[PHAR05] Active fraction (F16) from *Eurycoma longifolia* Jack induces apoptotic-cell death of MCF-7 cells

Tee Thiam Tsui, Azimahtol Hawariah Lope Pihie

School of Biosciences & Biotechnology, Faculty of Science & Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

Introduction

Breast cancer is one of the most commonly diagnosed cancers in women. In a continuing collaborative search for naturally occurring medicinal agents, the roots of *Eurycoma longifolia* Jack were selected for investigation. *E. longifolia*, a plant in the family of Simaroubaceae, is a tall slender shrub-tree commonly found as an understory in the lowland forests at up to 500 m above sea level (Kuo *et al.*, 2004).

E. longifolia is known to be a promising natural source of biologically active compounds (Kuo et al., 2004). From the roots, several classes of compounds have been identified and they include quassinoids, canthin-6-one alkaloids, β-carboline alkaloids, squalene derivatives, and biphenylneolignans (Kardono *et al.*, 1991; Morita *et al.*, 1992, 1993; Kuo *et al.*, 2004). Some of these constituents were shown to possess cytotoxic, antitumour, antimalarial, anti-HIV, and plant growth inhibitory activities (Kardono *et al.*, 1991; Jiwajinda *et al.*, 2001; Kuo *et al.*, 2004).

To date, only a few studies have been carried out to verify the direct cytotoxic activity of the extracts from the root of *E. longifolia*. Therefore, the main aim of the present study is to investigate the direct antiproliferative effects of the methanolic extract and chromatographic fractions from the roots of *E. longifolia* on the growth of MCF-7 cells, using a bioassay-guided fractionation method.

Materials and Methods

Plant material

E. longifolia roots were provided by Prof. Dr. Azimahtol Hawariah Lope Pihie (Universiti Kebangsaan Malaysia).

Plant extraction

Approximately 300 g of the powdered root was extracted with methanol (5 X 500 ml) using a Soxhlet system under reflux for 48 hours. The methanol solution was evaporated under reduced pressure to give 4 g of methanol extract (F2). 2 g of methanolic extract was subjected to column chromatography over silica gel (400 g, 230-400 mesh), using chloroform and chloroform containing increasing amounts of methanol as solvents. This resulted in 11 fractions (F3 - F13).

On the basis of the data obtained from bioassays carried out with all the 11 fractions collected, three active fractions, viz, fraction F5, F6, and F7 were further analyzed using thin layer chromatography in $CHCl_3$:MeOH ratio of 80:20 and pure eurycomanone as standard, to identify the presence of eurycomanone in the fractions. Previous studies indicated that eurycomanone is one of the bioactive compound present in the root of *E. longifolia* and is a potent cytotoxic agent. From the thin layer chromatography results, fractions F5 and F6 contained eurycomanone, while eurycomanone was not detected in fraction F7. With the hope of finding new compound(s), F7 was further partitioned with hexane and chloroform, successively, to afford three individual portions, hexane layer (F14), chloroform layer (F15), and water layer (F16). All the extracts and their fractions were tested for antiproliferative activity.

Cell culture conditions

MCF-7 and MCF-10A cells were obtained from the American Type Culture Collection (ATCC). MCF-7 and MCF-10A cells were maintained in DMEM and MEGM, respectively. Both the cell lines were routinely grown as a monolayer culture in respective medium and supplemented with 5% fetal calf serum (FCS) and penicillin-streptomycin, in 125 cm^2 plastic flasks at 37°C in a humidified atmosphere with 5% CO_2 and passaged weekly. For the experiments, cells were removed from the flasks by using a 0.025% Trypsin solution.

Cell proliferation experiments

The antiproliferative effect was evaluated by obtaining the IC_{50} values for these cell lines, as previously described (Lin & Hwang 1991). Briefly, cells were trypsinized, counted manually by means of a hemocytometer while simultaneously determining cell viability by Trypan blue exclusion, and added to 96-well tissue culture plates (Falcon, NJ, USA) at a concentration of 5,000 cells per well in respected medium supplemented with 5% FCS. The cells were added to each well in a volume of 200 µl and the cells were allowed to attach for 24 hours at 37°C, and then the seeding medium was removed and replaced with fresh medium containing varying concentrations of each compound. The compounds added were first dissolved in DMSO at the required concentration and then added to the culture medium. Control wells received only DMSO. Each concentration of the compounds under study was assayed in triplicate. Cells were maintained for 3 days and the antiproliferative activity of each fraction was determined by the procedure using methylene blue staining. The absorbance at 660 nm was read on a spectrophotometric plate reader and the proportion of surviving cells was calculated by dividing the average of nontreated wells. All experimental data were derived from at least 6 independent experiments.

Quantitation of apoptosis

DNA fragmentation that is characteristic of apoptotic cells was quantified by Tdt-mediated dUTP nick end labeling (TUNEL) with the Apoptosis Detection Kit, Fluorescein (Promega) according to the manufacturer's instructions. To calculate the percentage of TUNEL positive cells, we counted all of the cells from four random microscopic fields at 100X and 400X magnifications.

Nuclear staining assay

Staining with Hoechst 33258 was performed, as described elsewhere (Hishikawa *et al.*, 1999). Briefly, the floating and trypsinized-adherent treated cells were collected and washed with PBS. The cells were then fixed with 4% paraformaldehyde for 30 min. After washing, the cells were incubated in Hoechst 33258 (Sigma) at a final concentration of 30 µg/ml at room temperature for 30 min. Nuclear morphology was then examined with a Zeiss fluorescent microscope.

Western Blotting

The equal amounts of protein (20 µg per lane) from both the treated and untreated cells were loaded and electrophoresed on a 12% or 15% SDS-polyacrylamide gels. After electrophoresis, the proteins were blotted onto polivynyl-difluoride membranes (PolyScreen, Nen Life Science). The blotted membranes were dried, preblocked with 5% non-fat milk in phosphate-buffered saline and 0.1% Tween-20, then incubated with a primary antibody for Bax, Bcl-2, caspase-7 or PARP (Pharmingen) diluted 1: 2000, and detected with secondary antibody conjugated to horseradish peroxidase (1: 30 000). The proteins were detected by enhanced chemiluminescence system (ECL; Amersham) and exposed to X-ray film. Densitometry analysis was performed with a GS 670 Imaging Densitometer with the Molecular Analyst software (Bio Rad, Hercules, USA). The membranes were reprobed with β-actin (Sigma) antibodies to ensure equal loading.

Statistical analysis

All values are expressed as the mean ± S.D. Statistical analyses were evaluated by Student's *t*-test. Probability values $P < 0.05$ were considered statistically significant.

Results

Antiproliferative activitiy

Table 1 shows the antiproliferative activity of methanolic (F2) extracts from *E. longifolia* and their fractions (F3-F13). The methanolic extract has an IC_{50} value of (7.80±0.45) µg/ml. Within the eleven fractions (F3 - F13) obtained by silica gel permeation chromatography of the methanolic extract, the three main active fractions (F5, F6, and F7) gave IC_{50} values of (6.17±0.38) µg/ml, (4.40±0.42) µg/ml, and (20.00±0.08) µg/ml respectively. The rest of the fractions showed IC_{50} values of more than 30 µg/ml. Although fractions F5 and F6 displayed IC_{50} values less than F7, F7 was chosen for further purification. This was due to the fact that F5 and F6 contained eurycomanone, a potent cytotoxic agent found in the root of *E. longifolia*. Study of eurycomanone as a

cytotoxic agent has been well established; hence F5 and F6 need no further elucidation.

Partitioning of F7 resulted in F16 which gave an IC_{50} value of (15.23±0.66) µg/ml towards MCF-7 cells. In fact, F14 and F15, obtained by partitioning F7 with hexane and chloroform, respectively, showed very similar dose-effect curves, with IC_{50} values exceeding 99.99 µg/ml. In order to study the specificity of cell killing by F16, a normal breast cell line MCF-10A was also tested. F16 treatment displayed a certain extent of cytoselectivity towards MCF-10A whereby IC_{50} values were (66.31±0.47) µg/ml.

TABLE 1 Antiproliferative activity of methanolic extract and fractions from the roots of *Eurycoma longifolia*. Results are presented as means ± SD of 6 independent experiments.

Sample	IC_{50} (µg / ml)	
	MCF-7	**MCF-10A**
F2	7.80 ± 0.45	-
F3	47.35 ± 0.87	-
F4	34.00 ± 0.71	-
F5	6.17 ± 0.38	-
F6	4.40 ± 0.42	-
F7	20.00 ± 0.08	-
F8	46.84 ± 0.11	-
F9	> 99.99	-
F10	> 99.99	-
F11	> 99.99	-
F12	> 99.99	-
F13	> 99.99	-
F14	> 99.99	-
F15	> 99.99	-
F16	15.23 ± 0.66	66.31 ± 0.47

Determination of apoptosis

To determine the mechanism of cell killing by F16, we employed two types of assays for apoptosis. One of these assays is designed to measure DNA fragmentation, a hallmark of programmed cell death. We found that F16 induces a large increase in the percentage of cells with fragmented DNA; representative results are shown in Fig. 1. The fragmented DNA generates 3'-OH DNA ends, which can be labeled with fluorescein-12-dUTP using the enzyme Terminal Deoxynucleotidyl Transferase (TdT), which forms a polymeric tail using the principle of the TUNEL assay. Here, we labeled the treated cells to visualize the extent of DNA fragmentation in a time-course manner. The labeled DNA was then visualized directly by fluorescence microscopy (Figure 2); the percentage of apoptotic cells was quantitated from the mean of at least six independent experiments. Treatment with F16 resulted in a change from 0% (no treatment) to ~50% of apoptotic cells by 24 h, confirming that conventional apoptosis occurred.

Another property of cells dying by apoptosis is the ability to bind with a nuclear fluorochrome, Hoechst 33258. When stained with Hoechst 33258, the chromatin of the F16-treated MCF-7 cells can be seen as condensed into lumps, thus exhibiting the punctuated morphology typical of apoptotic cells (Figure 3), again confirming the mechanism of killing as apoptosis. In summary, MCF-7 cells exposed to F16 exhibit DNA fragmentation. These data confirmed that F16-mediated cell killing occurs via apoptosis.

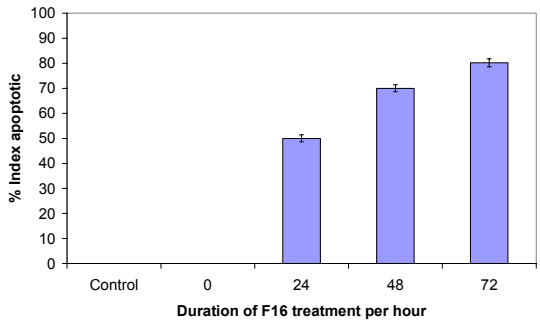

FIGURE 1 Apoptosis levels as determined by TUNEL assay. F16 treatment significantly increased the level of apoptosis in MCF-7 cells as compared to untreated controls. Augmented levels were observed till 72 h of F16 treatment as observed by Tdt-mediated dUTP nick end-labeling assay. Results are presented as means ± SD of 6 independent experiments.

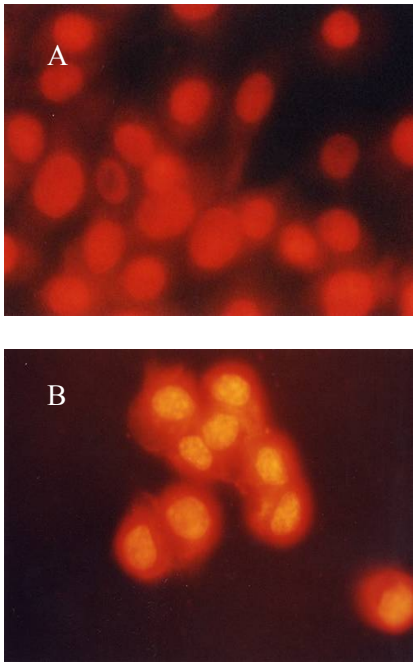

FIGURE 2 TUNEL labeling of MCF-7 cells. F16-treated and control cells were identified for DNA fragmentation by TUNEL assay as described in Materials and Methods. (A) Untreated cells. No fluorescence was detected in the nucleus, as the cells were not apoptotic and did not exhibit DNA fragmentation. (B) In cells treated with F16, fluorescence was detected in the nuclear region of the MCF-7 cells indicating DNA fragmentation and nuclear condensation, characteristic of apoptosis.

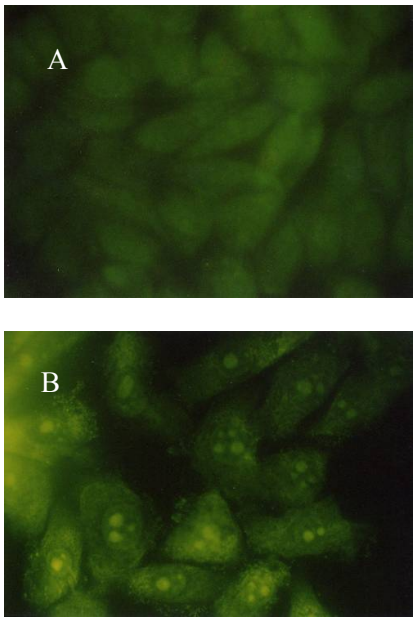

FIGURE 3 Nuclear staining of MCF-7 cells with Hoechst 33258. Cells were treated with F16 and compared to untreated controls. Nuclear morphology was observed by staining with the nuclear fluorochrome Hoechst 33258 as described in Materials and Methods. (A) Untreated control MCF-7 cells remained uniformly stained with round and unpunctured nucleus. (B) F16 treated MCF-7 cells showed apoptotic morphology; cell shrinkage, DNA condensation, and nuclear fragmentation.

Western blot analysis

Bcl-2 protein expression was down-regulated in F16-treated MCF-7 cells. An early event in the cell that sensitizes it to apoptosis is the desuppresion of the antiapoptotic protein Bcl-2. It is notable that western blot is a highly specific method for measuring down-regulation of proteins. Here, we detected a significant decrease in the Bcl-2 expression following treatment with western blotting (Figure 4), while the proapoptotic protein Bax levels were not altered and remained at the basal level throughout the treatment period (Figure 4). We found that F16 treatment decreased the Bcl-2 expression in a time-dependent manner, which was evident at 24 hours. Reduced cell viability and increased levels of apoptosis, together with a marked decrease in the level of antiapoptotic Bcl-2 protein, suggests a Bcl-2-dependent apoptotic pathway by the active fraction F16.

To confirm F16 induces apoptosis in MCF-7 cells, caspase-7 and PARP were investigated. From immunoblot analysis, untreated MCF-7 cells exhibited the ~35 kDa proform of caspase-7. When MCF-7 cells were treated with F16, the observed zymogen of caspase-7 slowly diminished in the course of the experiment (Figure 5) although the p17 kDa fragment was not detected. The disappearance of the procaspase-7 band reflects the processing of the zymogen to generate the active form of caspase-7, as has been interpreted in previous reports.

Measurement of PARP cleavage was used as a direct measure of apoptosis. Figure 5 clearly shows that MCF-7 cells that are induced by F16 undergo apoptosis through the disappearance of the uncleaved PARP (116 kDa), although its cleaved 85 kDa fragment was not detected. By assessing both caspase-7 and PARP cleavage, we concluded that F16 induces apoptosis in MCF-7 cells.

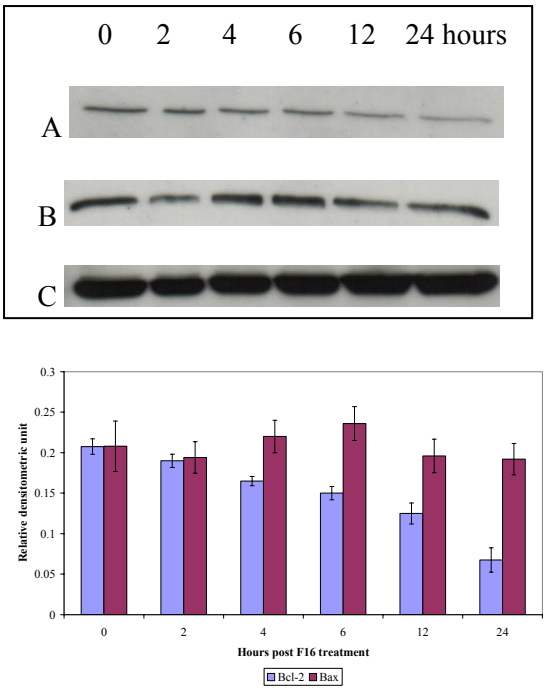

FIGURE 4 Western blot analysis of (A) Bcl-2, (B) Bax, and (C) β-actin protein in MCF-7 cells. MCF-7 cells treated with F16 for the indicated times were resolved on a 15% SDS-PAGE and submitted to western blotting. Bcl-2 protein expression decreased as early as 2 h following F16 treatment. Bax levels were not altered and remained at the basal level throughout the experiment. Results are presented as means ± SD of 6 independent experiments.

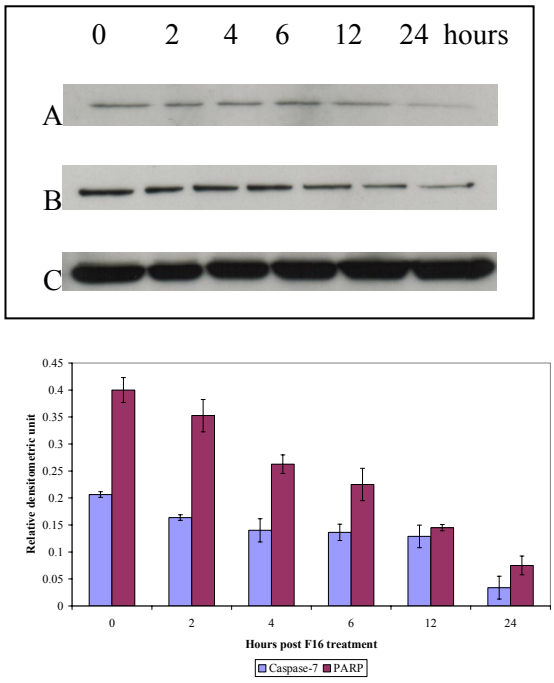

FIGURE 5 Western blot analysis of (A) Caspase-7, (B) PARP, and (C) β-actin protein in MCF-7 cells. MCF-7 cells treated with F16 for the indicated times were resolved on a 12% SDS-PAGE and submitted to western blotting. Caspase-7 and PARP protein expression decreased as early as 2 h following F16 treatment. Results are presented as means ± SD of 6 independent experiments.

Discussion

Our data indicated that, methanolic extract and fractions from *E. longifolia* exert a direct antiproliferative activity on the growth of MCF-7, the most studied human breast cancer cell line. In fact, the bioassay-guided fractionation of *E. longifolia* extracts from roots, resulted in the isolation of three active fractions (F5, F6, F7), which displayed IC_{50} values of (6.17±0.38) µg/ml, (4.40±0.42) µg/ml, and (20.00±0.08) µg/ml, respectively. Further purification of F7 resulted in F16, which displayed an IC_{50} value of (15.23±0.66) µg/ml. To our knowledge, this is the first time that the direct antiproliferative activity of this fraction towards MCF-7 cells has been experimentally verified.

There is an increasing realization that chemotherapeutic agents act primarily by inducing cancer cell death through the mechanisms of apoptosis. In the present study, we provide evidence that F16, an active fraction from *E. longifolia,* is able to induce apoptosis in the human breast cancer cell line, MCF-7. To understand the mechanism of F16 induced apoptosis in MCF-7 cells, Bcl-2 and Bax proteins were investigated.

From the results in this study, we observed that the Bax protein levels in the F16 treated MCF-7 cells were maintained at a basal level throughout the experiment. However, the Bcl-2 protein expression, decreased as early as 2 h following treatment with F16 and was maintained at a markedly low level than the controls throughout the experiment. Prior to treatment, the MCF-7 cells showed a high Bcl-2 protein expression. This is consistent with previous reports that Bcl-2 protein levels are elevated in a broad range of many human cancers including carcinomas of breast, prostate, ovary, colon, lung, and follicular B-cell lymphoma. However, when the Bcl-2 expression was down regulated in cells with transfected Bcl-2 antisense oligonucleotide, sensitivity towards apoptosis strongly increased (Yang *et al.*, 2004). Thus, the effectiveness of chemotherapy might depend on the level of Bcl-2 expression in the tumor cells.

Therefore, in the F16 treated MCF-7 cells, the decreased level of Bcl-2 expression may

play a positive role in increasing the susceptibility of these cells to undergo apoptosis. Treatment resulted in massive apoptotic-cell death, which may be explained by the low level of Bcl-2 protein in these cells, while Bax remains essentially unchanged. Bcl-2 is a dominant negative inhibitor of Bax, and the decreased expression of Bcl-2 sensitizes the MCF-7 cells to apoptose. Thus, when Bax expression level is conserved and the Bcl-2 expression level was low, homodimers of Bax will always be formed and apoptosis will be stimulated. Previous studies also found that the decreased expression of Bcl-2 sensitizes MCF-7 and non-breast-derived cells to undergo apoptosis.

To confirm F16 induced apoptotic cell death in MCF-7 cells, caspase-7 and PARP were investigated. Here we found that PARP was activated, following cleavage of caspase-7. In MCF-7 cells treated with F16, the 35 kDa proenzyme of caspase-7 was cleaved into its active 17 kDa subunit, by the reducing amount of the proenzyme with time duration. Synthesized as inactive precursors, caspases must be proteolytically cleaved to become active enzymes. PARP was revealed as the downstream target in the F16 mediated mechanism, linking caspase-7 activation and nuclear apoptosis. Cleaved fragment of PARP supports the finding that the processing of procaspase-7 resulted in its catalytic activation. PARP binds to DNA breaks and generates polymers of ADP-ribose bound to chromatin-associated proteins, including itself, thus making it accessible to DNA repair enzyme.

Here, our results provide evidence that the active fraction F16 of *E. longifolia* inhibited the proliferation of MCF-7 cells, a breast cancer cell line, by inducing apoptotic cell death. F16 has the ability to modulate Bcl-2 expression by decreasing the level of this proapoptotic protein. In conclusion, F16 induced apoptotic cell death in MCF-7 cells by down-regulating Bcl-2 and cleavage of caspase-7 and PARP.

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