Morphological studies of apoptotic HeLa cells death induced by eurycomanone

Kajian morfologi apoptosis pada sel HeLa yang terinduksi eurikomanon

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Abstract

Eurycomanone is a cytotoxic ingredient found in *Eurycoma longifolia* Jack. Previous studies have noted its activity against many epithelial cell lines. In this study, eurycomanone had obvious cytotoxic effect on HeLa cells by methylene blue staining assays. After HeLa cells were treated with eurycomanone, typical morphological changes, including cytoplasm shrinkage and decrease of cell volume were observed by light microscope. Chromatin condensation and nuclear fragmentation could be observed by fluorescence microscope after staining with Hoechst 33258 nuclear staining. DNA fragmentation and apoptotic body formation that is characteristic of apoptosis could be determined after treated cells were assayed with TUNEL (terminal deoxynucleotidyl transferase mediated d-UTP nick end labeling). The externalization of phosphatidyl serine (PS) could be determined by flow cytometry analysis using Annexin-V/PI double staining. The result suggested that eurycomanone exerted antiproliferative activity on HeLa cells by inducing apoptosis.
Key words : eurycomanone, cytotoxic, apoptosis, HeLa

Abstrak

Eurikomanon adalah senyawa yang bersifat sitotoksik yang ditemukan dalam *Eurycoma longifolia* Jack. Kajian terdahulu telah melaporkan aktivitas sitotoksiknya terhadap beberapa turunan sel-sel kanker. Pada kajian ini, eurikomanon menunjukkan efek sitotoksik terhadap sel HeLa yang ditunjukkan dengan pengecatan metilen biru. Setelah perlakuan dengan eurikomanon, perubahan-perubahan morfologi yang khas seperti pengecutan sitoplasma dan pengecilan ukuran sel teramati dengan pengamatan mikroskop cahaya. Kondensasi kromatin dan fragmentasi inti teramati setelah pengecatan dengan Hoechst 33258. Fragmentasi DNA dan pembentukan jasad apoptotik yang merupakan karakteristik apoptosis dapat teramati setelah sel yang diberi perlakuan eurikomanon dideteksi menggunakan TUNEL (terminal deoxynucleotidyl transferase mediated d-UTP nick end labeling). Eksternalisasi fosfatidil serin (PS) dapat teramati menggunakan analisis flowcytometri menggunakan pengecatan berganda Annexin V/PI. Hasil kajian menunjukkan bahwa eurikomanon mempunyai aktivitas antiproliferatif dengan menginduksi apoptosis.
Kata kunci : eurikumanon, sitotoksik, apoptosis, HeLa

Introduction

Apoptosis is the process of programmed cell death through a tightly controlled program that plays important roles in many normal processes (Reed, 2001). During apoptosis, the nucleus and cytoplasm condense to produce membrane-bound apoptotic bodies that are

phagocytosed by macrophages or adjacent cells. Compounds that block or suppress the proliferation of tumor cells by inducing apoptosis are considered to have potential as anti tumor agents (Frankfurt and Krishan, 2003).

Apoptosis is associated with a distinct set of biochemical and morphological changes involving the cytoplasm, nucleus and plasma membrane (Allen *et al*., 1997). Early in apoptosis, the cells round up, losing contact with their neighbors, and shrink. In the nucleus, chromatin condenses and aggregates into dense compact masses, and is fragmented internucleosomally by endonucleases (Lawen, 2003). The nucleus becomes convoluted and buds off into several fragments, which are encapsulated within the forming apoptotic bodies (Gewies, 2003; Kerr *et al.,* 1994). Under physiological conditions, certain modifications in the plasma membrane occur, which enable the recognition of apoptotic bodies by phagocytic cells (Savill and Fadok, 2000).

Eurycomanone is one of promising compound to be developed as antitumor agent. The previous studies had noted its cytotoxicity against various cancer cell lines (Kardono *et al.,* 1991; Kuo *et al.,* 2004; Cheah and Azimahtol, 2004) and apoptosis had noted as the important mechanism involves in its cytotoxicity.

Cervical carcinoma is one of the most common neoplastic diseases among women, with a combined worldwide incidence of almost half a million new cases annually, second ranking after breast cancer (Franco *et al.*, 2003). Therefore, the development of chemotherapeutic/ chemopreventive agents against cervical carcinoma is very important to reduce the incidence and mortality of this disease. The main aim of this paper was to study the cytotoxic effect of eurycomanone on human cervical carcinoma (HeLa) cells and explore through morphological and biochemical observation whether the effect was mediated through the apoptotic mechanism.

Methodology

Plant materials

E. longifolia roots were provided by Prof. Dr. Azimahtol Hawariah Lope Pihie (National University of Malaysia).

Compound extraction

Eurycomanone was extracted from the root of *E. longifolia* as previously described (Darise *et al.,* 1982). The root of the plant was dried, ground and extracted with methanol. The methanolic extract was then concentrated to dryness. A suspension of the dry extract in water was separated with diethyl ether using separating funnel. The water fraction was then separated with n-buthanol saturated with water. The buthanol layer was then evaporated to dryness and the residue was subjected to column chromatography over silica gel using a mixture of ethyl acetate-ethanol-water (100:10:1) as the mobile phase. The isolated compound was confirmed as eurycomanone by comparing the NMR spectral data with published value (Kardono *et al.,* 1991; Chan *et al.,* 1986).

Cell culture conditions

All cell lines (CaOv-3, HeLa, HepG2, HM3KO, MCF-7, Vero and MDBK) were obtained from American Type Culture Collection (ATCC). The cell lines were maintained in DMEM medium supplemented with 5 % fetal bovine serum (FBS) and 1 % penicillin streptomycin. The cells were grown at 37 °C in humidified atmosphere of 5 % CO_2.

Cell proliferation assay

The antiproliferative effect of eurycomanone was evaluated by obtaining the IC_{50} values for the above mentioned cancer cell lines as previously described (Lin and Hwang, 1991; Lee and Azimahtol, 2003). In the experiment, logarithmically growing HeLa cells were seeded at a density of 5.10^3 cells/well into 96-well plates and allowed to adhere for 24 hrs at 37 °C. Then the seeding medium was discarded and replaced with fresh medium containing varying concentration of compound. The cells were maintained for 3 days and the antiproliferative activity of eurycomanone was determined using the methylene blue staining.

Glutaraldehyde was added to each well to a final concentration of 2.5 % (v/v). Following of 15 minutes incubation, the cells were washed with 0.15 M NaCl. The cells were then stained with 0,1 mL of 0.05 % methylene blue solution for 15 minutes. The excess dye was washed out and 0.2 mL of 0.33 M HCl was added into each well. The absorbance was read at 660 nm.

Giemsa staining

Giemsa staining was assayed according to the method previously described (Song and Zhang, 2006). The eurycomanone treated HeLa cells were tripsinized and washed twice with PBS using centrifugation. The cells were overlaid on the slides and allowed to dry in room temperature. Then the cells were fixed with 100 % ethanol for 15 min and stained with Giemsa for 30 min. Following staining, the slides were washed with distilled water. After drying, the slides were observed under light microscope.

Nuclear staining assay

Staining with Hoechst 33258 was performed according to the method previously described (Hishikawa *et al.*, 1999; Tee and Azimahtol, 2005). HeLa cells were seeded onto poly-L-lysin slide. After 24 hrs adherence the old medium was discarded and replaced with fresh medium containing eurycomanone (2 μg/mL). The treated cells were incubated for 24 and 72 hrs. Following incubation, the cells were washed with PBS and fixed with 4 % methanol free para formaldehyde for 30 minutes at 4 °C. The fixed cells were washed with PBS and stained with Hoechst 33258 (Sigma) at a final concentration of 30 μg/mL. The slides were observed under *Leica* florescence microscope.

TUNEL assay

DNA fragmentation, that is characteristic of apoptotic cells, was examined by TdT-mediated dUTP nick end labeling (TUNEL). HeLa cells were seeded onto a poly-L-lysine slide in a petri dish. After 24 hrs adherence, the cells were incubated without and with eurycomanone (2 μg/mL) for 24 and 72 hrs. At the end of the treatment, the cells were fixed with 4 % methanol free paraformaldehyde in PBS at 4 °C for 30 minutes and then washed with PBS for further analysis. The cells were stained with TUNEL assay kit Florescein (Promega) according to the manufacturer's instruction. This assay specifically detects apoptotic cells when visualized through the fluorescent microscope.

Flow cytometry analysis

The externalization of PS in the apoptotic cells can be assessed by measuring the binding of FITC-conjugated annexin-V to cells and the number of apoptotic cell death could be measured by flow cytometer. In this study, the number of HeLa cell death induced by eurycomanone was analysed with flow cytometer (Dakocytomacion) using the APOPTEST^{TM}-FITC according to manufacturer's protocol. The treated and untreated HeLa cells were harvested and washed with cold PBS. An aliquot of 100 μL of 10^6/mL cell suspension was added with 1 μL Annexin-V FITC and 2.5 μL propidium iodide (250 μg/mL). After 10 minutes incubation on ice, the cells were measured immediately.

Results and Discussions

The ability of certain compound to suppress proliferation of tumor cells and induce tumor cell apoptosis is the important properties should have by an anticancer drug candidate (Frankfurt and Krishan, 2003).

Eurycomanone (Fig. 1) significantly reduced the viability and proliferation of cancerous cells (CaOv-3, HeLa, HepG2, HM3KO and MCF-7) in comparison with control in a dose dependent manner (Fig. 2). The IC_{50} values of eurycomanone on cancerous cell lines were found less than 20 μg/mL. These findings suggested the

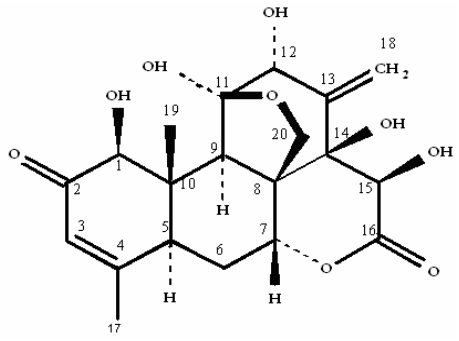

Figure 1. Chemical structure of eurycomanone.

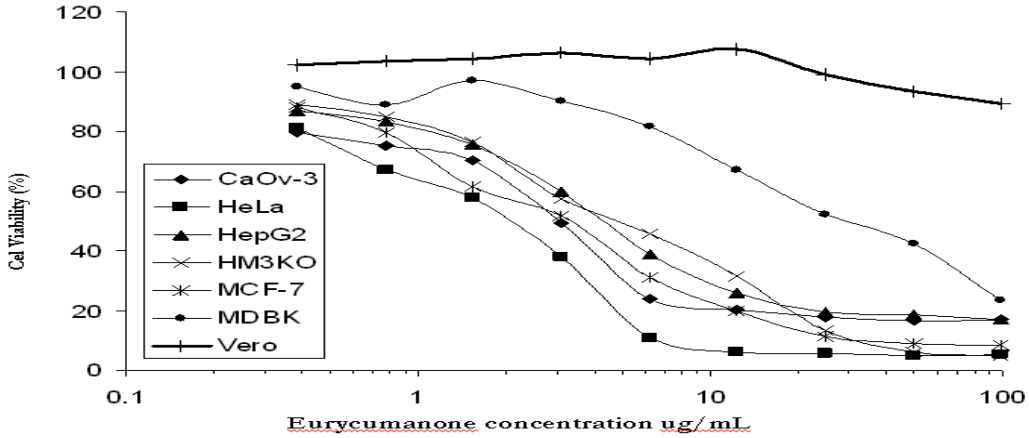

Figure 2. Cytotoxic effects of eurycomanone on cell viability. Treatment of eurycomanone on cancerous cells (CaOv, HeLa, HepG2, HM3KO and MCF7) significantly reduced the number of viable cells. It was relatively nontoxic against non cancerous cells (MDBK, Vero). Results were represented as means ± SEM of at least 3 independent experiments.

potency of eurycomanone as antiproiferative agent since the IC_{50} is less than 20 μg/mL (Wall *et al.*, 1987). Eurycomanone also showed the ability to act as a cytoselective anticancer agent since eurycomanone was relatively nontoxic towards non-cancerous MDBK and Vero cells. Previous research also showed the minimum effect of eurycomanone on non cancerous breast cells (MCF-10A) (Cheah and Azimahtol, 2004). The cytoselective properties of eurycomanone led to expectation that eurycomanone was potential to develop as anticancer agent. Since, the important problem in cancer treatment is non-selectivity of chemotherapy on normal cells. This problem limited the effect and responsible for many adverse effect of chemotherapy (Kerr *et al.*, 1994).

Morphological observation by inverted microscope

Under inverted microscope, cell shape and its changes can be observed clearly. As shown in Fig. 3a, cells in the control group showed regular polygonal and cell antennas were short and there were very few round cells. Cells treated with eurycomanone showed obvious morphological changes, including the loss of adhesion, rounding, and sporadical distribution (Fig. 3b-3c), and there was clear concentration-dependent response, indicating the possibility of apoptosis occurrence.

Morphological observation with Giemsa staining

Giemsa staining can detect apoptotic cells (Song and Zhang, 2006). Apoptotic cells and apoptotic bodies will stain dark blue as the chromatin condensed, while healthy cells will stain brightly (Oka *et al.*, 1997; Song and Zhang, 2006). Giemsa stain attaches itself to regions of DNA where there are high amounts of adenine-thymine bonding. As shown in Fig. (3e-3f), eurycomanone treated HeLa cells were stained dark blue, indicating the condensation of chromatin. The cells ize was also found to reduce. At igher consentration treatment, membrane blebbing was also observed.

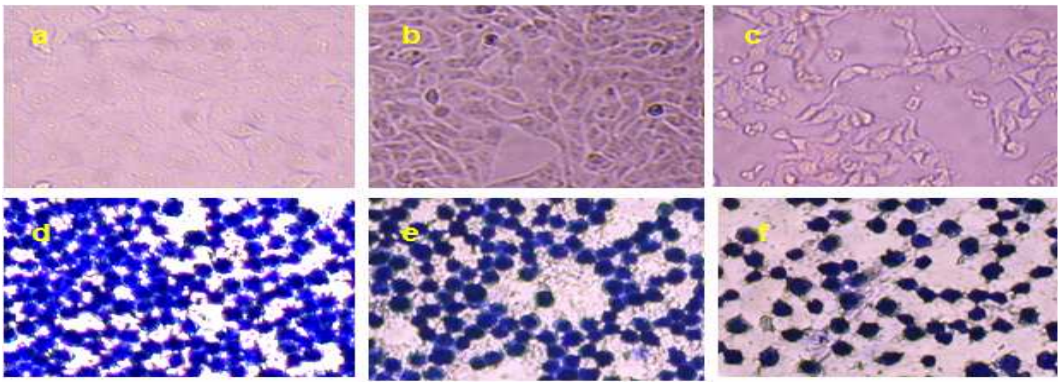

Figure 3. Morphological observation with light microscope on untreated HeLa cells (a), eurycomanone treated cells of 2 μg/mL (b) and 4 μg/mL (c); Giemsa staining of untreated HeLa cells (d), HeLa cells were treated with 2 μg/mL (e) and 4 μg/mL (f) eurycomanone.

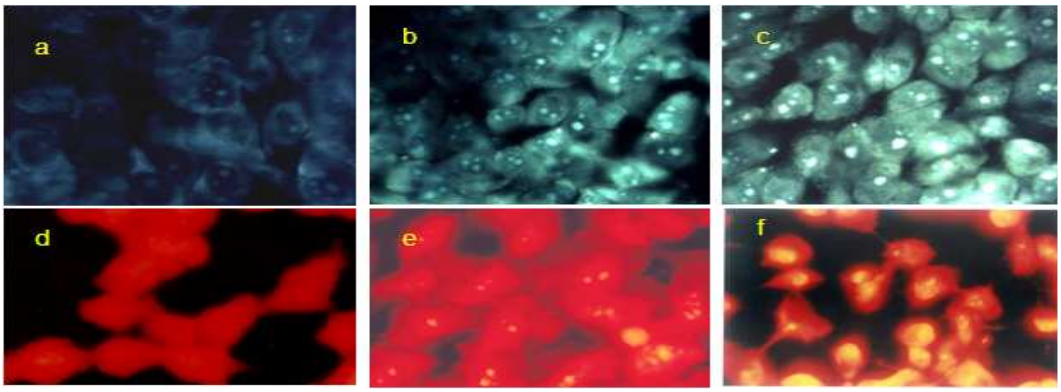

Figure 4. Nuclear staining with Hoechst 33258 on untreated cells (a), HeLa cells were treated with 2 μg/mL eurycomanone for 24 hrs (b) and 72 hrs (c); TUNEL assay of untreated HeLa cells (d), HeLa cells treated with 2 μg/mL eurycomanone for 24 hrs (e) and 72 hrs (f).

Nuclear morphology observation

Cells undergoing apoptosis display a profound destruction of the nucleus that results in the formation of nuclear blebs containing DNA. Staining of apoptotic cells with fluorescent DNA binding stain allows for easy detection of this phenomenon, because DNA-filled dye stain brightly, and are easily distinguished from intact nuclei (Dibartolomeis and Mone, 2003). The morphology of apoptotic cells including chromatin condensation and formation of apoptotic bodies could be determined after staining with Hoechst 33258. Hoechst 33258 bind at specific site of double stranded DNA and display a green fluorescence. However, Hoecsht 33258 stain both of live cells and apoptotic cells. Live cells could be easily distinguished from apoptotic cells, because live cells will stain homogenously while apoptotic cells will stain brightly (Brown, 2003).

Staining with Hoechst 33258 on eurycomanone treated HeLa cells for 24 hrs (Fig. 4b) showed intense fluorescence in the

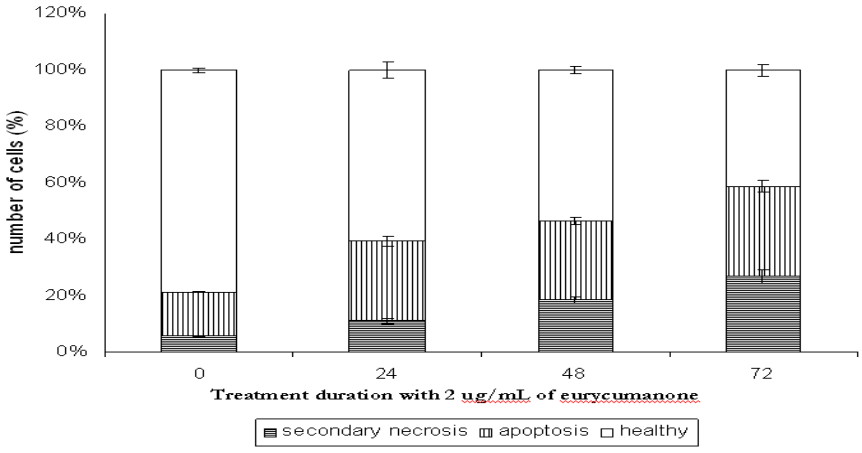

Figure 5. Flow cytometry analysis of HeLa cells. The increasing of the number of apoptotic cells were followed by the increasing of secondary necrotic cells number in eurycomanone treated HeLa cells, indicating the apoptotic cells with no phagocytic clear-up enter into secondary necrotic stage.

nucleus, thereby indicating chromatin condensation and fragmentation of the DNA in the nucleus. At 72 hrs after treatment, the florescence occurred more intensively and the formation of apoptotic bodies was detected (Fig. 4c), indicating the late stage of apoptosis.

DNA fragmentation, biochemical characteristic of apoptosis

The DNA fragmentation of apoptotic cells generates 3'-OH DNA ends, which can be labeled with fluorescein-12-dUTP using the enzyme Terminal Deoxynucleotidyl Tranferase (TdT). The fluorescein-12-dUTP-labeled DNA could be visualized by fluorescence microscope. DNA fragmentation is the primary biochemical characteristics (Allen *et al.*, 1997) which indicate an early event of apoptosis and it represents a point of no return from the path to cell death. This due to no more new cellular protein will be synthesized for cell survival.

HeLa cells were treated with eurycomanone for 24 hrs (Fig. 4e) showed active apoptosis and the fragmented DNA were labeled with fluorescence 12 dUTP in the nuclei. At 72 hrs after treatment more intensive fluorescence were detected, thereby indicating more DNA fragmentation of treated cells. The formation of apoptotic bodies in these cells indicated the late stage of apoptosis (Fig. 4f). In HeLa cells treated with DMSO as negative control, no fluorescence was detected in the nuclei, due to the absence of fragmented DNA (Fig. 4d).

Flow cytometry analysis

In many cell types, the induction of apoptosis is associated with plasma membrane changes where PS is translocated from the inner layer of plasma membrane to the outer leaflet, a process which required for phagocytosis by macrophages (Savill and Fadok, 2000). The externalization of PS can be assessed by measuring the binding of FITC-conjugated annexin-V to cells by flow cytometry. The combination between annexin-V and DNA-binding flouresence dye (PI) can distinguish the apoptotic and necrotic cells, as the apoptotic cells exclude PI. A typical cytogram is shown where cells stained negative for both annexin-V and PI (PS−/PI−) were live cells. Annexin-V-

positive and PI-negative (PS+/PI−) stained cells undergo early stages of apoptosis where the plasma membrane were still intact and excluded PI. In late stages of apoptosis, dying cells can no longer exclude PI and displayed both annexinV-positive and PI-positive (PS+/PI+). PI-positive and annexinV-negative (PS−/PI+) stained cells were necrotic cells. Our data confirms that eurycomanone was capable of inducing cell death in HeLa cells (Fig. 5).

Flow cytometric analysis of eurycomanone treated cells showed externalization of PS as assessed by annexin-V binding. PS is normally restricted to the inner-membrane leaflet, however dying cells expose these phospholipids as it is one of the key signals for phagocyte recognition (Fadok and Henson 2003). The concentration of eurycomanone used for treating the cells was 2 μg/mL (IC_{50} values of eurycomanone detected for 3 days treatment were 2.13 ± 0.09 μg/mL). As shown in Fig. 5, the background level of apoptosis in HeLa cells were 15.51 ± 0.33 % and background level of necrosis were 5.636 ± 0.17 %. After exposure with eurycomanone the level of apoptotic cells was increased from 15.51 ± 0.33 % to 22.54 ± 2.19 %, 28.06 ± 0.89 % and 26.96 ± 0.49 % after exposure with eurycomanone for 24, 48 and 72 hrs. The cells in the secondary necrosis stage were also found to increase from 5.54 ± 0.19 % to 9.78 ± 1.02 % at 24hrs, 14.71 ± 0.85 % by 48hrs and 25.72 ± 2.77 % after 72hrs treatment. There is no significant difference of apoptotic cells after 72 hrs treatment compared to 48 hrs while the secondary necrotic cells were significantly increased. These result suggested that treatment of eurycomanone on HeLa cells induced apoptosis and the apoptotic cells with no phagocyte surrounding them in the in vitro study then enter to secondary necrotic stage.

Therefore, there were no more increasing of apoptotic level after 72 hrs since the apoptotic cells entered into secondary necrotic stage.

Conclusions

Eurycomanone is cytotoxic on HeLa cells by inducing apoptosis and less toxic on normal cells. This finding may suggest further investigation of eurycomanone as anticancer agent.

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