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Cytotoxicity of Aqueous and Ethanolic Extracts of *Ficus deltoidea* on Human Ovarian Carcinoma Cell Line

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Research Article

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ABSTRACT

Aims: This study was to investigate the cytotoxicity of both plant extracts from *Ficus deltoidea* (locally known as Mas Cotek), aqueous and ethanolic extracts on human ovarian carcinoma cells using standard colometric MTT assay.

Study design: Cell based assay

Place and Duration of Study: Institute of Bioproduct Development and Department of Bioprocess Engineering, Universiti Teknologi Malaysia, Johor Bahru, Malaysia between January 2007 and December 2009.

Methodology: The biochemical responses of cells after plant sample treatment were observed and have been reported through several assays such as trypan blue exclusion assay for cell viability, analysis of glucose uptake and lactate release, cell survival evaluation and genomic assay through DNA fragmentation.

Results: Both aqueous and ethanolic extracts of the plant sample gave IC₅₀ value of 224.39 ± 6.24 µg/ml and 143.03 ± 20.21 µg/ml, respectively. The detachment capability of the plant aqueous extract was observed in the cell viability assays. DNA fragmentation was not observed in the aqueous extract, but in ethanolic extract (1000 µg/ml). The DNA was fragmented around 200 Kbp. Morphological observation was carried out and apoptosis body was observed at 1000 µg/ml of both extract.

Conclusion: A2780 cancer cells behaved differently on cell growth profile upon

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treating with different concentrations of the aqueous and ethanolic extracts of *F. deltoidea*. Even though both extracts could cause apoptosis at 1000 µg/ml, the aqueous extract prompted to promote cell detachment, and the ethanolic tried to inhibit cell proliferation through DNA fragmentation.

Keywords: *Ficus deltoidea*; human ovarian carcinoma cells; cytotoxicity; DNA fragmentation;

1. INTRODUCTION

Nowadays, herbal plants have been widely used for diseases treatment and immunological enhancement. The increasing trend of herbal application in traditional herbal industry is mainly due to numerous beneficial effects of natural sources compared to single synthetic drug. Natural herbal medicines usually offer less undesirable side effect, more efficiency and less toxic to consumers.

However, a very limited scientific data can be accessed regarding the beneficial effect of herbal medicine, especially herbal plants from South East Asian countries. Therefore, the effect of *F. deltoidea* extract on human ovarian carcinoma cells was studied as a preliminary exploration. *F. deltoidea* or Mas Cotek as local name from the family of Moraceae was chosen because this fig tree is widely used in cancer therapy traditionally in the Malay women community.

Cancer is the major health problem worldwide. It claims more than six million people lives a year. Ovarian cancer is the first leading cause of death from gynaecologic cancer besides breast cancer. Usually, ovarian cancer patients have high response rate to initial chemotherapy after cytoreductive surgery. Most of them will then develop resistant to anticancer drug at the latter stage of treatment (Mi and Hong, 2003). The survival rate of ovarian cancer patients is reported to be 30% only. Therefore, this study is crucial as a stepping stone to better understanding to the behaviour of *F. deltoidea* extract in the inhibition of human ovarian carcinoma cells before proceed to animal toxicology study.

In the present study, cell based assay is used to determine cell growth by measuring cell viability and cell cytotoxicity after treated with plant extract. The glucose uptake and lactate release were also monitored to measure the glycolysis rate and by-product formation from cell growth. The result of the assay was then confirmed with the survival observation through microscope. The cytotoxicity effect of plant extract was evaluated at gene level based on genomic assay such as DNA fragmentation from gel electrophoresis.

2. MATERIALS AND METHODS

2.1 Plant Material and Chemicals

The plant material, *F. deltoidea* was bought from Malaysian Agriculture and Research Development Institute (MARDI), Pahang. The specimen of the plant, MFD4 has been deposited in MARDI (Musa and Lip, 2007).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, USA. Phosphate buffer saline (PBS) was prepared from the analytical grade of sodium chloride (Sigma, USA). The other chemicals include potassium chloride (Sigma, USA), disodium hydrogen phosphate (Fluka, Switzerland) and potassium dihydrogen phosphate (Sigma, USA), DNA purification kit (Promega), Ethidium Bromide (Sigma, USA) and agarose powder (Promega).

2.2 Sample Preparation

The leaves of *F. deltoidea* were cut and dried in oven at 60°C before ground into powder. The sample powder (100 g) was double-boiled in distilled water (1 L) at 60°C for 3 days. This aqueous extract was then filtered and freeze-dried. The yield of the aqueous extract was 4.75 g.

Another 100 g of powdered sample was macerated in 95% denatured ethanol (1 L) for 24 hours at ambient temperature. The solution was filtered and the sample was macerated again 95% denatured ethanol (1 L). The procedures were repeated for 3 times. A total volume of 2.85 L solution was collected and dried using rotary evaporator (Buchi Rotavapor R114, Switzerland) under reduced pressure. The yield of the ethanolic extract was 1.98 g.

2.3 Cell Line Culture

Human ovarian carcinoma cell line, A2780 was obtained from the European Collection of Cell Culture (ECACC). Cells were cultured in RPMI 1640 media supplemented with 10% foetal bovine serum, glutamine (2mM) and 1 % penicillin-streptomycin in static 75 cm² T-Flask (GIBCO, USA). The cells were incubated in a humidified atmosphere with 5 % CO₂ at 37°C.

2.4 Cell Cytotoxicity Assay

Cells were plated in a 96-well-plate with 1 X 10⁵ cells/well of concentration. The cells were left to adhere for 48 hours before exposed to the plant extracts (0-1000 µg/ml) administered in media containing 1% of FBS and returned to the incubator for 48 hrs. Subsequently, MTT reagent (0.5 mg/mL in sterile PBS) was added directly to the wells. Cells were returned to the incubator for 4 hrs. The formation of insoluble purple formazan from yellowish MTT by enzymatic reduction was dissolved in DMSO after removal of supernatant. The optical density of solution was measured at 590 nm using a microplate reader (ELx808, BioTek, USA).

2.5 Cell Viability Assay

After treatment with the plant extracts, the cells were pooled together and the remaining attached cells were detached from the culture plates by exposure to trypsin-EDTA. The resultant cells were then stained with trypan blue at the concentration of 0.2%. Then, the trypan blue-excluded viable cells were counted using a hemacytometer (FORTUNA® GERMANY) under microscope.

2.6 Apoptosis Observation

The morphology of cells was monitored during cell growth after treatment with the plant extract under an inverted microscope (Axiovert100, Zeitz, Germany). The cell morphology was also evaluated by adding a mixture of acridine orange and ethidium bromide (2 μ l) before checking under the fluorescence microscope (BX51, Olympus, USA). Pictures were taken at 400x magnification with excitation filter 480/30 nm, dichromatic mirror cut-on 505 nm LP and barrier filter 535/40 nm.

2.7 Analysis of Glucose Uptake

Glucose uptake analysis was carried out on supernatant collected after treatment, based on enzymatic reaction of hexokinase to produce NADPH, which was then detected photometrically in C111 Cobas analyzer (Roche, Switzerland).

2.8 Analysis of Lactate Release

The concentration of lactate in supernatant was analysed by Biochemistry Analyzer (YSI 27000, SELECT, USA). This analyser uses immobilised oxidase coated on the probe to catalyse substrate and produce hydrogen peroxide, which was the electrochemically detected as signal.

2.9 DNA Electrophoresis

The post-treatment cells were pooled together. The cells were palleted and washed twice with cold PBS. Cell pallets were incubated in lysis buffer (1 ml) for 30 minutes at 60°C. The clear lysates were separated by centrifugation and re-incubated with RNase (3 μ l) for 30 min at 37°C. A mixture of solvents consisted of phenol, chloroform and isoamyl alcohol was added and vigorously vortex for a few seconds before centrifugation. This procedure was repeated twice. The layer of clear lysates was transferred into 100% ethanol (1 ml) and kept at 4°C. The mixture was re-centrifuged to discard the supernatant. The remaining pallet was washed with 70% ethanol and dried before dissolved in Tris-EDTA (TE) for DNA electrophoresis.

2.10 Statistical Analysis

Statistical software, Design Expert 6.0.8 has been used to analyze the difference between the control and the plant extracts with different concentrations to the cell line.

3. RESULTS AND DISCUSSION

3.1 Cell Growth Profile in MTT Assay

MTT assay is a rapid and high accuracy colorimetric approach that widely used to determine cell growth and cell cytotoxicity, particularly in the development of new drug. It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction on the reduction of MTT to formazan.

The profile of cell growth after treated with the plant extracts is presented in Fig. 1(a). From this figure, it was found that both extracts only showed a significant reduction in the number of viable cells at the concentration higher than 250 $\mu\text{g/ml}$. The reduction because of the treatment with the ethanolic extract was more than the aqueous extract. Therefore, the IC_{50} value was 224.39 and 143.03 $\mu\text{g/ml}$ for the aqueous and ethanolic extract, respectively (Fig.1 (b)).

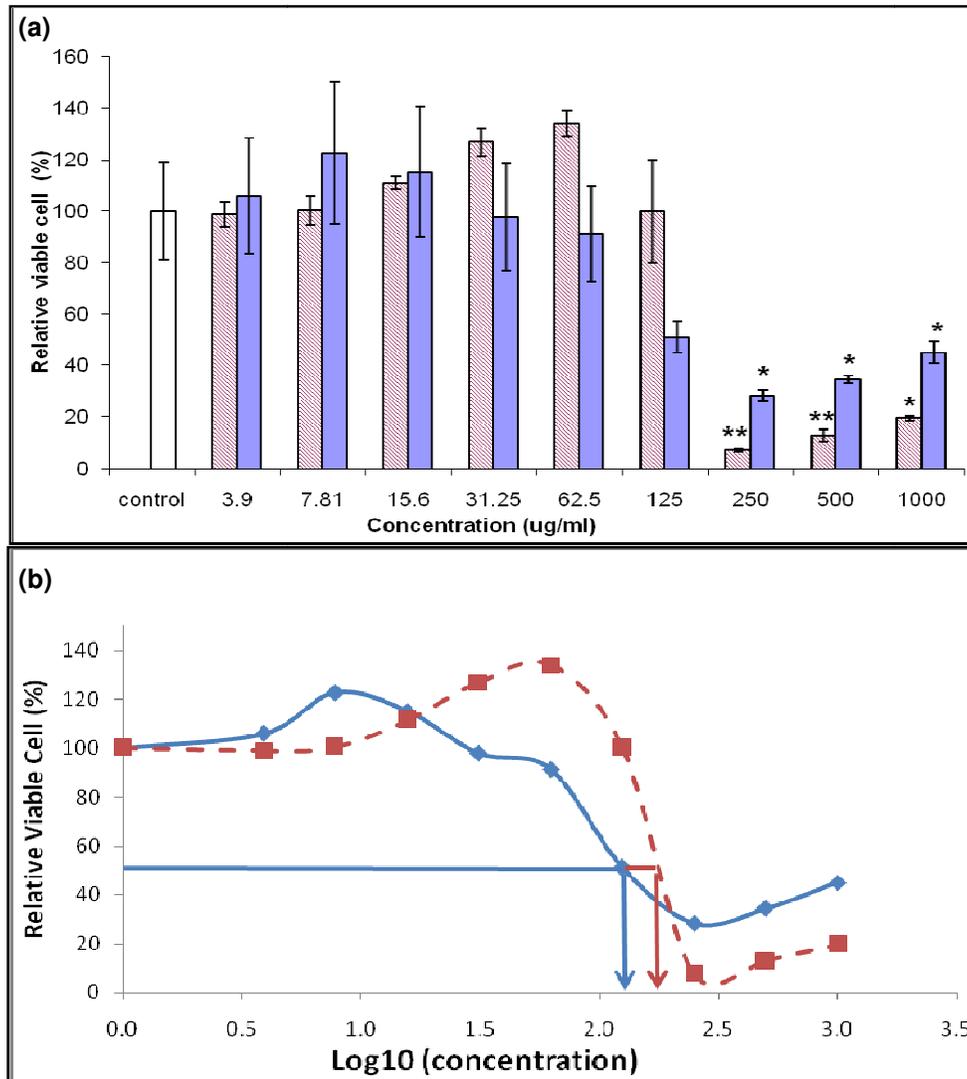


Fig. 1. (a) Effect of various concentration of aqueous (solid bar) and ethanolic (line bar) extracts of *F. deltoidea* on the cell viability in MTT assay.

All values are recorded based on the six replications of tests and analysed statistically, where * and ** represent the confident level at 95% ($P < 0.05$) and 99% ($P < 0.01$), respectively.

(b) Cell cytotoxicity of aqueous (dash line) and ethanolic (solid line) extracts of *F. deltoidea*.

The IC_{50} values are determined from the cytotoxicity curve at 50% of viable cells after 48 hours of plant extract treatment.

3.2 Cell Growth Determination Using Trypan Blue Exclusion Assay

Trypan blue exclusion assay was then carried out to further confirm the viable cell count at the concentrations of extract ranging from 125 to 1000 µg/ml based on the previous result from MTT assay. However, the results of trypan blue assay were contradictory to the results of MTT assay as presented in Fig. 2.

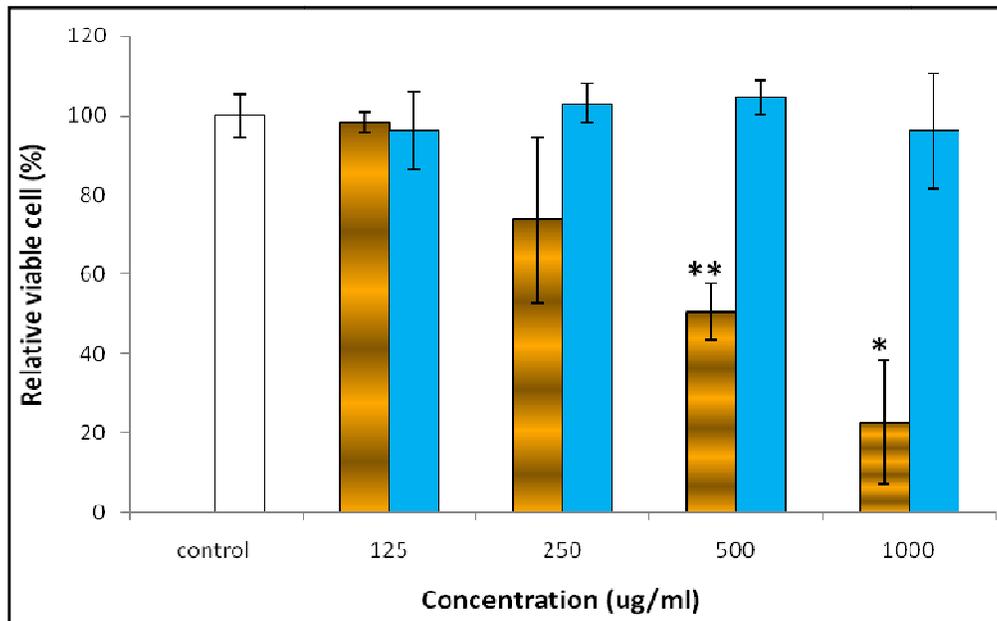


Fig. 2. Effect of aqueous and ethanolic extracts of *F. deltoidea* at the concentration from 125 to 1000 µg/ml on the cell viability in trypan blue exclusion assay.

All values are recorded based on the six replications of tests and analysed statistically, where * and ** represent the confident level at 95% ($P < 0.05$) and 99% ($P < 0.01$), respectively.

The contradiction could be explained by the detachment capability of aqueous extract of *F. deltoidea* in cell culture media. The aqueous extracts might promote cell detachment by interacting with intercellular junctions or extracellular matrix. The modification of cell surface might be due to the neoplastic transformation (Hynes, 1978), binding of plant lectins (Laferte and Loh, 1992), change of glycoprotein in cell surface (Bruyneel, 1990) and cell adhesion molecules (Yang, 2004) which might be correlated to the invasion of metastasis in vivo. Hynes (Hynes, 1978) reported that fibronectin or large extracellular transformation-sensitive (LETS) protein was lost from the surface of transformed fibroblast due to the alterations in integrins. This loss might contribute to a decrease in cell-cell and cell-substrate adhesion (Yamada, 1991) and lead to the reduction in cell attachment as well as cell spreading for proliferation. Therefore, the aqueous extract of *F. deltoidea* might be an anchorage-independent-cell inducer.

The detachment caused the number of viable cell counted in MTT assay less than the actual value. Only the attached cells were considered as live cells in MTT assay. In fact, the detached cells were still alive because they could proliferate into normal cancerous cell, if

they were re-supplied with fresh medium. However, the viable cells in trypan blue exclusion assay were counted based on the number of stained cells in the medium. Hence, the number of viable cells either from the attached or the detached cells was taken into consideration in cell counting.

The ethanolic extract treatment on the cells in trypan blue exclusion assay produced almost similar results as in the MTT assay. The significant reduction in the number of viable cells was increased from 250 µg/ml in the MTT assay (Fig. 1(a)) to 500 µg/ml in the trypan blue exclusion assay (Fig. 2). The increase could be explained by the staining technique used in cell counting for trypan blue exclusion assay. Anyhow, the ethanolic extract of *F. deltoidea* has significant effect on cell growth inhibition compared to the aqueous extract.

3.3 Determination of Glucose Uptake and Lactate Release

The glucose consumption of the cells was monitored after plant extract treatment. The ethanolic extract at 1000 µg/ml caused a significant reduction in glucose uptake as presented in Fig. 3(a). This observation was in line with the cell viability assay, where the number of viable cells was higher for the cells treated with aqueous extract compared to the ethanolic extract. The uptake of glucose was determined because glucose consumption plays a key role in cancer cell proliferation. According to Ortega (2009), glycolysis is the 'selfish' pathway used for cellular proliferation, providing both the metabolic precursors and the energy required for biosynthesis, in the context of a plethora of substrates. The glucose avidity of carcinomas is thus presented as the result of both the instalment of glycolysis for cellular proliferation and of the impairment of mitochondrial activity in the cancer cell. At the end, the repression of mitochondrial activity affords the cancer cell with a cell-death resistant phenotype making them prone to malignant growth. The rate of glucose consumption by the cells is dependent on the demand of carbon skeletons that used for the accretion of new biological matter and/or on the energy provided in the form of ATP by mitochondrial oxidative phosphorylation (Ortega, 2009). Somehow, the excessive consumption of glucose was neither used for synthesis nor oxidation, but rather secreted as lactate (Elstrom, 2004).

Besides glucose uptake, the release of lactate was also monitored after 48 hours of treatment in this study. This is because the content of lactate in the medium will affect the cell growth profile. Schneider (1996) reported that the toxic action of lactate was probably due to the acidic pH and osmolarity activity on the cells, particularly at high concentration (>20 mM). In the presence of c-myc, the genes of glycolytic enzymes, namely lactate dehydrogenase-A (LDHA) and GLUT1 would be transactivated to enhance both glucose uptake and lactate production (Shim, 1997). LDHA was also reported could be upregulated in several tumors and it is essential for c-myc-mediated transformation (Shim, 1997). Actually, lactate was released as the by-product during cell growth. However, if the concentration of lactate in the medium was too high, it would affect the cell growth. Therefore, the content of lactate is crucial to monitor in order to avoid the side effect of lactate to the growth process of cells. The lactate release profile showed that in fact, the amount of lactate present in the medium did not significantly affect the cell growth (Fig. 3(b)). Hence, the growth profile of the cells was mainly influenced by the plant extracts, but not because of the content of lactate released into the medium.

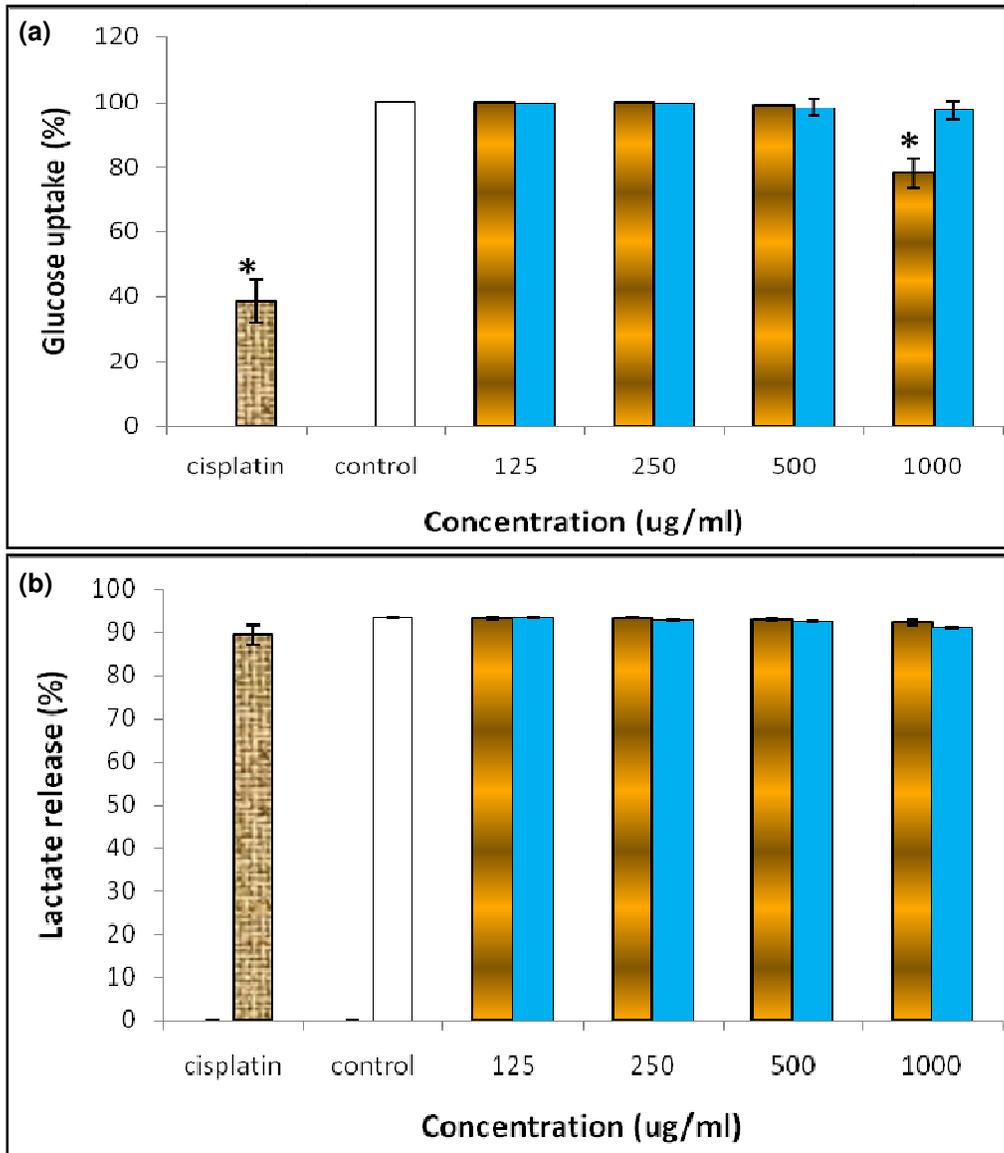


Fig. 3. (a) Glucose uptakes of cells after treated with the aqueous (solid bar) and ethanolic (line bar) extracts of *F. deltoidea* compared to the positive control result from cisplatin (texture bar) and control cells (unfilled bar),
Where * represent the confident level at 95% ($P < 0.05$).

(b) Lactate content of the cells after treated with the aqueous (solid bar) and ethanolic (line bar) extracts of *F. deltoidea* compared to the positive control result from cisplatin (texture bar) and control cells (unfilled bar).

3.4 DNA Fragmentation on the Treated Cells

The conventional agarose gel electrophoresis was performed on the cells treated with 1000 $\mu\text{g/ml}$ of plant extract for 48 hours. The result showed that internucleosomal DNA cleavage

produced no ladder pattern for the aqueous extract treated cells (Fig. 4). The DNA might be intact and no DNA fragmentation was detected.

According to Walker (1998), cells and untreated cells could produce a discrete band from 700 to 1000 kbp, which was unrelated to apoptotic DNA cleavage, but attributed to the migration of any DNA fragment larger than 700 kbp (Walker, 1998). This indicates that the DNA might be cleaved after treatment but in a large number of base pair. The explanation also describes the presence of apoptotic bodies in the cell morphological study. This observation was also happened to the positive control cells treated with cisplatin. An extensive DNA fragmentation might be occurred which could not be detected in this study.

It was found that the ethanolic extract could cause DNA degradation at 1000 µg/ml. The fragmented DNA was observed around 5 to 8 kbp, which was smaller than the typical fragmentation of DNA at 20 to 300 kbp when entering early stage of apoptosis (Cohen, 1992). However, there was no fragmented DNA observed at the concentration less than 1000 µg/ml.

According to Wyllie (1980), the biochemical hallmark of apoptosis is cleavage of the nuclear DNA into ~200 base pair multiples. This specific DNA cleavage is due to the activation of endogenous endonuclease that cleaves at the exposed linker regions between nucleosomes. It is worthy to highlight that necrosis was not happened in this study because it associates with the random form of DNA cleavage (Darling, 2000).

3.5 Cell Morphology Observation

The morphology of the treated cells has been observed under an inverted microscope as presented in Fig. 5(a). Cell detachment was observed for the aqueous extract treated cells. They were clumped together on the surface of the medium. This phenomenon was also observed for the ethanolic extract treated cells, but not significant as the aqueous extract treated cells. There was a lot of empty space among the clumped cells. However, the cell detachment was not occurred to the cells treated with cisplatin. Oppositely, the cells shrunk at the bottom of the medium.

The cell morphology was also carried out using ethidium bromide and acridine orange (EB/AO) as staining agent. The apoptotic cells are stained in orange, the live cells are stained in green and the necrotic cells are stained in red as presented in Fig. 5(b). It was found that only early stage of apoptosis was observed for the cells treated with aqueous extract. Owing to that, there was no 180 bp of DNA laddering being observed in Fig. 4. Although DNA fragmentation into oligonucleosomal ladders is the characteristic of apoptosis, recent evidence indicates that not all cells undergo such extensive DNA fragmentation (Cohen, 1992). In fact, the fragmentation of DNA into kilo base-size fragments appears to be an early stage of apoptosis before preceding the complete digestion of DNA into multiples of nucleosomal size fragments (Sun and Cohen, 1994). Besides, the early stage of apoptosis on the cells treated with 1000 µg/ml of aqueous extract also did not reduce the number of viable cell significantly in the trypan blue exclusion assay. This assay was applied as it is easier than apoptosis staining method in cell counting.

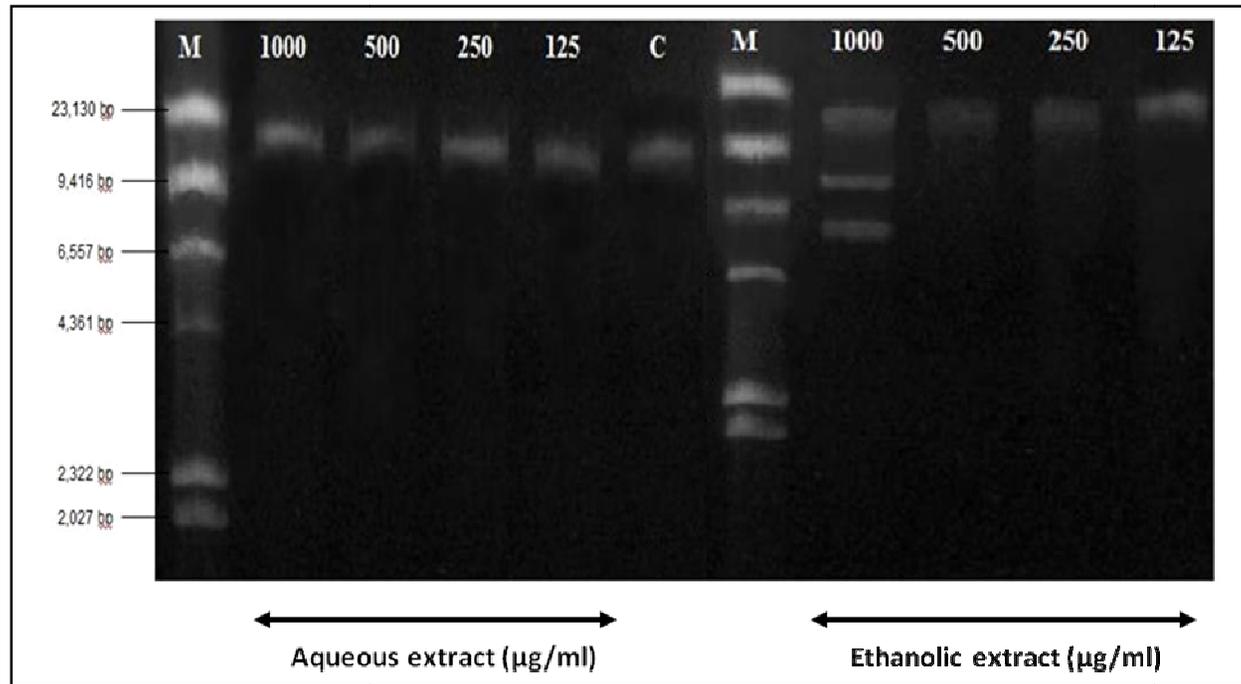


Fig. 4. DNA fragmentation after treatment with *F. deltoidea* extracts compared to the control (C) and marker (M) values

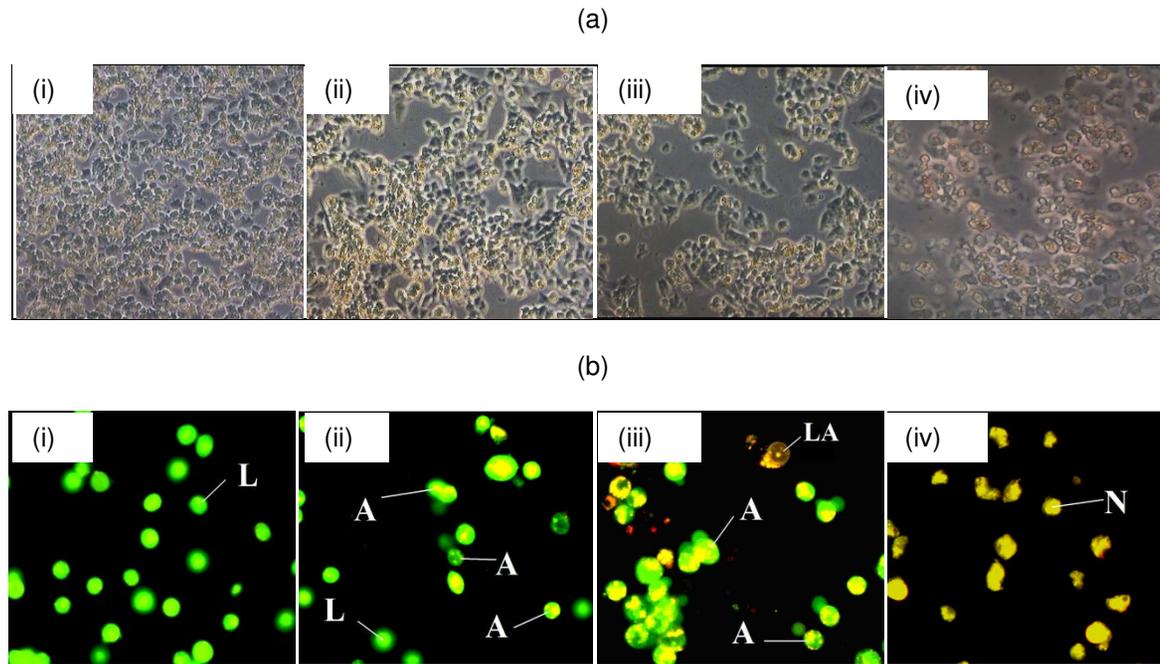


Fig. 5. (a) Comparison of cell morphology under inverted microscope for the control cells (i), cells treated with 1000 µg/ml of aqueous extract (ii), cells treated with 1000 µg/ml of ethanolic extract (iii) and cells treated with 25 µg/ml of cisplatin as positive control (iv).

(b) Morphology of live cells (L), apoptotic cells (A) and necrotic cells (N) after stained with ethidium bromide and acridine orange and observed under fluorescence microscope for the control cells (i), cells treated with 1000 µg/ml of aqueous extract (ii), cells treated with 1000 µg/ml of ethanolic extract (iii) and cells treated with 25 µg/ml of cisplatin as positive control (iv).

The cells; the floating and adhered cells which was treated with the ethanolic extract were blabbing. The cell membrane became out of the shape and the condensation of chromatin was observed. In addition to the chromatin aggregation, the cells treated with ethanolic extracts were also formed kidney shaped nuclei. It was also observed that the control cells did not have apoptotic and necrotic cells. However, the positive control experiment showed necrotic condition to the cells. They were shrinking and visible orange-stained cells with kidney shaped nuclei were observed under fluorescent microscope. However, the morphology of the cells did not resemble to the cells treated with cisplatin. They were derived from empty cell membrane and then being phagocyte by other viable cells.

4. CONCLUSION

F. deltoidea is well known for its medicinal therapeutic value, especially in cancer treatment among Malay practitioners. Therefore, the effect of *F. deltoidea* extracts on human ovarian carcinoma cell line was studied by using cell based assay and supported by morphological data. The findings of this study could be concluded as below.

- Both aqueous and ethanolic extracts of the plant have different effects on cell growth, DNA fragmentation and cell morphology due to the difference in their phytochemical profiles.
- The aqueous extract of the plant prompted to promote cell detachment, whereas the ethanolic extract tried to stop cells from proliferation.
- Both extracts could cause apoptosis at the concentration of 1000 µg/ml, but in aqueous extract, the apoptosis effect was slower than the cells treated with ethanolic extract.
- DNA fragmentation was found in the cells treated with the ethanolic extract at around 200 Kbp.

The crude extract of the plant should be further fractionized into at least semi-purified sample in order to determine the type of phytochemicals inhibiting the growth of cancerous cells. The cell detachment property of the aqueous extract should also be studied as this phenomenon might be the cause of metastasis clinically.

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