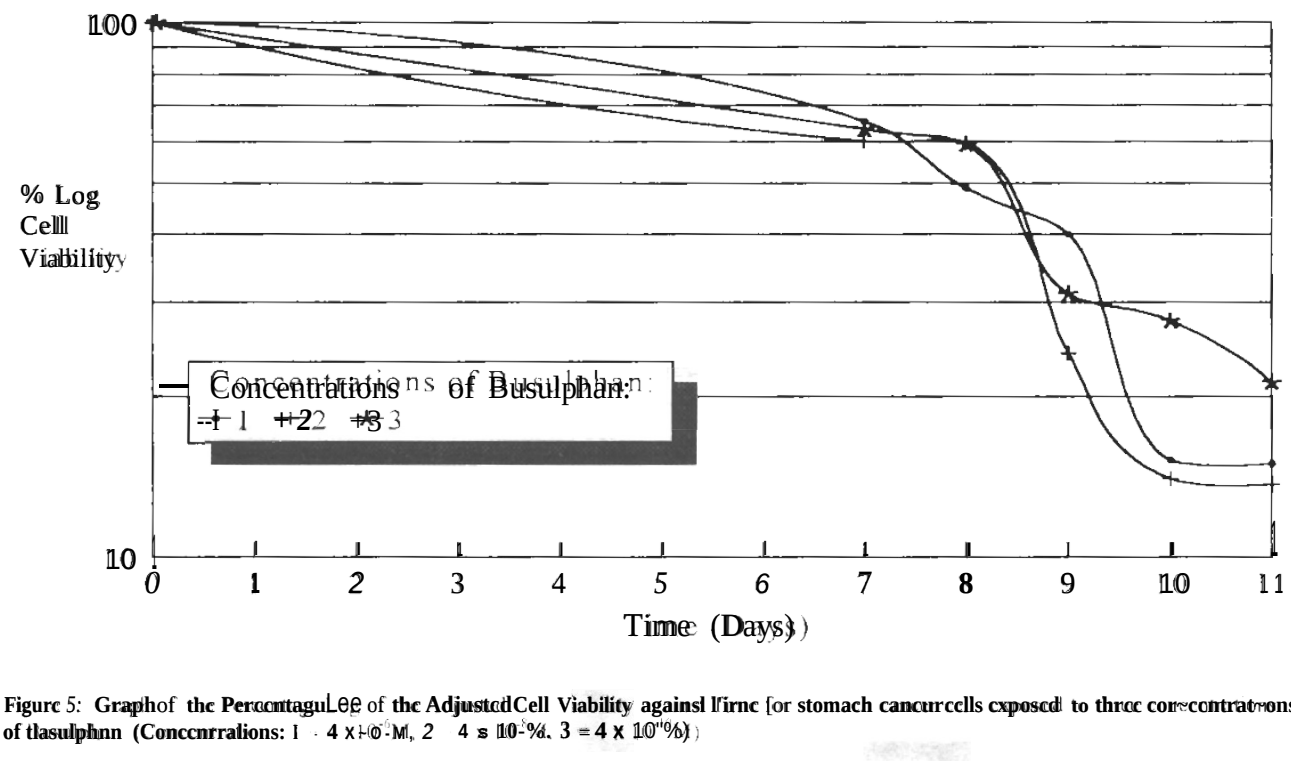

Figure 5: Graph of the Percentage of the Adjusted Cell Viability against Time for stomach cancer cells exposed to three concentrations of busulphan (Concentrations: 1 = 4 x 10^-1 M, 2 = 4 x 10^-2 M, 3 = 4 x 10^-3 M).

that at the highest concentration of both cytotoxic compounds used (1.8 x 10^-1 M cucurbitacin E and 4 x 10^-1 M busulphan), a similar pattern was observed: characterized by no further cytotoxicity at day 11. In the stomach cancer cells, a greater percentage cell death was obtained with busulphan than with cucurbitacin E.

Minimum Median Lethal Concentration. Table 1 shows the minimum median lethal concentration for the two different cell lines treated with the two cytotoxic drugs. Cucurbitacin E showed a minimum LC50 of 2.7 x 10^-1 M for the ovarian cancer cells at day 10, and a minimum LC50 of 0.526 M for the stomach cancer cells at day 7. For busulphan, the minimum LC50 for ovarian cancer cells was 9.1 x 10^-1 M at day 9, while that for the stomach cancer cells was 2.1 x 10^-1 on day 10.

From the values one may note that cucurbitacin E has a greater activity on ovarian cancer cells than busulphan (mLC50 E < mLC50 B). On the other hand, busulphan showed a greater effect on the stomach cancer cells although this occurred on day 10 as opposed to the

	Ovarian Cancer Cells	Stomach Cancer Cells
	Cucurbitacin E	
mLC50	2.72x10^{-7} M	0.5269 M
It value*	0.8795	0.7575
	Busulphan	
mLC50	9.14x10^{-4} M	2.14x10^{-8} M
R value**	0.9006	0.9540

Table 1. Table showing the mLC50 for the two different compounds and cell lines. All R values are taken at p<0.05, n = 4.

mLC50 of cucurbitacin E which was found on day 7. The LC50 for cucurbitacin E-treated stomach cancer cells is too high (0.5269M) to be considered as an effective compound.

Discussion

Since cucurbitacin E has been shown to have an effect on DNA by alkylation (Kupchan *et al.*, 1973), and on the cell membrane by the process of pinocytosis (Gallily *et al.*, 1962), it was of interest in this study to compare its effects on cancer cells with those of busulphan and to draw some conclusions from the results obtained.

Tumour Cell Growth Inhibition. The results show that the *in vitro* cytotoxic effect of cucurbitacin E was best observed on ovarian cancer cell lines while busulphan showed a greater effect on stomach cancer cells.

If one considers that cucurbitacin E is taken up by the tumour cell by a rate limiting process, there might be sufficient uptake at low concentrations to have an alkylating effect on the DNA. This might explain the greater cytotoxic effect observed for cucurbitacin E on ovarian cancer cells at the lowest concentration (1.8×10^{-8} M) used. Whether this effect is due to the process of pinocytosis is still to be determined. However, this process is greatly influenced by high cucurbitacin E concentrations, where an increase in the uptake of fluid inside the cell leads to cell blistering and eventually cell death. This was observed by Gallily and co-workers (1962) on four cell lines, using elaterin A and B. At high concentrations, the effect on the cell membrane is more pronounced.

Busulphan, at a concentration of 4×10^{-4} M and 4×10^{-8} M, did not have an effect on the ovarian cancer cells. This is known as the tumorostatic effect. At these two concentrations, insignificant cytotoxicity was observed until day 10 after which a decrease in cell viability was observed. At the highest concentration (4×10^{-4} M) used, the same effect was observed until day 9 after which there was a better response. This might be explained by the fact that busulphan did not appear to affect the pinocytic activity of the tumour cells since cell morphological changes were not observed. The high rate of cell death observed for the high concentration might be due to the effects on the DNA by alkylation.

At the lowest concentration (1.8×10^{-8} M) of cucurbitacin E used, a small effect on the stomach cancer cell line was observed, probably due to the limited amount of drug in solution. However, at the highest concentration (1.8×10^{-6} M) a marked effect on cell viability was observed. It can be concluded that stomach cancer cells showed marked resistance towards cucurbitacin E, as was shown by the ovarian cancer cells toward busulphan. On the other hand, busulphan showed effective cytotoxicity in the stomach cancer cells. Although there was a great reduction in the viable count at the highest concentration (4×10^{-4} M), the 4×10^{-8} M concentration showed a lower end-point. However, at these two concentrations, after day 10, no further significant inhibition was observed.

It would appear that the activity of the cytotoxic compounds on stomach cancer cells does not depend on the pinocytic activity but on the alkylating effect on the DNA since busulphan had a greater activity than cucurbitacin E on these cancer cells.

It can be concluded from these results that cucurbitacin E lacks the pronounced alkylating effect of busulphan but the latter lacks the pinocytic activity of cucurbitacin E. It might also be postulated that cucurbitacin E increases the uptake of busulphan (and other alkylating agents) while the latter exerts its effects inside the cell. The effect of cucurbitacin E on the permeability of cell membranes (Shohat *et al.*, 1962) could be due to its steroid-like structure which is similar to that of the cell membrane. It should also be stressed that cucurbitacin E has an effect on both cell lines, although a minimal one on the stomach cancer cells.

Minimum Medium Lethal Concentration. The median lethal concentrations (LC50) for cucurbitacin E on the ovarian and stomach cancer cells show that for the ovarian cells the LC50 was quite satisfactory and hence merits further attention while for stomach cancer cells the high LC50 indicates a lack of sensitivity of these cells for the cytotoxic compound.

For busulphan the LC50 for the ovarian cancer cells is quite high and so it can be regarded as ineffective for the treatment of ovarian cancer. In fact the mLC50 for busulphan is 3361 times greater than that for cucurbitacin E in these cells. However, the low LC50 for stomach cancer cells suggests that it can be used. On the contrary, the LC50 for busulphan is much smaller than that for cucurbitacin E, i.e. the mLC50 of the latter, being about 2.46×10^{2} times greater than the mLC50 of busulphan.

To substantiate the above finding, further investigations would have to be carried out to determine the extent of the activity of the two cytotoxic agents *in vivo*, to determine morphological changes and to detect any DNA aberrations.

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References

Cini G (1991) Faqqus il-Hmir li faqqas it-Tmintas Irbigha. *L-Eghjun. Lehen il-Moviment Civiku Xoghra fil-Qrun 20.*

Farnsworth NR (1992) Napralert (SM) Issue 2: Department of Medicinal Chemistry and Pharmacognosy. College of Pharmacy, University of Illinois. Chicago, U.S.A.

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

Gallily R, Shohat B, Kalish J, Gitter S and Lavie D (1962) Further Studies on the Antitumor Effect of Cucurbitacins. *Cancer Research*, **22**, 1038.

Gitter S, Gallily R, Shohat B and Lavie D (1961) Studies on the antitumour effect of Cucurbitacins. *Cancer Research*, **21**, 516.

Kupchan SM and Tsou G (1973) The Structure and Partial Synthesis of Fabacein. *Journal of Organic Chemistry*, **38**, 1455 - 6.

Kupchan SM, Smith RM, Aynehchi Y and Maruyama M (1970) Tumour Inhibitors. LVI. Cucurbitacins O, P and Q, the Cytotoxic Principles of *Brandgea higelowii*. *Journal of Organic Chemistry*, **35**, 2891.

Lanfranco G (1975) Duwa u Semm il-Hxejjex Maltin. In: Edizzjoni Klabb Kotba Maltin Valletta, Malta 33-40.

Lavie D and Szinai S (1958) The constituents of *Ecballium elaterium* L. II, a Diacetate. *Journal of the American Chemical Society*, **80**, 707.

Penza C (1969) Flora Maltija Medicinali. Progress Press, Malta, 29.

Roger H and Spector R (1976) *An Introduction to Medicines in Pharmacology and Therapeutics. First Edition*. William Heinemann Medical Books Ltd., Great Britain. 383-385

Shohat B, Gitter S and Lavie D (1962) Antitumour Activity of Cucurbitacins: Metabolic Aspects. *Cancer Chemotherapy Reports*, **23**, 19.

Shohat B, Gilter S, Lwy B and Lavie D (1965) The combined effect of cucurbitacins and X-ray treatment of transplanted tumours in mice. *Cancer Research*, **25**, 1828.

Sigma Cell Culture Catalogue (1994) Sigma Chemical Company. 205-206.

Yesilada E, Tanaka S, Sezik E and Tabata M (1988) Isolation of an anti-inflammatory principle from the juice of Ecballium elaterium. *Journal of Natural Products*, **51**, 504.