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Full Length Research Paper

Methanol extract of *Oroxylum indicum* leaves induces G_1/S cell cycle arrest in HeLa cells via p53-mediated pathway

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Oroxylum indicum **is a Malaysian edible plant with traditional claims for some medicinal properties. This research was done to investigate the methanol extract of *O. indicum* leaves (MEOIL) for antiproliferative activity against human cervical cancer cell line, HeLa and its mode of cell death. Results from methylene blue assay showed that MEOIL exhibited antiproliferative activity on HeLa cells with IC_{50} of 3.87 µg/ml without affecting normal cells. Phase contrast microscopy indicated the confluency reduction in HeLa cells and changes on the cell shape. Nuclear staining with Hoechst 33258 displayed the formation of apoptotic bodies and fragmented nuclei. MEOIL was shown to induce early apoptosis event in HeLa cells as confirmed by FITC-Annexin V/propidium iodide staining by flow cytometry analysis. Protein study by flow cytometry indicated the increment of p53, slight increase of Bax and unchanged level of Bcl-2. Cell cycle analysis indicated the growth arrest of HeLa cells at G_1/S phase. In conclusion, MEOIL demonstrated an antiproliferative activity in HeLa cell by inducing G_1/S cell cycle arrest via p53-mediated pathway.**

Keywords: *Oroxylum indicum*, apoptosis, p53, G_1/S cell cycle, antiproliferative activity, HeLa cells.

INTRODUCTION

Cervical cancer is the third world commonest cancer and has been the leading cause of deaths among women particularly in many developing countries (Saslow et al., 2002). In Malaysia, a total of 8.4% cervical cancer cases among women have been reported in National Cancer Registry Report 2007 (Zainal Ariffin and Nor Saleha, 2011). This situation is worrying given the current lack of awareness among the people regarding the cause and endurance of the disease while the selective and safe treatment is still indeterminate to this day.

Generally, chemotherapy or neoadjuvant chemotherapy is always the first strategy for treating a patient with an advanced cervical cancer. However, the use of some chemotherapeutic drugs in the cancer treatment has been limited by the lack of selectivity and specificity as well as numerous side effects (Sultana et al., 2003). The present cancer treatment and prognoses with them have somehow improved but most of the success comes with the people refraining from the cause of the disease rather than the effectiveness of the new treatments.

Epidemiological studies suggest that a reduced risk of cancer is associated with the consumption of phytochemical-rich diet including fruits and vegetables (Riboli and Norat, 2003). Therefore, even though of late, much works were carried out to search for plant bioactive compounds with potential use in the treatment of cancer. Until today, plenty of plants and their derived agents either in Malaysia or throughout the world have been used for the search of novel anticancer agents to combat cervical cancer. For example, the eurycomanone (from *Eurycoma longifolia* roots) and the vitamin C component (from *Lycium barbarum* fruit) have been found to reduce

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the viability of cervical carcinoma cells via apoptosis pathway (Mahfudh and Lope Pihie, 2008; Zhang et al., 2011). Furthermore, plant extracts containing active compounds such as xanthorrhizol (Ismail et al., 2005) and clitocine (Ren et al., 2008) have been identified to exhibit antiproliferative activity in HeLa cells with significant activity.

The antiproliferation or the killing activity is characterized as one of the anticancer properties which is necessary for anticancer drug in cancer treatment because this could inhibit the uncontrolled proliferation of cancer cells and create the antiproliferative activity through induction of apoptosis.

Apoptosis is a form of programmed cell death controlled by the activation of caspase cascade and regulation of apoptotic proteins such as p53, Bax, Bad, Bcl-2, Bcl-xl and other molecules (Jourdain and Martinou, 2009). Early sign of apoptosis can be recognized by surface changes that mediated by the exposure of phosphatidylserine to the outer leaflet of plasma membrane. This enables the engulfment and removal of apoptotic cells by phagocyte as soon as they appear to prevent the accumulation of late apoptotic and necrotic cells (Poon et al., 2010). A series of biochemical events occurs in apoptosis including the cell shrinkage, nuclear fragmentation, chromatin condensation and formation of apoptotic bodies (Kerr et al., 1972), as well as the involvement of apoptotic proteins which have been investigated in this study.

Oroxylum indicum is a medium-sized tree and belongs to the family of Bignoniaceae. It is called Midnight Horror tree and known as 'beko' in Malaysia (Gokhale and Bansal, 2006). It grows mainly near the rivers and in swampy areas in most Asian country like India, Sri Lanka, South China, Philippines and Malaysia. In Malaysia, the young leaves and fruits of *O. indicum* is consumed raw or cooked as vegetables. *O. indicum* have been applied traditionally to treat stomach ache, rheumatism, jaundice, to name a few (Gokhale and Bansal, 2006) and possess some biological activities such as antiulcer (Hari Babu et al., 2010), anticancer (Ashok Kumar et al., 2010) and anti-inflammatory (Siriwatanametanon et al., 2010).

It has been reported that *O. indicum* contains chrysin, baicalein, oroxylin B, baicalin (Yuan et al., 2008) and dihydrooroxylin A-7-O-methyl glucuronide (Hari Babu et al., 2010).

Nonetheless, very few of those compounds were reported to pose the apoptosis inducing activity towards cervical cancer either in the *in vitro* or *in vivo* systems. Instead, those activities have been studied in other types of cancer using the crude extracts of young pod, stem bark and fruit of *O. indicum*. Thus, the present study sought to investigate the potential use of *O. indicum* leaves in the *in vitro* antiproliferative and apoptosis inducing activity on HeLa cells.

MATERIALS AND METHODS

Plant materials

The leaves of *O. indicum* were collected from Kelantan, Malaysia. The voucher specimens (PID 321212-24) were identified by Mr. Kamarudin Saleh from Forest Research Institute Malaysia (FRIM) and deposited in the herbarium.

Preparation of organic extracts

Dried plant leaves were grinded and extracted with petroleum ether and methanol using soxhlet apparatus as described by Hasmah et al. (2008). Each plant extract was concentrated through vacuum using rotary evaporator to remove the solvent residue. A series concentration of plant extracts (0.039-10 mg/ml) were prepared by dilution with DMSO and stored at 4°C until use.

Cell culture

Cervical cancer cells (HeLa), kidney epithelial African green monkey cells (Vero), Madin-Darby canine kidney cells (MDCK) and mouse connective tissue cells (L929) were obtained from American Type Culture Collection (ATCC). Cells were cultured in cell culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were incubated at 37°C in humidified incubator supplemented with 5% (v/v) CO_2.

Antiproliferative assay

HeLa, Vero, MDCK and L929 cells were seeded in 96-well plates (5×10^4 cells/well) and maintained at 37^oC humidified incubator supplied with 5% (v/v) CO_2. After 80% confluency, cells were treated with petroleum ether and methanol extracts of *O. indicum* at final concentration of 0.39-99 µg/ml. After 72 hours, the cells viability were determined using methylene blue assay (Lin and Hwang, 1991). The assay procedure was also followed by Tan et al. (2005). Cisplatin was used as positive control. The most active plant extract with lowest IC_{50} value was chosen for subsequent experiments.

Morphological examination by phase contrast microscopy

HeLa, Vero, MDCK and L929 cells were treated with methanol extract of *O. indicum* leaves (MEOIL) at concentration of IC_{50} value for 24, 48 and 72 hours.

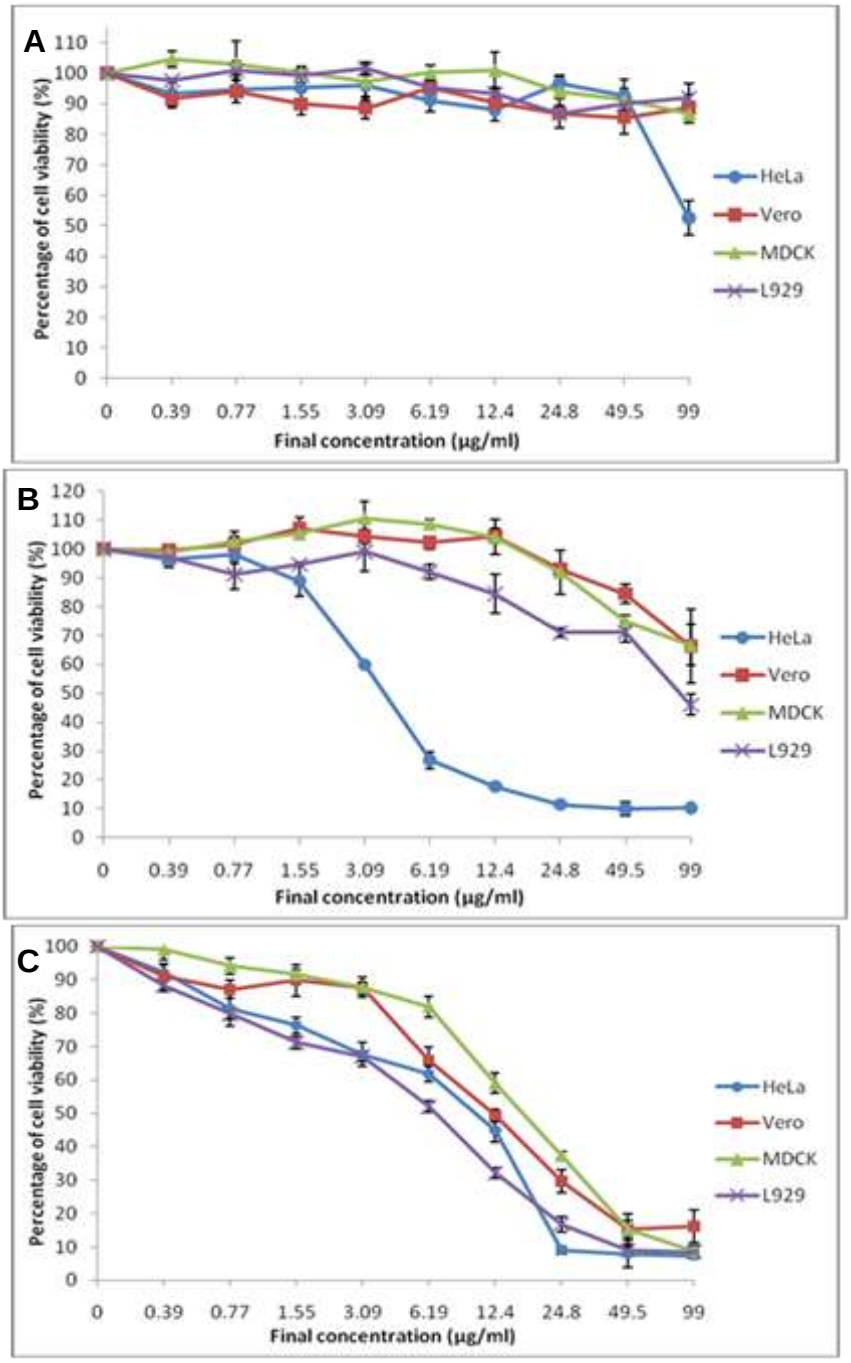

Figure 1. Antiproliferative activity of petroleum ether (A) and methanol (B) extracts of *O. indicum* and cisplatin (C) on HeLa, Vero, MDCK and L929 cell lines.
Each point showed the percentage of viable cells compared to negative control, DMSO. Each point represented as mean ± S.E.M. n = 3.

Morphology and confluency changes of treated cells were observed under phase contrast microscope. Untreated cells were used as control.

Hoechst 33258 nuclear staining assay

Nuclear staining with Hoechst 33258 was performed as previously described (Hishikawa et al., 1999) with slight modifications. HeLa cells were treated with IC_{50} concentration of MEOIL for 24, 48 and 72 hours. Briefly, the treated and untreated HeLa cells were washed and fixed with cold methanol for 20 minutes. After washing, cell membrane was permeabilized with 0.1% triton X-100 for 1 minute followed by incubation with 0.5 µg/ml of Hoechst

Table 1. IC_{50} value of *O. indicum* and cisplatin on the tested cell lines.

Test agents	Type of agents	IC_{50} values (µg/ml) HeLa	Vero	MDCK	L929
Oroxylum indicum	Petroleum ether extract	NA	NA	NA	NA
	Methanol extract	3.87 ± 1.10	NA	NA	89.72 ± 3.63
Cisplatin	Positive control	10.07 ± 3.60	12.45 ± 1.79	17.05 ± 3.06	6.97 ± 1.75

Values are mean ± S.E.M, n = 3 in each group. NA, not acquired.

Hoechst 33258 for 1 hour. Nuclear morphology was examined under fluorescence microscope. Cisplatin was used as positive control.

Measurement of early apoptosis event by flow cytometry

The number of early apoptotic event which induced by MEOIL was measured by flow cytometry using the BD Pharmingen FITC Annexin V Apoptosis Detection Kit I. Assay was carried out according to manufacturer protocol. Briefly, the treated and untreated HeLa cells were washed with cold PBS and pelleted by centrifugation at 1000 rpm. The cell pellet was resuspended in 1× binding buffer at a concentration of 1 × 10^6 cells/ml. 100 µl of cell suspension was transferred into flow cytometry tube and added with 5 µl of Annexin V-FITC and 5 µl of PI followed by thoroughly mixing. The solution was incubated for 15 minutes at room temperature in dark. Finally, 400 µl of 1× binding buffer was added. Samples were analyzed using BD FACS Canto II equipped with BD FACSDiva Version 6.1.2 software.

Detection of apoptotic proteins by flow cytometry

The numbers of apoptotic and non-apoptotic proteins expressed by HeLa cells after treated with MEOIL were determined by flow cytometry (Zakaria et al., 2009). Treated cells were harvested by trypsinization and c centrifuged at 1800 rpm. Cells were washed with PBS and fixed with cold 70% (v/v) ethanol (≤ 1 hour, 4°C). After washing, cells were resuspended in blocking buffer (2% BSA) for 10 minutes. Cells pellet were resuspended in PBS and 100 µl of cells suspension (1 × 10^6 cells/ml) were transferred into sample tube and 20 µl of FITC conjugated antibodies (p53, Bax and Bcl-2) were added followed by gentle mixing. The cells were incubated at room temperature for 30 minutes in the dark. After washing, cell pellets was resuspended in 500 µl PBS and analyzed using BD FACS Canto II installed with BD FACSDiva Version 6.1.2 software.

Cell cycle analysis

Analysis of cell cycle was performed according to manufacturer protocol (BD Biosciences, USA). HeLa cells were treated with IC_{50} concentration of MEOIL for 24, 48 and 72 hours. Untreated cells were used as control. Briefly, cell pellet was washed with PBS and centrifuged at 1000 rpm for 10 minutes. While vortexing, 5 ml of cold 70% ethanol was dropped into the pellet followed by incubation at -20°C for at least 2 hours. Cells were firstly washed with PBS and secondly wash with BD Pharmingen stain buffer. Next, 1 × 10^6 cells were resuspended in 0.5 ml of PI/RNase staining buffer (BD Biosciences, USA) followed by incubation in dark for 15 minutes at room temperature. Samples were analyzed using BD FACS Canto II and data was later analyzed with ModFit *LT* software.

Statistical analysis

All values were expressed as mean ± standard error mean (S.E.M). Statistical analysis was evaluated by student-t test. Probability values $P<0.05$ were considered statistically significant.

RESULTS

Antiproliferative activity

The antiproliferative activity for both petroleum ether and methanol extract of *O. indicum* leaves was evaluated through the IC_{50} value, which represents the concentration

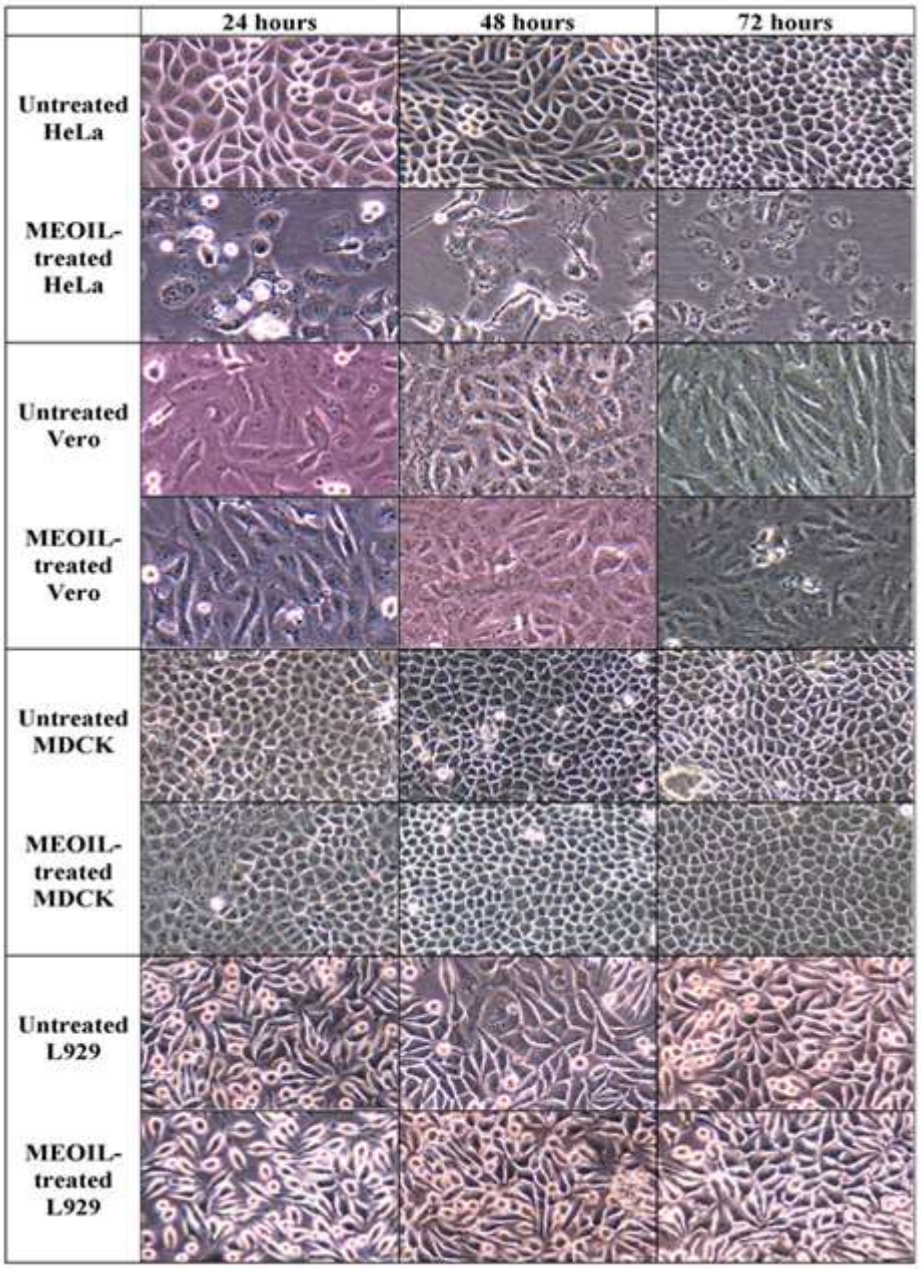

Figure 2. Morphology of HeLa, Vero, MDCK and L929 cells by phase contrast microscopy. Cells were left untreated for negative control. All cells were treated with 3.87 µg/ml of MEOIL for 24, 48 and 72 hours. Similar cellular morphology was observed in three independent experiments (n = 3). Magnification: 20x.

of drug that is required for the inhibition of 50% of cell population *in vitro*. The criteria for good antiproliferation for the crude extracts, as guided by the National Cancer Institute (NCI), is an IC_{50} value ≤ 20 µg/ml. Result showed that there was no antiproliferative activity were acquired from petroleum ether extract of *O. indicum* on HeLa, Vero, MDCK and L929 cells as the IC_{50} value was more than 99 µg/ml (Figure 1A). In contrast, the methanol extract of *O. indicum* exhibited good antiproliferative activity toward HeLa cells with IC_{50} 3.87 µg/ml without affecting the proliferation of Vero and MDCK cells (IC_{50} > 99 µg/ml). However, the methanol extract of *O. indicum* showed a slight effect on L929 cell proliferation with IC_{50} 89.72 µg/ml (Figure 1B). The positive control, cisplatin exhibited antiproliferative activity on both HeLa cells (IC_{50} 10.07 µg/ml) and normal cells; Vero, MDCK and L929 cells with IC_{50} values of 12.45 µg/ml, 17.05 µg/ml

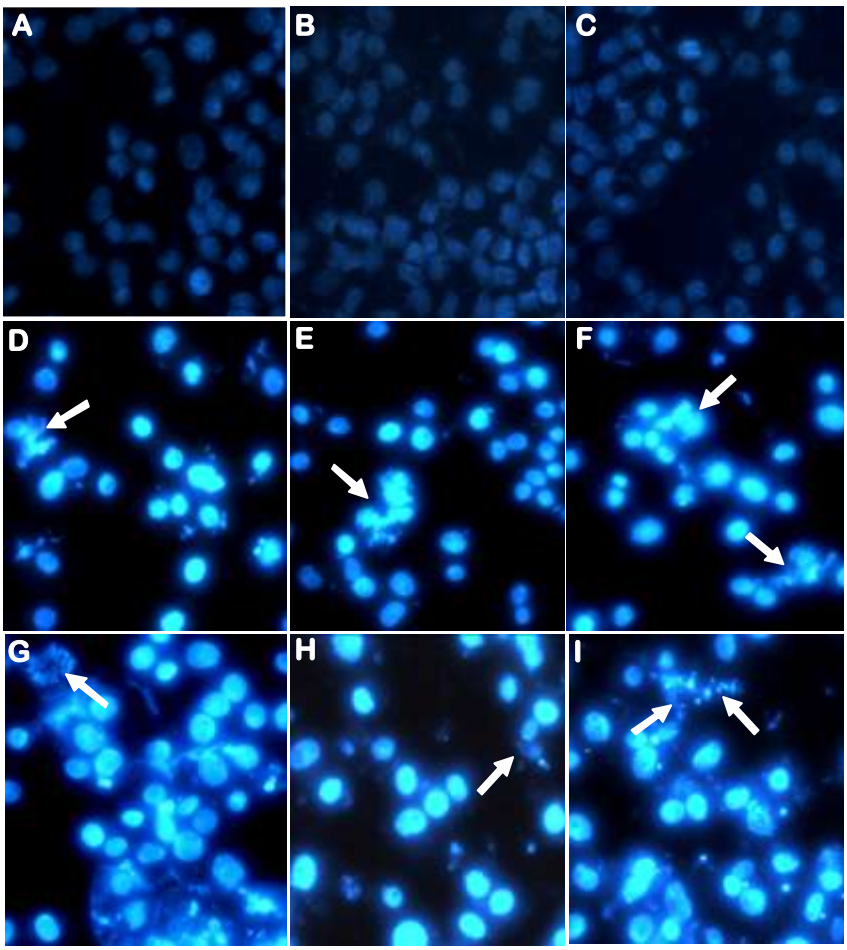

Figure 3. Nuclear staining of HeLa cells by Hoechst 33258 dye. Cells were left untreated for 24, 48 and 72 hours as negative control (A, B, C). All cells were treated with 3.87 µg/ml of MEOIL for 24, 48 and 72 hours (D, E, F). For positive control, cells were treated with 10.07 µg/ml of cisplatin for 24, 48 and 72 hours (G, H, I). Similar cellular morphology was observed in three independent experiments (n = 3). Magnification: 20x.

and 6.97 µg/ml respectively (Figure 1C). Table 1 summarized all the IC_{50} values for *O. indicum* extract and cisplatin on all tested cell lines. With the above results, methanol extract of *O. indicum* was used in the subsequent experiments and cells were treated with the IC_{50} value of the extract (3.87 µg/ml).

Morphology of cells under phase contrast microscope

The effects of methanol extract of *O. indicum* leaves (MEOIL) on the morphology and confluency of HeLa and normal cells were observed under the phase contrast microscope. Results indicated that the untreated HeLa cells showed high confluency of cells and normal morphology through all the treatment hours (Figure 2) but n MEOIL-treated HeLa cells, the cells showed morphological and confluency changes. The treated HeLa cells indicated a dramatically declination of confluency as the incubation time increased. Morphologically, the treated HeLa cells became shrank and reduced in size and shape (Figure 2).

The morphology of the untreated normal cells (Vero, MDCK and L929) during all treatment hours were in the form of confluent monolayer as were seen in cells treated with MEOIL for 24, 48 and 72 hours (Figure 2). This indicated that MEOIL was not affecting the proliferation of Vero, MDCK and L929. Hence, the present study suggested that MEOIL has successfully promoted death in

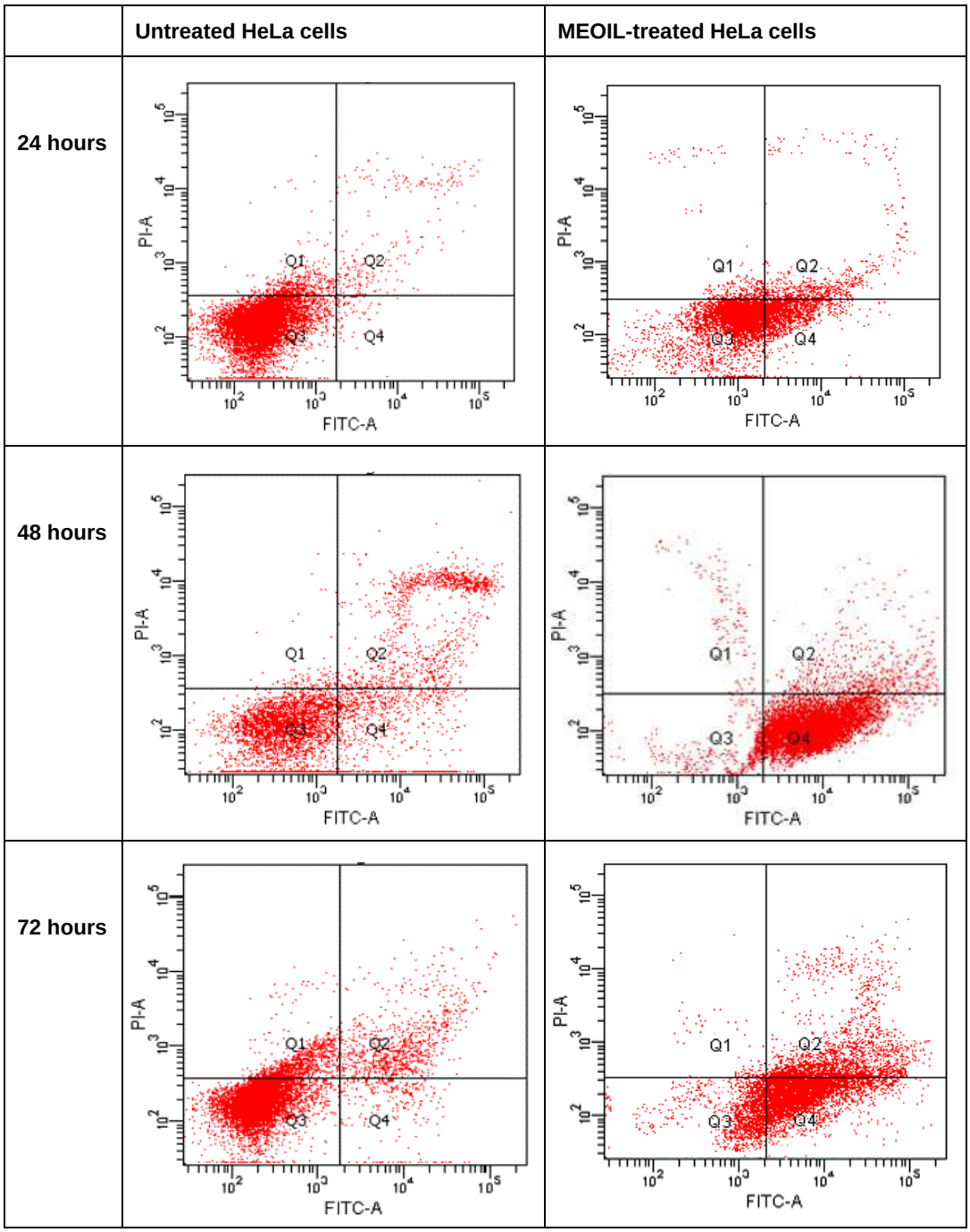

Figure 4. Scatter plots of HeLa cells stained with PI/FITC-Annexin V under four situations in quadrant analysis; viable cells (Q3), early apoptotic cells (Q4), late apoptotic cells (Q2) and necrotic cells (Q1). Cells were left untreated for negative control. All cells were treated with 3.87 µg/ml of MEOIL for 24, 48 and 72 hours. Similar plots were observed in three independent experiments (n = 3).

in HeLa cells without interfering the proliferation of all normal cells.

Morphology of apoptotic cells by Hoechst staining

To determine the ability of MEOIL to induce death in HeLa cell via apoptosis, the morphology of cell nucleus were studied using Hoechst 33258 staining. At all treatment hours, the untreated HeLa cells (Figure 3A-C) remained homogeneously stained and neither bright fluorescence nor fragmentation in the nucleus was detected.

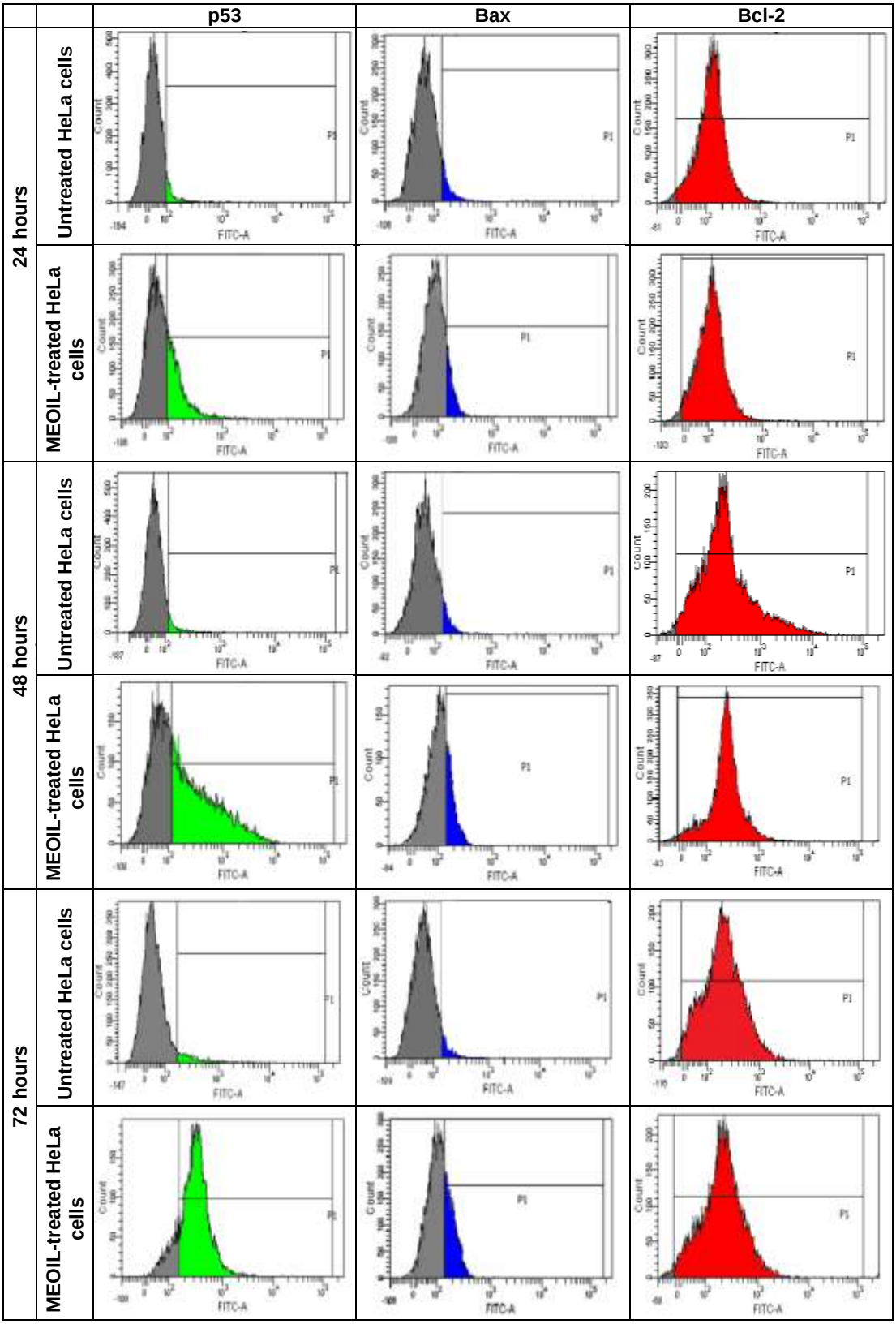

Figure 5. The profile of p53, Bax and Bcl-2 proteins detected by flow cytometry in untreated HeLa cells (negative control) and MEOIL-treated HeLa cells for 24, 48 and 72 hours. Unstained cells, Cells stained with FITC-p53, Cells stained with FITC-Bax, ells stained with FITC-Bcl-2.
n = 3.

However at 24 hours treatment, cells began to display apoptotic morphology as bright fluorescence was detected in the nuclear region, indicating the presence of chromatin condensation (Figure 3D).
At 48 hours, nuclear condensation and fragmentation was more apparent as seen by the condensation of chromatin into pieces (Figure 3E). At 72 hours, apoptotic bodies were finally formed, seen as smaller intense spots (Figure 3F).
Cisplatin-treated cells (Figure 3G-I) displayed a similar nuclear fluorescence and apoptotic events indicating that the MEOIL-treated cells might die via apoptosis pathway as in the cisplatin-treated cells.

Induction of Apoptosis Event by EMOIL

The apoptotic death mode in MEOIL-treated HeLa cells was further determined through the double staining with FITC-Annexin V and propidium iodide (PI) by flow cytometry analysis. This enabled the differentiation towards four cell types in four quadrants analysis; Q1 (PI+/FITC-Annexin V-: dead or necrotic cells), Q2 (PI+/FITC-Annexin V+: late apoptotic cells), Q3 (PI-/FITC-Annexin V-: viable cells) and Q4 (PI-/FITC-Annexin V+: early apoptosis cells) (Figure 4).
In the untreated group, the number of viable cells (Q3) was high with percentage of 77.6%, 72.5% and 78.4% at all treatment hours while in MEOIL-treated group, the number of viable cells lessened from 70.7% to 11.7% and 2% during 24, 48 and 72 hours treatment respectively. For early apoptotic cells (Q4), there was no increment of the cells in the untreated group where only 6.3%, 10.4% and 6.5% of cells were detected at all incubation periods. In MEOIL-treated group, the number of early apoptotic cells was initially increased to 20.3% and 63.7% during 24 and 48 hours treatment respectively. However, after 72 hours treatment, the number of early apoptotic cells was decreased to 45.3% (Figure 4). For late apoptotic cells (Q2), in the untreated group, the number of the cells was low with 11%, 12.5% and 11% at all incubation hours. In the MEOIL-treated group, the number of late apoptotic cells was originally low with 5.4% at 24 hours but after 48 and 72 hours, the cell number raised up to 23% and 46.4% respectively. The final quadrant analysis; dead or necrotic cells (Q1) in the untreated group was maintain low with 5.1%, 4.6% and 4.1% during all incubation periods. In the MEOIL-treated group, the number of necrotic cells was also detected at low level with 3.6%, 1.4% and 2.9% during all incubation periods (Figure 4).

The effect of EMOIL to apoptotic proteins expression

The mechanism of apoptosis in MEOIL-treated HeLacells was further examined through the inspection of p53, Bax and Bcl-2 proteins which involved in the controlling of the apoptosis pathway using flow cytometry analysis. Results indicated that the level of p53 protein in HeLa cells was dramatically increased as the cells were treated with MEOIL over the time of exposure. The p53 levels of 29.7%, 57.1% and 88.5% were observed at 24, 48 and 72 hours respectively. On the contrary from untreated cells, the levels of p53 were low at all treatment hours with percentage of 5.3%, 5.5% and 5.5% at all treatment hours (Figure 5). Bax protein was detected in MEOIL-treated HeLa cells at low level and no major increment was seen throughout experiment. The levels of Bax were 12.4% during 24 hours and increased to 22.8% at 48 hours followed by slight increase to 29.6% at 72 hours treatment. As compared to untreated cells, the percentage of Bax was much lower where the values of 5.9%, 5.8% and 5.6% were observed during all incubation times (Figure 5).
For Bcl-2, result indicated the expression of the protein was unaffected although the incubation time was increased. In MEOIL-treated cells, Bcl-2 was expressed with higher level of 98.1%, 98.7% and 98.6% at 24, 48 and 72 hours respectively. The Bcl-2 levels were comparable to the untreated cells with the values of 98%, 98.5% and 98.5% at 24, 48 and 72 hours respectively (Figure 5).

The effect of EMOIL to DNA content in the cell cycle

The relative DNA content in untreated and MEOIL-treated HeLa cells was represented as three distinct stages within the interphase of the cell cycle, called G_0/G_1, S and G_2/M phases. From the results, there were no changes in the DNA content in each phase of the untreated cells. For G_0/G_1 phase, the DNA content was 52.15% at 24 hours, 54.33% at 48 hours and 53.87% at 72 hours. During S phase, the DNA content was 18.96%, 17.19% and 16.22% at 24, 48 and 72 hours respectively while in G_2/M phase, the DNA content was 33.9%, 35.53% and 33.22% at 24, 48 and 72 hours respectively (Figure 6).
In MEOIL-treated HeLa cells, the profile of cell cycle was altered after the treatment was done. During 24 hours treatment, the DNA content in G_0/G_1 phase was shown to increase up to 96.6%, while in S phase, the DNA content was declined to 0.87%, whereas in G_2/M phase, DNA content was also declined to 0.49%. At 48 hours treatment, the DNA content in G_0/G_1 phase slightly increased to 97.8%, while in S phase, the DNA content as in G_2/M phase, the DNA content became lower with percentage of 0.09%.
At 72 hours treatment, the DNA content in G_0/G_1 phase continued to increase to 99.31% whereas in S phase, the DNA content continued to decrease to 0.7% as well as in G_2/M phase, the DNA content decreased to 0.08% (Figure 6).

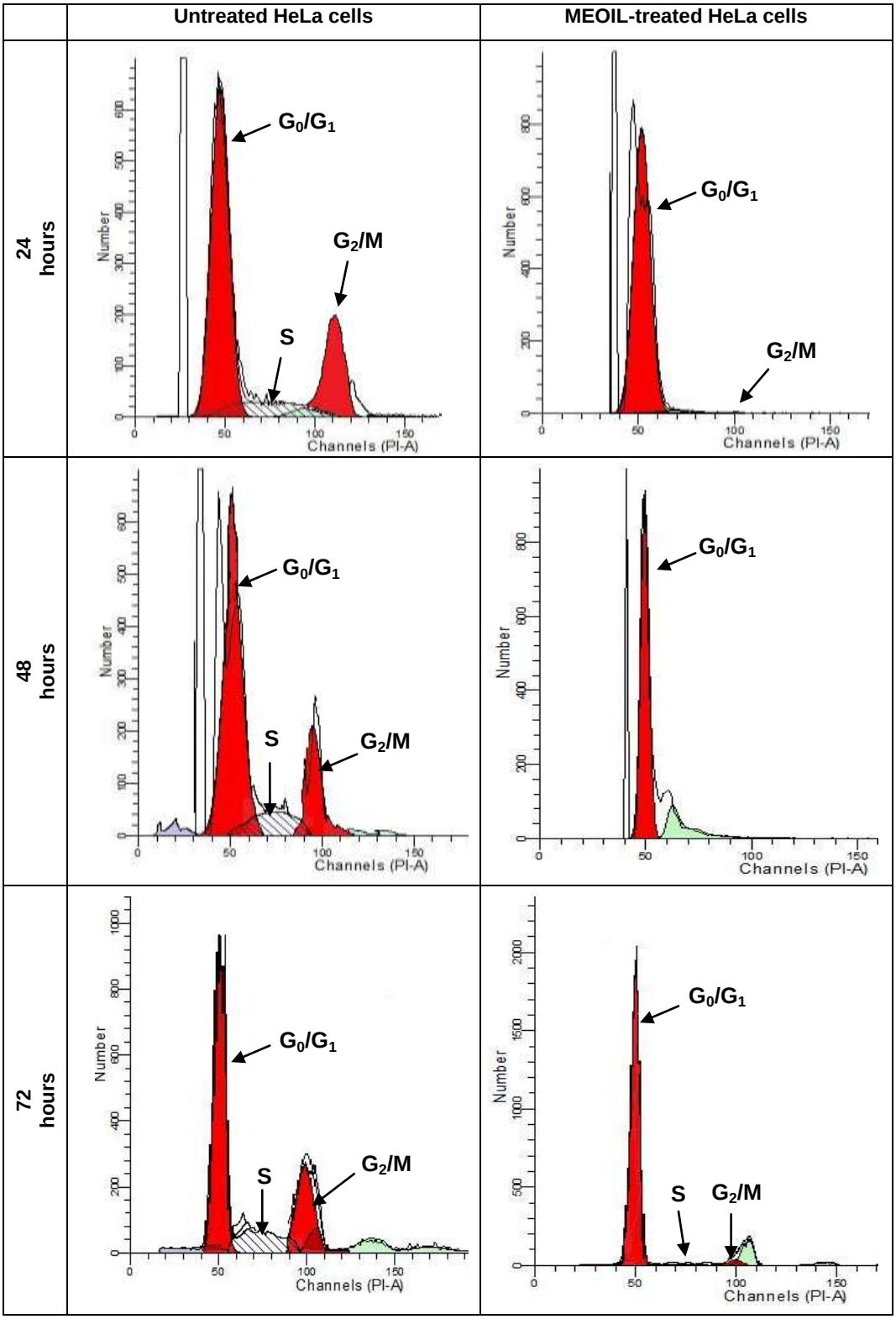

Figure 6. DNA profile in each phase of cell cycle of HeLa cells indicating the G_0/G_1, S and G_2/M phases. Cells were left untreated (negative control) and treated with 3.87 µg/ml of MEOIL for 24, 48 and 72 hours. G_0/G_1 or G_2/M phase, S phase, aggregates, debris.
n = 3.

DISCUSSION

For thousand years, the plant products have been widely used in the medicinal preparations among the folks. These preparations were initially in the form of powder, water decoction, paste and others (Barnes et al. 2007). With scientific information, the uses of those formulations were then developed into the production of therapeutic drugs. Until today, plenty of drugs produced from the plants sources have been used in the treatment of many diseases including cancer (Topliss et al., 2002). Regardless, the hunting for plant species as a source for anticancer drugs still continues since most of the available drugs produce side effects (Harvey, 2008).

The methanol extract of *O. indicum* leaves (MEOIL), is one of the possible candidates with promising potencies to be developed as a new chemotherapeutic agent. In this study, MEOIL was shown to inhibit the proliferation of HeLa cells (IC_{50} 3.87 µg/ml) with lower dose than cisplatin (IC_{50} 10.07 µg/ml) without interfering the proliferation of all tested normal cells. In previous study, cisplatin was reported to exhibit a cytotoxic effect on normal Vero cells with IC_{50} 9.06 µg/ml (Sharifah Sakinah et al., 2007) which was near to the IC_{50} value obtained from this study (12.45 µg/ml). The present study suggested that MEOIL has successfully exerted a selective antiproliferative effect on HeLa cells. Thus, MEOIL is much less cytotoxic than cisplatin.

The search for anticancer property from the leaves of *O. indicum* is scarcely reported. However, some studies have conducted the anticancer research using the other parts of *O. indicum*. For example, methanol extract of the young pod has been reported to promote a strong cytotoxic activity against Epstein-Barr virus in Raji cells (Murakami et al., 1995). The ethanol extract of the stem bark has also been identified having the highest toxicity towards murine melanoma, human colon carcinoma and leukemia cell lines (Costa-Lotufo et al., 2005). However, no significant antiproliferative activity was observed in HeLa cells treated with petroleum ether and methanolextract of the stem bark (Siriwatanametanon et al., 2010). Furthermore, there was no antiproliferative effect observed in HEp2 cells after treatment with ethanol extract of the fruit (Kamkaen et al., 2006). With this information, it is a good move if the leaves of *O. indicum* are highlighted for anticancer studies since there is no study using the plant leaves for the development of anticancer drugs. MEOIL was shown to induce death in HeLa cells via apoptosis as initially proved by nuclear staining with Hoechst 33258, indicating the classical apoptosis characteristics; chromatin condensation, nuclear fragmentation and formation of apoptotic bodies as described by Kerr et al. (1972). This observation was also seen in cisplatin-treated cells indicating that the data obtained can be accepted. The untreated HeLa cells remained uniformly stained and no bright fluorescence was detected in the nucleus indicating that the cells were not undergoing death or apoptosis. The results provided evidence that apoptotic pathway occurred in HeLa cells following MEOIL treatment. The nuclear staining with Hoechst 33258 is relatively stains the late apoptotic cells only (Close et al., 1999). The identification of apoptotic cells which in the initial stage was done through the staining with FITC-Annexin V and PI.

Apoptosis pathway in MEOIL-treated HeLa cells has been determined in this study through the inspection of early apoptosis event. Early event of apoptosis was accessed by targeting the membrane phospholipid phosphatidylserine (PS) which was exposed at the outer leaflet of plasma membrane due to the loss of plasma membrane asymmetry during the early event of apoptosis (Henson et al., 2001). The FITC-Annexin V used in this study bound to that PS while propidium iodide (PI) entered to a damage cell membrane and stained DNA. Thus, when apoptosis was measured over time, cells can be distinguished between FITC Annexin V negative and PI negative (viable, no measurable apoptosis), FITC Annexin V positive and PI negative (early apoptosis, membrane integrity is present), FITC Annexin V positive and PI positive (end stage of apoptosis, no membrane integrity) and FITC Annexin V negative and PI positive (death or necrosis, no membrane integrity). The movement of cells through all these stages suggested apoptosis (Schutte et al., 1998).

In control cells, the number of viable cells remained high (average of 76.2%) while the number of cells in early apoptosis, late apoptosis and necrosis were very low in all treatment hours indicating that in untreated cells, cells continuously grew and apoptosis process occurred as a normal process in every normal cell lives (Zakaria et al., 2009). In MEOIL-treated cells, as the time of treatment increased, the cells were shown to undergo apoptosis as proved by the regression of viable cells and the progression of early apoptotic cells to 20.3% and 63.7% during 24 and 48 hours respectively. However, during 72 hours, the early apoptotic cells decreased to 45.3% indicating that early apoptosis event took place in HeLa cell at less than 48 hours. After 48 hours, cells gradually underwent the late apoptosis event. This is supported by the results of late apoptotic cells which indicated lower events at 24 hours (5.4%), but increased to 23% and 46.4% after 48 and 72 hours. Normally, cultured cells which were induced to undergo apoptosis exhibit signs of the incident within 5-10 hours compared to cells in the body tissues (within 11-14 days) (Sundquist et al., 2006). There have been reported that the number of early apoptotic cells in HeLa cells treated with clitocine (isolated from mushroom *Leucopaxillus giganteus*) elevated up to 28.45% after only 8 hours treatment suggesting that the compound was able to induce apoptosis in HeLa cells in a short time period (Ren et al., 2008). The numbers of necrotic cells in this study remained

low with average of 2.6% as more than 60% of the cells have died via apoptosis.

Apoptotic genes regulate apoptosis by the action of their pro- and anti-apoptotic products. Among the most studied apoptotic proteins are p53, Bax and Bcl-2 proteins. These proteins are among the most important models in determining the successful or failure of apoptosis (Haupt et al., 2003; Jourdain and Martinou, 2009).

The present study indicated that MEOIL caused high accumulation of p53 protein in HeLa cells up to 88.5% during 72 hours treatment. The accumulation of p53 might be induced by the activation of DNA damage that caused by cytotoxic agents that present in MEOIL. Stresses such as oncogene stimulation, DNA damage, hypoxia, radiation and medication can activate p53 (Elmore 2007).

When activated, p53 is responsible to activate the critical genes involved in regulating cell cycle. In this study, the cell cycle profile was examined to determine the patent of cell division in HeLa cells after being treated with MEOIL. Cell cycle is required to monitor and maintain the genomic integrity of cells by means of a complex network of DNA repair pathways or cell cycle checkpoints. Cell cycle checkpoints detect various types of structural defects in DNA, or in chromosome function and induce a multifaceted cellular response that activates DNA repair and delays cell cycle progression (Bartek and Lukas, 2001). During 24 hours treatment, the DNA amount in G_0/G_1 phase in MEOIL-treated HeLa cells raised up (98.16%) followed by declination of DNA amount in both S phase (1%) and G_2/M phase (0.63%). This indicating that the growth of HeLa cells was impeded at G_1/S checkpoint, causing the interruption of cells to enter the S phase to synthesis the DNA and thus, no growth occurred during G_2 phase.

Cell cycle arrest in HeLa cells might be stimulated by stress or DNA damage that triggered by the MEOIL. DNA damage checkpoints are positioned before S phase (G_1/S checkpoint) or after S phase (G_2/M checkpoint). At the G_1/S checkpoint, regulation of cell cycle arrest is controlled by p53 and other target proteins. The checkpoint arrest at G_1/S of the cell cycle is pivotal to let the DNA to be repaired and prevents the replication of damaged cells (Vermeulen et al., 2003). In this study, the growth arrest at G_1 checkpoint took place until 48 and 72 hours indicating that the cell division has stopped completely. Thus, p53 has successfully played its role in preventing HeLa cells from further dividing by terminating the cell cycle progress.

The growth arrest that was caused by MEOIL was permanent indicating that DNA repair system in HeLa cells cannot be performed. In this situation, p53 may induce apoptosis by up-regulating Bax or suppressing the Bcl-2 expression (Jourdain and Martinou, 2009). The expression levels of Bax increased slightly to 29.6% after the maximum treatment period of 72 hours with MEOIL. The production of Bax might be activated by p53 that highly accumulated in HeLa cells. Bax starts the apoptosis pathway by stimulating the release of cytochrome c from mitochondria and subsequently activates the caspase cascades (Chipuk and Green 2008). Several studies have reported the high expression of p53, followed by the accumulation of Bax that activated the mitochondrial apoptosis pathway (Ismail et al. 2005; Mahfudh and Lope Pihie 2008).

Beside the action on Bax and cell cycle, p53 may also suppress the Bcl-2 and hinder its anti-apoptosis function. However, the results of this study showed the expression levels of Bcl-2 unchanged and remained high (average 98.5%) at all treatment periods. This indicating that p53 was unable to prevent the transcription of Bcl-2 in MEOIL-treated HeLa cells. The same result has also been reported in HeLa cells treated with methanol and chloroform extracts of *Nigella sativa* seeds which showed the expression of p53 was not affecting the expression of Bcl-2 (Shafi et al., 2009). Nevertheless, the mechanism behind the up-regulation of p53 without affecting Bcl-2 level remained unknown (Johnstone et al., 2002).

CONCLUSION

Apoptosis is the main system for anticancer agents to kill tumor cells, thus the increase in p53 and Bax protein expression may restore sensitivity to apoptotic stimuli in cervical cancer cells. The results of this study provided evidence that MEOIL was able to inhibit the proliferation of HeLa cells by inducing apoptotic cell death. Additional studies are necessary to determine the downstream molecules in the apoptotic pathway especially the E6 protein, a type of onco-protein expressed by HPV16 which deactivates p53.

The active compound that present in MEOIL need to be isolated to ensure the apoptosis induction is more effective and this could lead to the discovery of a novel compound. Understanding the functional role and regulation of apoptotic events will provide new foresight into mechanisms involved in cancer cell proliferation and in combating malignancy.

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REFERENCES

Ashok Kumar R, Rajkumar V, Guha G, Mathew L (2010). Therapeutic potentials of *Oroxylum indicum* bark extracts. Chin. J. Nat. Med. 8(2): 121-126.

Barnes J, Anderson LA, Phillipson JD (2007). Herbal medicines, third edition. London, UK: Pharmeceutical Press. p. 4.

Bartek J, Lukas J (2001). Mammalian G1- and S-phase checkpoints in response to DNA damage. Current Opinion in Cell Biology 13: 738-747.

Chipuk JE, Green DR (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization?. Trends in Cell Biology 18,(4): 57-164.

Close AH, Guo TL, Shenker BJ (1999). Activated human T lymphocytes exhibit reduced susceptibility to methylmercury chloride-induced apoptosis. Toxicological Sciences 49(1): 68-77.

Costa-Lotufo LV, Hassan Khan MT, Ather A, Veras Wilke D, Jimenez PC, Pessoa C, de Moraes MEA, de Moraes MO (2005). Studies of the anticancer potential of plants used in Bangladeshi folk medicine. J. Ethnopharmacol. 99: 21-30.

Elmore S (2007). Apoptosis: a review of programmed cell death. Toxicologic Pathology 35: 495-516.

Gokhale M, Bansal YK (2006). An avowal of importance of endangered tree *Oroxylum indicum* (Linn.) Vent. Natural Product Radiance 5(2) 112-114.

Hari Babu T, Manjulatha K, Suresh Kumar G, Hymavathi A, Tiwari AK, Purohit M, Madhusudana Rao J, Suresh Babu K (2010). Gastroprotective flavonoid constituents from *Oroxylum indicum* Vent. Bioorganic & Medicinal Chemistry Letters 20:117-120.

Harvey AL (2008). Natural products in drug discovery. Drug Discovery Today 13(19-20): 894-901.

Hasmah A, Hohmann J, Azimahtol Hawariah LP, Molnar J, Forgo P (2008). Antiproliferative compounds from *Hydnophytum formicarium*. J. Trop. Med. Plants 9(2): 366-371.

Haupt S, Berger M, Goldberg Z, Haupt Y (2003). Apoptosis: the p53 network. J. Cell Sci. 116:4077-4085.

Henson PM, Bratton DL, Fadok VA (2001). Apoptotic cell removal. Current Biology 11: R795-R805.

Hishikawa K, Oemar BS, Tanner FC, Nakaki T, Lűscher TF, Fuji T (1999). Connective tissue growth factor induces apoptosis in human breast cancer cell line MCF-7. J. Biol. Chem. 274,(54): 37461-37466.

Ismail N, Lope Pihie AH, Nallapan M (2005). Xanthorrhizol induces apoptosis *via* the up-regulation of Bax and p. 53 in HeLa cells. Anticancer Research 25: 2221-2228.

Johnstone RW, Ruefli AA, Lowe SW (2002). Apoptosis: a link review between cancer genetics and chemotherapy. Cell 108:153-164.

Jourdain A, Martinou JC (2009). Mitochondrial outer-membrane permeabilization and remodelling in apoptosis. Int. J. Biochem. Cell Biol. 41(10):1884-1889.

Kamkaen N, Wilkinson JM, Cavanagh HMA (2006). Cytotoxic effect of four Thai edible plants on mammalian cell proliferation. Thai Pharm. Health Sci. J. 1(3): 189-195.

Kerr JF, Wyllie AH, Currie AR (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Bri. J. Cancer 26: 239-257.

Lin L, Hwang PL (1991). Anti-proliferative effects of oxygenated sterols: positive correlation with binding affinities for the anti-estrogen binding sites. Biochemica Biophysica Acta 1082:177-184.

Mahfudh N, Lope Pihie AH (2008). Eurycomanone induces apoptosis through the up-regulation of p53 in human cervical carcinoma cells. J. Canc. Mol. 4(4): 109-115.

Murakami A, Jiwajinda S, Koshimizu K, Ohigashi H (1995). Screening for in vitro anti-tumor promoting activities of edible plants from Thailand. Cancer Letters 95: 139-146.

Poon IKH, Hulett MD, Parish CR (2010). Molecular mechanisms of late apoptotic/necrotic cell clearance. Cell Death and Differentiation 17: 381-397.

Ren G, Zhao YP, Yang L, Fu CX (2008). Anti-proliferative effect of clitocine from the mushroom *Leucopaxillus giganteus* on human cervical cancer HeLa cells by inducing apoptosis. Cancer Letters 262: 190-200.

Riboli E, Norat T (2003). Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. Am. J. Clin. Nutr. 78: 559S-569S.

Saslow D, Runowicz CD, Solomon D, Moscicki AB, Smith RA, Eyre HJ, Cohen C (2002). American cancer society guideline for the early detection of cervical neoplasia and cancer. CA: Canc. J. Clin. 52: 342-362.

Schutte B, Nuydens R, Geerts H, Ramaekers F (1998). Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells. J. Neurosci. Methods 86: 63-69.

Shafi G, Munshi A, Hasan TN, Alshatwi AA, Jyothy A, Lei DK (2009). Induction of apoptosis in HeLa cells by chloroform fraction of seed extracts of *Nigella sativa.* Cancer Cell International 9: p. 29.

Sharifah Sakinah SA, Tri Handayani S, Azimahtol Hawariah LP (2007). Zerumbone induced apoptosis in liver cancer cells via modulation of Bax/Bcl-2 ratio. Cancer Cell International 7: p. 4.

Siriwatanametanon N, Fiebich BL, Efferth T, Prieto JM, Heinrich M (2010). Traditionally used Thai medicinal plants: In vitro anti-inflammatory, anticancer and antioxidant activities. J. Ethnopharmacol. 130(2):196-207.

Sultana H, Kigawa J, Kanamori Y, Itamochi H, Oishi T, Sato S, Kamazawa S, Ohwada M, Suzuki M, Terakawa N (2003). Chemosensitivity and p53-Bax pathway mediated apoptosis in patients with uterine cervical cancer. Annual Oncology 14: 214-219.

Sundquist T, Moravec R, Niles A, O'Brien M, Riss T, P. Corporation (2006). Timing your apoptosis assays. Cell

Notes 16: 18-21.
Tan ML, Sulaiman SF, Najimuddin N, Samian MR, Tengku Muhammad TS (2005). Methanolic extract of *Pereskia bleo* (Kunth) DC. (Cactaceae) induces apoptosis in breast carcinoma, T47-D cell line. J. Ethnopharmacol. 96: 287-294.
Topliss JG, Clark AM, Ernst E, Hufford CD, Johnston GAR, Rimoldi JM, Weimann BJ (2002). Natural and synthetic substances related to human health (IUPAC technical report). Pure Appl. Chem. 74(10): 1975-1985.
Vermeulen K, Van Bockstaele DR, Berneman ZN (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Proliferation 36(3): 131-149.
Yuan Y, Hou W, Tang M, Luo H, Chen L, Guan YH, Sutherland IA (2008). Separation of flavonoids from the leaves of *Oroxylum indicum* by HSCCC. Chromatographia 68: 885-892.
Zainal Ariffin O, Nor Saleha IT (2011). Malaysia cancer statistics-data and figure 2007. National Cancer Registry Report, Ministry of Health, Kuala Lumpur:1-127.
Zakaria Y, Rahmat A, Lope Pihie AH, Abdullah NR, Houghton PJ (2009). Eurycomanone induce apoptosis in HepG2 cells via up-regulation of p53. Cancer Cell International 9: p. 16.
Zhang Z, Liu X, Wu T, Liu J, Zhang X, Yang X, Goodheart MJ, Engelhardt JF, Wang Y (2011). Selective suppression of cervical cancer Hela cells by 2-O-β-D-glucopyranosyl-L-ascorbic acid isolated from the fruit of *Lycium barbarum* L. Cell Biology and Toxicology 27(2): 107-121.