

Full Length Research Paper

Cytotoxic effect of 2', 3'-epoxy isocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol isolated from *Micromelum minutum* (G.Forst.) Wight and Arn. in human T-lymphocyte leukemia CEM-SS cells

B. K. Tan¹, N. B. Alitheen^{1*}, S. K. Yeap², A. M. Ali³ and R. Mawardi⁴

¹Institute of Bioscience, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

²Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

³Faculty of Agriculture and Biotechnology, University Darul Iman Malaysia (UDM), Kampus Kota, Jalan Sultan Mahmud, 20400 Kuala Terengganu, Malaysia.

⁴Department of Chemistry, Faculty of Science, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

Accepted 4 May, 2009

2',3'-Epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol are two bioactive compounds isolated from the leaves of *Micromelum minutum*. In this study, the induction of apoptosis by 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol on CEM-SS cells were investigated. The cytotoxicity of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol was screened on CEM-SS by using MTT assay. The apoptotic effect of both compounds on CEM-SS was evaluated by using fluorescence and electron microcopies morphological observation and DNA laddering analysis. The inhibition effect of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol at 50% of cell population (IC₅₀) was found to be 4.6 µg/ml (13.5 µM) and 3 µg/ml (7.8 µM) on CEM-SS cells, respectively. From DNA fragmentation, Acridine orange and Propidium iodide staining and electron microscope analyses, the compounds were confirmed to have ability in promoting apoptosis. However, the % of apoptosis induced is low and the event is time-dependent. At high concentration of 10 µg/mL, 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol induced necrosis. Furthermore, 8-hydroxyisocapnolactone-2',3'-diol also exhibited better cytotoxicity compared to 2',3'-epoxyisocapnolactone. The induction time for apoptosis by 8-hydroxyisocapnolactone-2',3'-diol in CEM-SS is earlier than 2',3'-epoxyisocapnolactone, which is 4 h and 12 h after treatment. Both 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol are potential as anticancer agent.

Key words: Cytotoxic, 2',3'-epoxy isocapnolactone, 8-hydroxyisocapnolactone-2',3'-diol, *Micromelum minutum*, CEM-SS.

INTRODUCTION

Natural products play an important role in discovery of chemotherapy drug. It contributes to over 50% of novel chemotherapy drugs since 1941 to 2002. (Newman et al., 2003). *Micromelum minutum* (G.Forst.) Wight and Arn. is

a shrub or small tree of the family rutaceae, which can grow up to 25 feet high. This plant normally found in the hilly parts of the northern half and in the north-west area of Peninsular Malaysia, locally known as "Chemama", "Cherek-cherek" or "Secherek". Traditionally, this plant was used as herbal medicine by local people, where the leaves are normally pounded with tamarinds and salt and applied to skin for pain relieves. In Peninsular Malaysia, it is traditionally used in the treatment of fever and giddiness and a poultice of the boiled roots is applied to treat

*Corresponding author. E-mail: noorjahan@biotech.upm.edu.my. Tel.: 603 89477471. Fax: 603 89747510.

ague (Bulkil, 1966). The ethanol extract of *M. minutum* was previously found to be active against MCF-7 cell line (Itharat et al., 2004).

A chemotaxonomic investigation on the leaves and stems of *M. minutum* has resulted in the isolations of pure compounds. Seven novel coumarin: 3",4"-dihydrocapnolactone, 2',3'-epoxyisocapnolactone, 8-hydroxyisocapnolactone-2',3'-diol and 8-hydroxy-3",4"-dihydrocapnolactone-2',3'-diol, 8,4"-dihydroxy-3",4"-dihydrocapnolactone-2',3'-diol, 8-methoxycapnolactone and stigmastrol and 2 known triterpenes, 5(6)-gluten-3 1 and 5(6)-gluten-3 α -ol were isolated from the leaves of *M. minutum* (Rahmani et al., 2003; Susidarti et al., 2006; Susidarti et al., 2007). Coumarins are highly active biological substances. They are used in the fields of biology, medicine, perfumes, cosmetics and laser dyes (Peng and Cai, 2008). They have spasmolytic, antioxidant, anticoagulant, antibacterial, antiviral and antifungal activities. There is a lot of data about antineoplastic action of coumarin derivatives. They act at different stages of cancer formation. Some of them have cytostatic properties and the others have cytotoxic activity (Cherng et al., 2008). Besides coumarins, mahanine had also been isolated from *M. minutum* leaves. Mahanine has shown wide range of pharmacological effects including anti-mutagenicity, anti-microbial activity on gram negative bacteria, anti-inflammatory effect and cytotoxic effect toward U937 cell (Roy et al., 2005).

Although the bioactivity of mahanine isolated from *M. minutum* had been studied intensively, the contribution of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol toward the cytotoxicity of *M. minutum* has not been tested yet. Thus, this study was carried out to investigate the cytotoxic and apoptotic effect of these compounds towards CEM-SS.

MATERIALS AND METHODS

Natural compounds

2',3'-Epoxyisocapnolactone (Figure 1A) and 8-hydroxyisocapnolactone-2',3'-diol (Figure 1B) which were isolated from the leaves of *M. minutum* as described previously (Rahmani et al., 2003) were kindly supplied by Prof. Dr. Mawardi Rahmani, Department of Chemistry, Faculty of Science and Environmental Studies, University Putra Malaysia, Serdang, Selangor. Both compounds were dissolved in RPMI-1640 (Sigma, USA) at 10 mg/ml as stock. (Figure 1 A and B)

Cell line

Suspension cell line, CEM-SS (human T-lymphoblastic leukemia) was obtained from the National Cancer Institute, Frederick, Maryland, USA. Anchorage dependent cell lines, HeLa (cervical carcinoma), HT29 (colon carcinoma), HepG2 (liver carcinoma) and Chang (transform liver cell) were obtained from animal tissue culture laboratory, department of biotechnology, university Putra Malaysia. Cell lines were then stored in liquid nitrogen before further used. CEM-SS and HeLa were maintained in RPMI-1640 (Sigma, USA) supplemented with 100 IU/ml penicillin (Flowlab, Australia) and 100 μ g/ml streptomycin (Flowlab, Australia) while

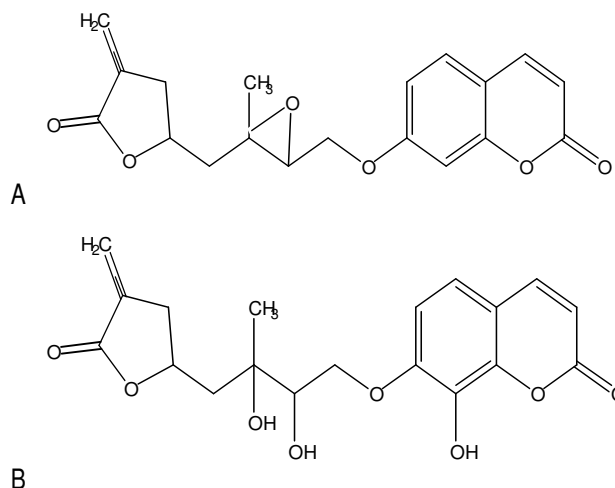


Figure 1. The chemical structure of (A) 2',3'-epoxyisocapnolactone (B) 8-hydroxyisocapnolactone-2',3'-diol isolated from the leaves of *M. minutum*.

HT29, HepG2 and Chang liver cell were maintained in DMEM (Sigma, USA) with 100 IU/ml penicillin (Flowlab, Australia) and 100 μ g/ml streptomycin (Flowlab, Australia). All the cells were grown at 37°C in a humidified incubator with a 5% CO₂ atmosphere and were used for assays during the exponential phase of growth.

Microtitration cytotoxicity assay

The cytotoxicity of both compounds on CEM-SS, HeLa, HT29, HepG2 and Chang liver cell were screened by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, 100 μ L of suitable medium were added into each well of flat-bottomed 96 well plate except row A. 100 μ L of diluted 2',3'-epoxyisocapnolactone or 8-hydroxyisocapnolactone-2',3'-diol (60 μ g/mL) was added into row A and row B. The solution was resuspended and serially diluted from row B to row G. The excess (100 μ L) was discarded. Row H was left untouched. 100 μ L of target cell were added into all the wells and incubated in 37°C, 5% CO₂, 90% humidity for 24, 48 and 72 h. A stock solution of 5 mg/mL MTT in PBS was prepared and 20 μ L of MTT was added into each well. The culture medium was discarded and added with 100 μ L of DMSO (Sigma, USA) to dissolve the purple crystal. Finally, the plate was read on an automated spectrophotometric EL 340 μ Quant ELISA reader (Bio-Tek Instruments, USA) using test and reference wavelength of 570 nm. The % of cytotoxicity was calculated by using the following equation:

$$\% \text{ Cytotoxicity} = (\text{OD sample} / \text{OD control}) \times 100$$

Fluorescent microscopy

The cell suspension was treated at 4 different concentrations (0 μ g/ml, IC₅₀, 1/2 of IC₅₀ and 10 μ g/ml) in 6 wells plates, and then incubated in 5% CO₂ humidified incubator at 37°C for 4, 12, 24, 48 and 72 h respectively. The cells were harvested by removing 500 μ L of cell suspension from plate into 1.5 ml microcentrifuge tube. The cells were then mixed with 2 μ L of staining solution containing 1 mg/ml acridine orange (Sigma, USA) and 1 mg/ml propidium iodide (Sigma, USA). After that, the cells were spun down at 1000 rpm for 2-3 min. 450 μ L of the supernatant were discarded and the pellet

Table 1. Inhibition concentration of 50% (IC_{50}) of both natural compounds isolated from *M. minutum* (2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol) against various cell lines determined by MTT assay.

Cell line	2',3'-epoxyisocapnolactone, $\mu\text{g/ml}$ (μM)	8-hydroxyisocapnolactone-2',3'-diol, $\mu\text{g/ml}$ (μM)
CEM-SS	4.6 \pm 0.9 (13.5 \pm 2.6)*	3 \pm 0.4 (7.8 \pm 1.1)*
HeLa	13.4 \pm 1.2 (39.2 \pm 3.5)*	9.0 \pm 2.2 (23.9 \pm 5.9)*
HT29	14.2 \pm 1.1 (41.5 \pm 3.2)*	7.7 \pm 0.8 (20.5 \pm 2.1)*
HepG2	7.4 \pm 1.7 (21.6 \pm 5.0)*	5.9 \pm 1.0 (15.7 \pm 2.7)*
Chang	6.5 \pm 2.0 (19.0 \pm 5.8)*	7.1 \pm 1.8 (18.9 \pm 4.8)*

Data are mean of triplicate determinations plus \pm SD.

* $p \leq 0.05$ compared with untreated cells.

was mixed vigorously with remain supernatant, dripped on the slide and observed under a fluorescence microscope (Nikon, Japan) with combination of excitation filter and barrier filter of 450 - 490 nm and long pass filter 520 nm. The % of viable (green intact cells), apoptotic (green shrinking cells with condensed or fragmented nucleus) and necrotic (red cells) were determined from > 200 cells for the data to be statistically significant. Results were expressed as a proportion of the total number of cells examined.

Scanning electron microscopy

Treated (IC_{50} and 10 $\mu\text{g/ml}$) and untreated cells were harvested and washed twice with PBS. In primary fixation, cell pellets were fixed in 4% buffered glutaraldehyde for 6-24 h at 4°C. The fixative was removed after centrifugation at 3000 rpm for 8 min. Cell pellets were washed 3 changes with 0.1 M sodium cacodylate buffer at 10 min interval each. After that, post fixation was carried out in 1% osmium tetroxide for 2 h at 4°C, followed by washing step with 3 changes of 0.1 M sodium cacodylate buffer again at 10 min interval each. Dehydration of specimens were carried with a series of acetone in the order of 35, 50, 75 and 95% each for 10 min at room temperature and lastly with absolute acetone (Merck, Germany) at the final stage of dehydration, allowing 10 min interval each. The specimens can be kept in 75% acetone if not assessed immediately. A drop of cell suspension was then coated with gold and viewed by using scanning electron microscope (JEOL JSM-35C). Specimen can also be stored in a silica gel desiccator before viewing.

DNA fragmentation assay

T-lymphoblastic leukemia (CEM-SS) cells at concentration 5×10^5 cells/ml in 5 ml culture were treated with natural compounds (2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol) at IC_{50} for 4, 12, 24, 48 and 72 h. Cells were then harvested and washed twice with PBS and resuspended in 500 μl of lysis buffer (150 mM NaCl, 100 mM Tris pH 8.0, 100 mM EDTA and 0.5% sodium dodecylsulphate). Then, 0.25 mg/ml proteinase K (promega, USA) was added and incubated in 50°C for 60 min. After the incubation, proteinase K was inactivated by heating up to 70°C for 15 min. Incubation was continued with 0.1 mg/ml RNase (promega, USA) for another 60 min. Then, cellular DNA was extracted by standard phenol/chloroform method. An equal volume of phenol/chloroform/ isoamyl (25:24:1) was added and the different solutions phase was separated by centrifugation at 2500 rpm for 8 min. The upper layer was then transferred into new microcentrifuge tube followed by extraction with chloroform/ isoamyl (24:1). The resulting upper layer was removed again into new microcentrifuge tube and

mixed with 1/10 volume of 3 M sodium acetate. The DNA was then precipitated with 2 volume of 100% cold ethanol. The mixture was then spun down at 14000 rpm for 10 min and supernatant was discarded. DNA pellet was air dried and dissolved in TE buffer (10 mM Tris-HCl pH 7.5 and 1 mM EDTA). The whole lysate was then analysed by gel electrophoresis on 1.5% agarose gel with DNA marker Lambda-Hind III (MBI Fermentas, Lithuania).

Statistical analyses

The effects of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol against T-lymphoblastic leukemic cells (CEM-SS) were compared using the unpaired student's t-test (version 11.5, SPSS inc., USA). Results were considered to be statistically significant when $p \leq 0.05$.

RESULTS

Microtitration cytotoxicity assay

For this study, microtitration cytotoxicity assay was used to evaluate the cytotoxic effect of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol on suspension cell line (CEM-SS) and 4 anchorage dependent cell lines (HeLa, HT29, HepG2 and Chang liver).

Both natural compounds isolated from *M. minutum* (2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol) were found to be more active against T-lymphoblast leukemia cell (CEM-SS). The IC_{50} of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol was 4.6 $\mu\text{g/ml}$ (13.5 μM) and 3 $\mu\text{g/ml}$ (7.8 μM) respectively. However, the inhibition effect of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol against HeLa, HT29, HepG2 and Chang cells was significant ($p \leq 0.05$). As shown in Table 1, IC_{50} of 2',3'-epoxyisocapnolactone against HeLa, HT29, HepG2 and Chang cells were 13.4 $\mu\text{g/ml}$ (39.2 μM), 14.2 $\mu\text{g/ml}$ (41.5 μM), 7.4 $\mu\text{g/ml}$ (21.6 μM) and 6.5 $\mu\text{g/ml}$ (19.0 μM), respectively. For 8-hydroxyisocapnolactone-2',3'-diol, IC_{50} values against HeLa, HT29, HepG2 and Chang cells were 9.0 $\mu\text{g/ml}$ (23.9 μM), 7.7 $\mu\text{g/ml}$ (20.5 μM), 5.9 $\mu\text{g/ml}$ (15.7 μM) and 7.1 $\mu\text{g/ml}$ (18.9 μM), respectively. From the above re-

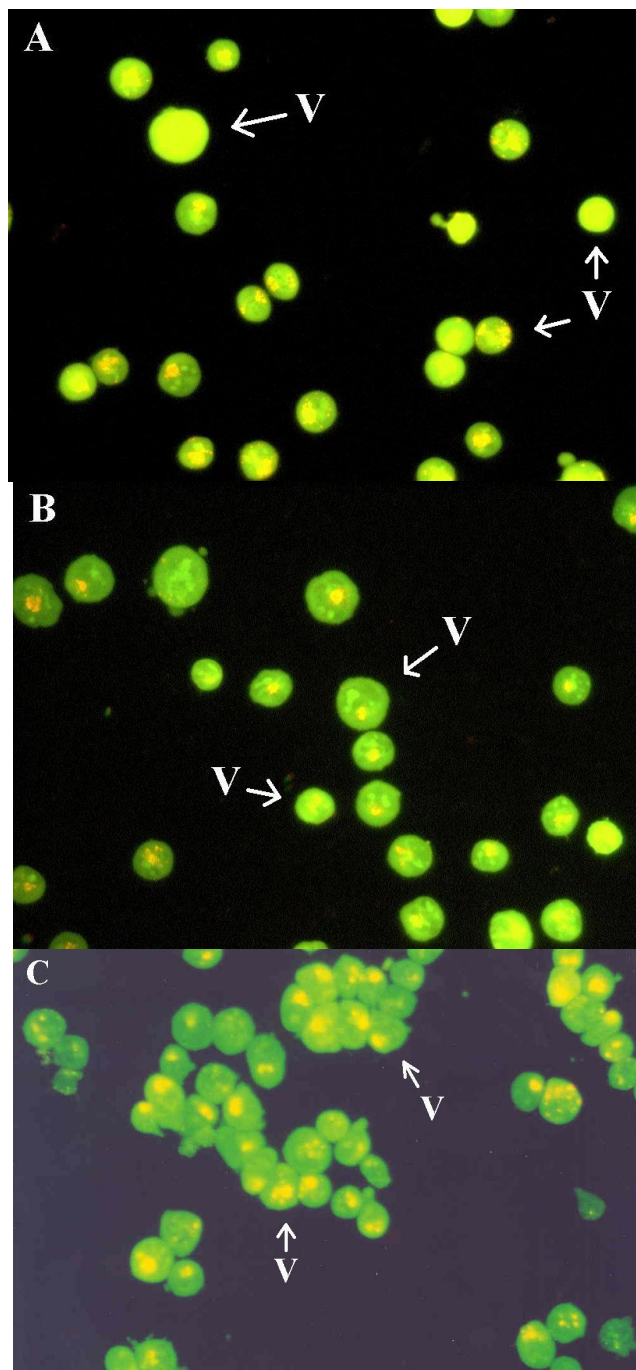


Figure 2. Fluorescence microscopy examination of untreated CEM-SS cells. The cells were stained with acridine orange (AO) and propidium iodide. Viable (V) cells were scored based on their different appearances. A: Untreated cells at 4 h (Magnification: 200X); B: Untreated cells at 12 h (Magnification: 200X); and C: Untreated cells at 24 h (Magnification: 200X).

sults, both natural compounds isolated from *M. minutum* (2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol) were more effective against CEM-SS cell lines. Therefore, CEM-SS was chosen for further study

(Table 1).

Microscopy evaluation

Based on morphological changes, the modes of cell death whether by apoptosis or necrosis, can be determined. In this study, the alteration of morphology induced by 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol were observed by three different kinds of microscopes, fluorescent and electron microscopy.

Fluorescent microscopy

In this study, the cell death mode was evaluated by applying AO/PI staining. Figures 2, 3 and 4 show the fluorescent images of untreated cells, 2',3'-epoxyisocapnolactone-treated cells and 8-hydroxyisocapnolactone-2',3'-diol-treated cells at various concentrations and time points. The % of these cells were then illustrated in Figure 5. When the cells were subjected to 2',3'-epoxyisocapnolactone at IC_{50} concentration, the % of apoptotic population increased significantly ($p \leq 0.05$) from $4.64 \pm 5.4\%$ at 4 h, $8.5 \pm 0.7\%$ at 12 h to $16.48 \pm 4.6\%$ at 24 h compared with untreated cells populations. After 24 h of incubation, the % of apoptosis cells slowly reduced while necrotic cells increased. This situation might be due to the change of apoptotic cell into secondary necrotic cells after certain period. At concentration of $10 \mu\text{g/ml}$, 2',3'-epoxyisocapnolactone induced necrotic cell death after 12 h of treatment and destroyed almost all the viable cells after 48 h. For the concentration $\frac{1}{2}$ of IC_{50} , 2',3'-epoxyisocapnolactone had no significant cytotoxic effect against CEM-SS cells ($p > 0.05$).

The result obtained at IC_{50} in Figure 6, indicated that 8-hydroxyisocapnolactone-2',3'-diol was a more potent apoptosis inducer compared with 2',3'-epoxyisocapnolactone. The % of apoptosis was increased significantly ($p \leq 0.05$) from $9.91 \pm 2.9\%$ at 4 h, $15.61 \pm 4.4\%$ at 12 h to $25.1 \pm 5.0\%$ at 24 h compared with untreated cells populations. Similar with 2',3'-epoxyisocapnolactone, apoptosis was induced by 8-hydroxyisocapnolactone-2',3'-diol reduced after 48 h of treatment. However, the % of viable cells started to increase at 48 h. It might be due to the loss of effect of 8-hydroxyisocapnolactone-2',3'-diol in culture after certain period of incubation. For $10 \mu\text{g/ml}$, the effect of 8-hydroxyisocapnolactone-2',3'-diol against CEM-SS started at 4 h and destroyed almost all the viable cells at 24 h. For the concentration half of IC_{50} , the effect of 8-hydroxyisocapnolactone-2',3'-diol against CEM-SS cells was not significant ($p > 0.05$). The cytotoxic effect of 0.1% DMSO against CEM-SS cells was not significant ($p > 0.05$) compared with untreated CEM-SS cells (Figure 7). Therefore, the cytotoxic effect shown by both natural compounds was not interfered with by the vehicle used to dissolve the compounds (Figure 2A, B, C; 3A, B, C; 4A, B, C; 5A, B, C; 6A, B, C; 7A, B).

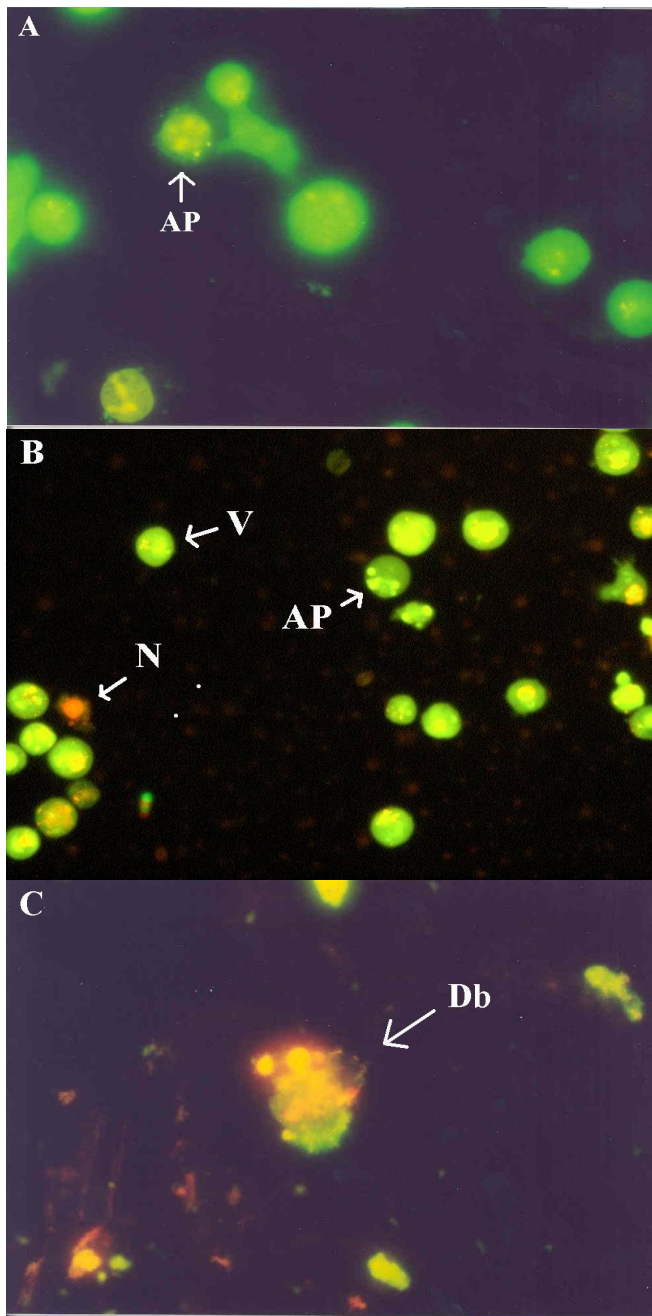


Figure 3. Fluorescence microscopy examination of CEM-SS cells treated with 2',3'-epoxyisocapnolactone apoptotic (AP), necrotic (N), viable (V) and debris (Db) cells were scored base on their different appearances. A: Cells treated with IC₅₀ (5 µg/ml) at 12 h (Magnification: 400X); B: Cells treated with IC₅₀ (5 µg/ml) at 24 h (Magnification: 200X); and C: Cells treated with high dose (10 µg/ml) at 24 h (Magnification: 400X).

Scanning electron microscopy

Ultra structural changes of CEM-SS cells especially at the plasma membrane can be readily evaluated by using scanning electron microscope. Apoptotic cells have their own typical features that are distinguishable from necrotic

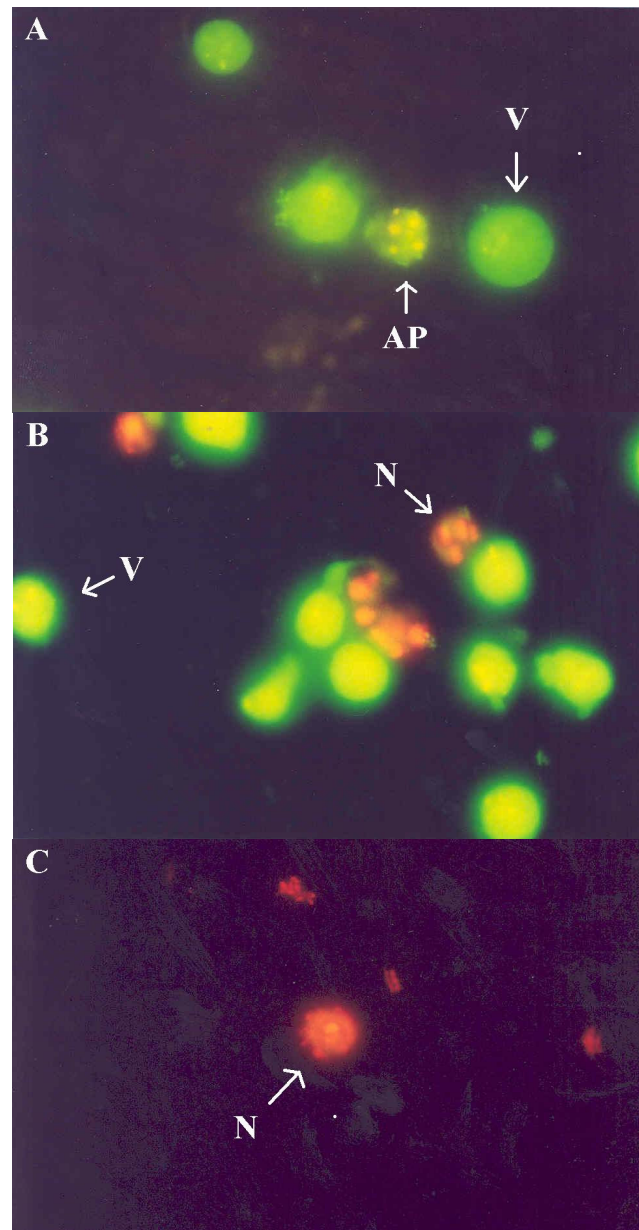


Figure 4. Fluorescence microscopy examination of CEM-SS cells treated with 8-hydroxyisocapnolactone-2',3'-diol. Apoptotic (AP), necrotic (N) and viable (V) cells were scored base on their different appearances; A: Cells treated with IC₅₀ (3 µg/ml) at 4 h (Magnification: 400X); B: Cells treated with IC₅₀ (3 µg/ml) at 24 h (Magnification: 400X); and C: Cells treated with high dose (10 µg/ml) at 24 h (Magnification: 400X).

cells using scanning electron microscope. In Figure 8, the membranes for untreated CEM-SS cells remain intact and smooth at 4 and 24 h. However, late apoptosis occurred in treatment with 2',3'-epoxyisocapnolactone at IC₅₀ after 24 h with apoptotic bodies foaming around the surface of cell (Figure 9). On the other hand, when the cell was treated with high dose in 2',3'-epoxyisocapnolactone at 24 h, formation of holes on the membrane

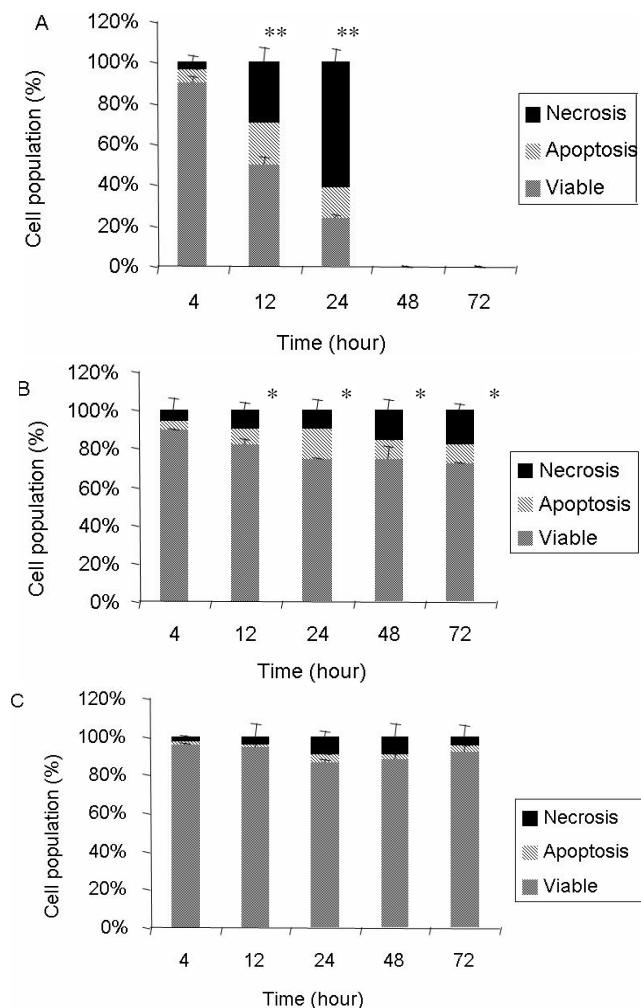


Figure 5. The % of viable, apoptotic and necrotic CEM-SS cell in the population after treated with 2',3'-epoxyisocaprolactone at various time courses. Data are mean of triplicate determinations \pm SD. * $p \leq 0.05$, ** $p < 0.001$ compared with untreated cells. A: Treated with high dose (10 $\mu\text{g/ml}$); B: Treated with IC_{50} (5 $\mu\text{g/ml}$); and C: Treated with half concentration of IC_{50} (2.5 $\mu\text{g/ml}$).

occurred and led the cell into necrosis pathway. For the treatment with 8-hydroxyisocaprolactone-2',3'-diol, apoptosis feature also occurred in treatment at 24 h with IC_{50} (Figure 10). In this treatment, the formation of membrane holes on cell surface appeared earlier at 4 h. (Figure 8A, B; Figure 9A, B; Figure 10A, B)

DNA fragmentation assay

Using a qualitative analysis of DNA fragmentation, CEM-SS were subjected to treatment with 2',3'-epoxyisocaprolactone and 8-hydroxyisocaprolactone-2',3'-diol, and the DNA of treated cells was harvested at 4, 12, 24, 48 and 72 h. Fragmentation analysis is shown in Figure 11 and the characteristic of DNA ladder pattern was observed. In

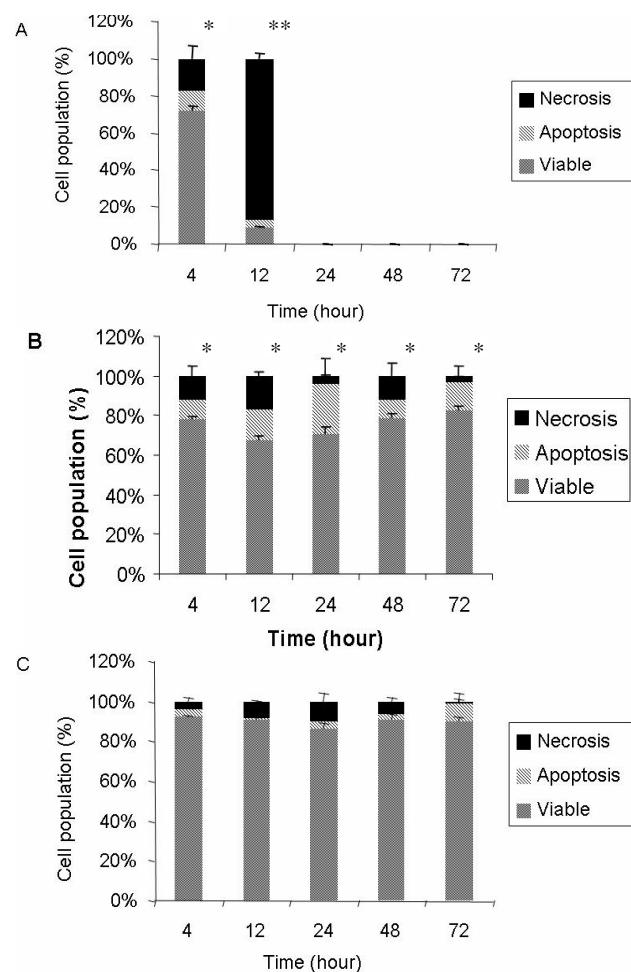


Figure 6. The % of viable, apoptotic and necrotic CEM-SS cell in the population after treated with 8-hydroxyisocaprolactone-2',3'-diol at various time courses. Data are mean of triplicate determinations \pm SD. * $p \leq 0.05$, ** $p < 0.001$ compared with untreated cells. A: Treated with high dose (10 $\mu\text{g/ml}$); B: Treated with IC_{50} (3 $\mu\text{g/ml}$); and C: Treated with half concentration of IC_{50} (1.5 $\mu\text{g/ml}$).

the treatment with 2',3'-epoxyisocaprolactone, the amount of DNA ladder occurs only after 24 h of incubation. While for the treatment with 8-hydroxyisocaprolactone-2',3'-diol, the activation of endonuclease enzyme seemed more early and rapid compared with 2',3'-epoxyisocaprolactone. The occurring of DNA ladder in treatment with 8-hydroxyisocaprolactone-2',3'-diol started at 4 h after incubation. However, the ladder pattern in both cases is not clear. It could be due to lack of internucleosomal DNA degradation in the present of necrosis. Double staining with acridine orange (AO) and propidium iodide (PI) showed a higher % of necrotic cells in comparison with apoptotic cells. This means that the lower % of apoptotic cells could remain undetectable as DNA fragment in the gel electrophoresis analysis (Figure 11).

CEM-SS cells were treated with 2',3'-epoxyisocaprolactone and 8-hydroxyisocaprolactone-2',3'-diol at IC_{50} at

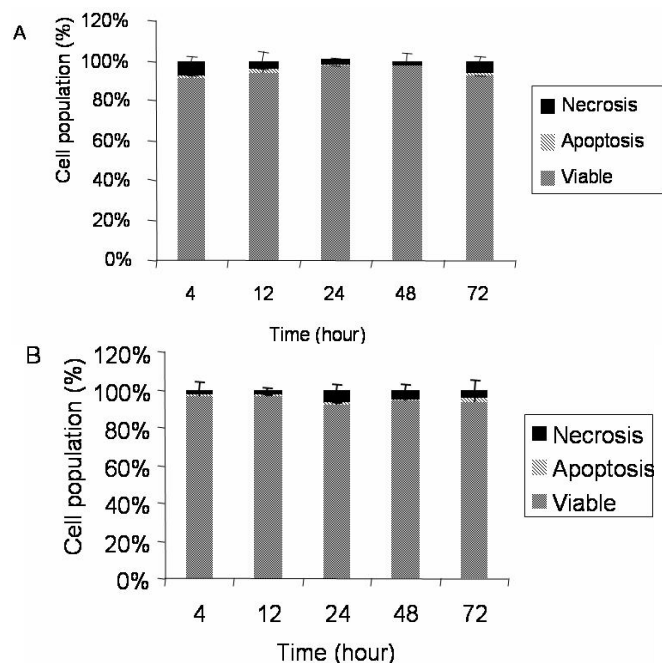


Figure 7. The % of viable, apoptotic and necrotic CEM-SS cell in the untreated population and population after treated with 0.1% DMSO at various time courses. Data are mean of triplicate determinations \pm SD. * $p \leq 0.05$, ** $p < 0.001$ compared with untreated cells. A: Treated with 0.1% DMSO; and B: Negative control (untreated cells).

different period. Lanes A, B, C, D and E were treated with 8-hydroxyisocapnolactone-2',3'-diol at 4, 12, 24, 48 and 72 h, respectively. Lane F, G, H, I and J were treated with 2',3'-epoxyisocapnolactone at 4, 12, 24, 48 and 72 h, respectively. Lane N, negative control (untreated CEM-SS cells). Lane M, Ladder mix marker

DISCUSSION

In the present work, we showed that 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol strongly cytotoxic on various cancerous cell line especially CEM-SS. Purified materials which require concentration lower than 10 $\mu\text{g/ml}$ to exhibit cytotoxicity were considered as promising cancer chemotherapy agents (Shier, 1991). As the IC_{50} against CEM-SS cell lines were less than 10 $\mu\text{g/ml}$, 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol considered as potential cancer chemotherapy agent. In this study, cytotoxic effects induced in cells may trigger by the coumarin structure (lactones of cis-o-hydroxycinnamic acid derivatives, belong to the phenolics with the basic skeleton of C6 + C3) of the compounds. Several authors had reported on the used of coumarin for treatment of some human carcinomas, many coumarin derivatives were also known as free radical scavengers and had been found to exhibit cytotoxicity

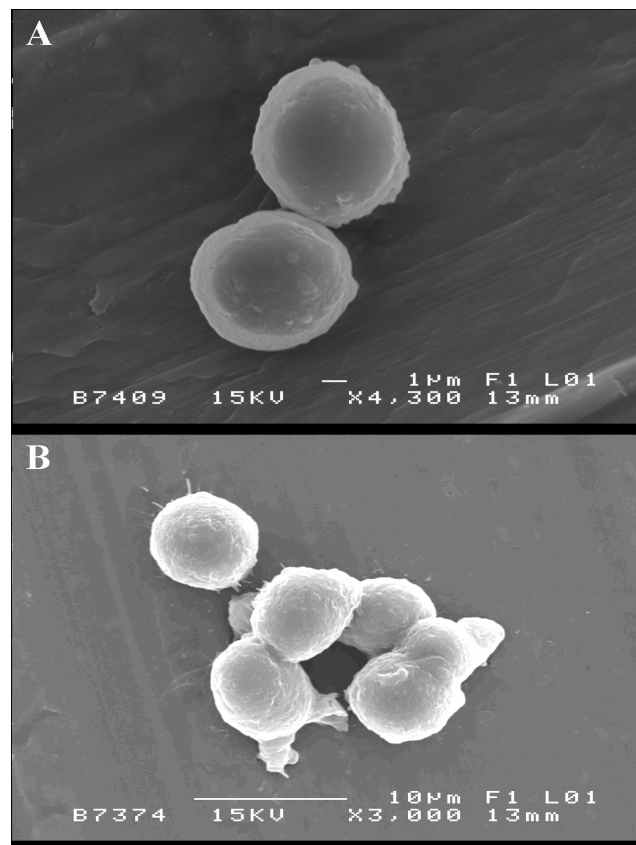


Figure 8. Scanning electron microscopy examination of untreated CEM-SS cells. A: Normal CEM-SS cells at 4 h; B: Normal CEM-SS cells at 24 h. Magnification: A: 4300X; B: 3000X.

against a panel of mammalian cancer cell lines (Peng and Cai, 2008). In this study, the effect of coumarin was proven to induce cell death in CEM-SS cells.

Our studied also indicated that effects of both compounds were stronger in suspension culture compared to anchorage dependent culture. This can be due to the exposure of larger cell surface in suspension culture by 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol during treatment. Ali et al. (2000) reported that cytotoxic compounds commonly exhibited to be more sensitive towards the suspension cell lines. This is due to the anchorage-dependent cell lines require attachment to a solid matrix in order to proliferate thus make them interact with each other through cell-cell contact and generates transmembrane signal that not only affected the cell proliferation, migration and differentiation but also survival of the cell that prevent it from undergoing apoptosis (Ali et al., 2000). Previous study showed that primary culture that require anchorage will undergo apoptosis in suspension culture, thus proving that anchorage-independent cell lines have undergone genetic changes through transformation that prevent expression of the apoptotic pathway in response to the lost of contact which

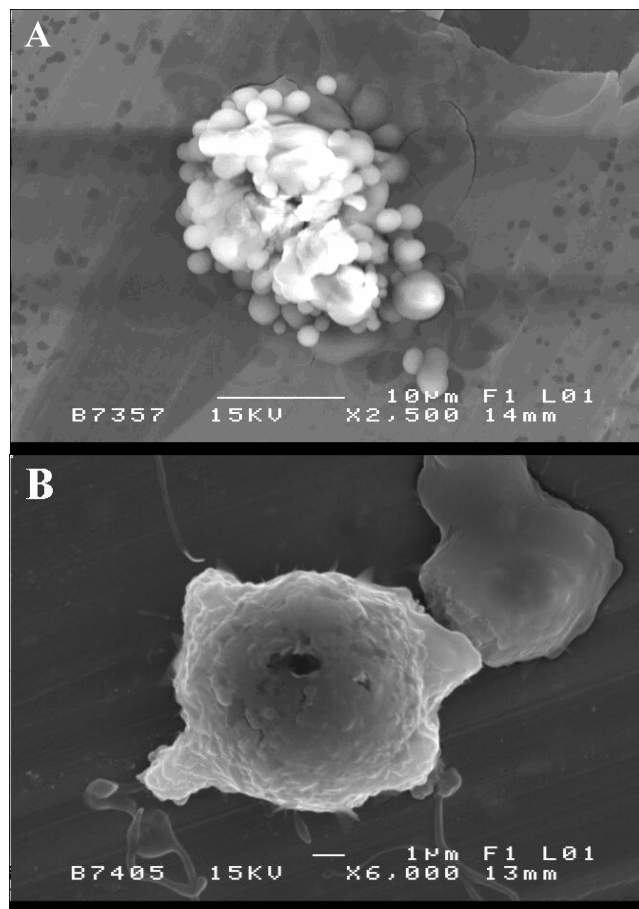


Figure 9. Scanning electron microscopy examination of CEM-SS cells treated with 2',3'-epoxyisocapnolactone. A: CEM-SS cells treated with IC_{50} (5 μ g/ml) at 24 h; and B: CEM-SS cells treated with high dose (10 μ g/ml) at 24 h. Magnification: A: 2500X; B: 6000X.

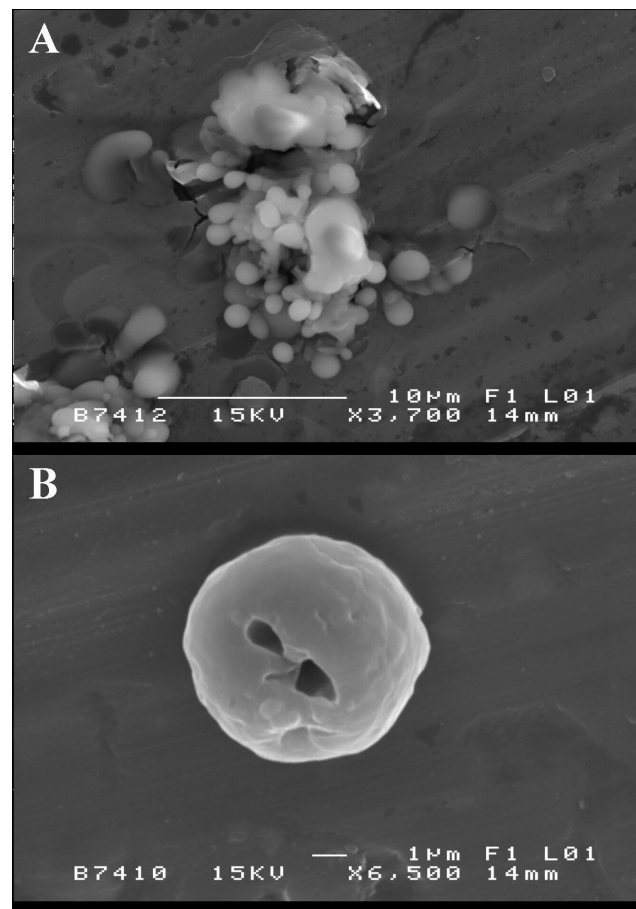


Figure 10. Scanning electron microscopy examination of CEM-SS cells treated with 8-hydroxyisocapnolactone-2',3'-diol. A: CEM-SS cells treated with IC_{50} (3 μ g/ml) at 24 h; and B: CEM-SS cells treated with high dose (10 μ g/ml) at 4 h. Magnification: A: 3700X; B: 6500X.

caused them to be more sensitive towards cytotoxic drug and chemical (Singh et al., 1996).

The induction of apoptosis by 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol were evident from the morphological examination of the treated cells stained with nucleoprotein/DNA-intercalating dye acridine orange and propidium iodide (Figures 2, 3 and 4). The cells that had nucleus stained with orange-green fluorescence and diffused chromatin were counted as viable cells and those with fragmented chromatin condensations were scored as apoptotic cells (Singh et al., 1996). This was observable because the membrane of the cells was able to exclude propidium iodide but allowed the acridine orange to enter the cell and intercalate in the DNA molecules which gave the orange-green fluorescence to the nucleus. On the other hand, the cells with nucleus stained orange-red, with no chromatin condensation, were considered as necrotic cells and those with red fluorescence were counted as secondary necrotic. For quantification of the mode of cell death, AO/PI is not a very good process because it only represents point

quantification and not the whole population of cells. Our S.E.M. results show another hallmark pattern of CEM-SS apoptosis which was surface blebbing due to a deep cytoskeleton rearrangement and formation of apoptotic bodies after treatment. Besides, our DNA fragmentation assay showed the presence of "DNA ladder". DNA ladder is the pattern in which degraded DNA occurs and it is considered as one of the hallmarks of induction of apoptosis. Chromatin condensation paralleled by DNA fragmentation is one of the most important criteria which are used to identify apoptotic cells. Activation of endonuclease was proposed to be the initiating event in the characteristic condensation of chromatin and preferentially cleave DNA at the internucleosomal sections (Cohen and Duke, 1984). The condensation of chromatin and cleavage of internucleosomal DNA at the linker regions between nucleosomes can be both examined in agarose gel electrophoresis. All of these results proposed that majority of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol treated CEM-SS were undergoing apoptosis rather than necrosis.

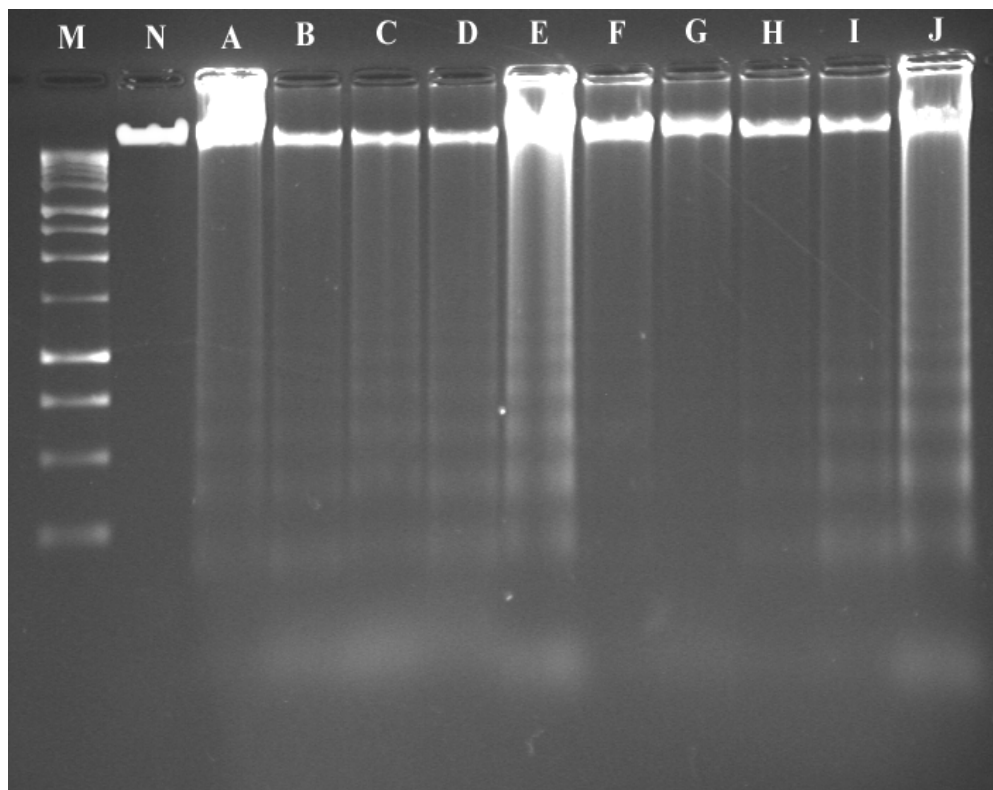


Figure 11. Effect of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol on DNA fragmentation.

However, the toxicity of most cytotoxic compounds was very unspecific, damaged neoplastic tissue as well as healthy tissue (Thurow et al., 2004). In light of the genetic complexity and heterogeneity of most common human cancers, the future use of a target selective agent is elusive. Agents must underscore the need to prove the dependence of tumour growth on the selected target and the difficulty of achieving absolute tumour selectivity (Broxterman and Georgopapadaku, 2004). So, the future use of targeted compounds might be in combination or in sequence with other novel agents and/or cytotoxins directed at signalling pathways critical for survival of tumour cells.

This current study has effectively demonstrated that 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol have a good potential anti cancer drug. Further studies on the molecular mechanism involved in the apoptosis induction by 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol ought be carried out and more work needed to be done to elucidate the factor that involved in cell death and develop the potential active compounds into clinically useful drugs.

REFERENCES

- Ali AM, Ismail N, Mackeen M, Yazan L, Mohamed S, Ho A, Lajis NH (2000). Antiviral, cytotoxic and antimicrobial activities of anthraquinones isolated from the roots of *Morinda elliptica*. *Pharm. Biol.* 38: 3791-3801.
- Broxterman HJ, Georgopapadaku NH (2004). New cancer therapeutics: target-specific in, cytotoxics out? *Drug Resist Updates*, 7: 79-87.
- Bulkil IH (1966). A dictionary of economic products of the Malay Peninsular. Ministry of Agriculture and cooperatives, Kulau Lumpur, Malaysia.
- Cherng JM, Chiang W, Chiang LC (2008). Immunomodulatory activities of common vegetables and spices of Umbelliferae and its related coumarins and flavonoids. *Food Chem.* 106: 944-950.
- Cohen JJ, Duke RC (1984). Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* 132: 38-42.
- Itharat A, Houghton PJ, Eno-Amoquaye E, Burke PJ, Sampson JH, Raman A (2004). In vitro cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. *J. Ethnopharmacol.* 90: 33-38.
- Mosmann T (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxic Assay. *J. Immunol. Meth.* 65: 55-63.
- Newman DJ, Cragg GM, Snader KM (2003). Natural products as sources of new drugs over the period 1981-2002. *J. Nat. Prod.* 66: 1022-1037.
- Peng MS, Cai J (2008). Synthesis and fluorescence of crown ethers containing coumarin. *Dyes Pigments*, 79: 270-272.
- Rahmani M, Susidarti RA, Ismail HBM, Sukari MA, Taufiq-Yap YH, Gwendoline ECL, Ali AM, Kulip J, Waterman PG (2003). Coumarins from Malaysian *Micromelum minutum*. *Phytochem.* 64: 873-877.
- Roy MK, Thalang VN, Trakoontivakorn G, Nakahara K (2005). Mahanine, a carbazole alkaloid from *Micromelum minutum*, inhibits cell growth and induces apoptosis in U937 cells through a mitochondrial dependent pathway. *B.J.P.* 145: 145-155.
- Shier WT (1991). *Mammalian Cell Culture on \$5 a Day: A Laboratory Manual of Low Cost Methods*. National Institute of Biotechnology and

- Applied Microbiology (BIOTECH). University of the Phillipines at LosBarios, College, Laguna.
- Singh RP, Al-Rubeai M, Emery AN (1996). Apoptosis: Exploiting novel pathways to the improvement of cell culture processes. *Gen. Eng. Biotech.* 16: 227-251.
- Susidarti RA, Rahmani M, Ali AM, Sukari MA, Ismail HBM, Kulip J, Waterman PG (2007). 8-Methoxycapnolactone and stigmasterol from *Micromelum minutum*. *Majalah Farmasi Indonesia*, 18: 105-109.
- Susidarti RA, Rahmani M, Ismail HBM, Sukari MA, Taufiq-Yap YH, Gwendoline ECL, Ali AM, Kulip J, Waterman PG (2006). A new coumarin and triterpenes from Malaysian *Micromelum minutum*. *Nat. Prod. Res.* 20: 145-151.
- Thurrow K, Entzian K, Eberlein G (2004). Toxicological and Pharmacological Evaluation of New Drug Candidates by In Vitro Robotic High-Throughput Cell Assays. *JALA.* 9: 159-62.