

## Induction of Apoptosis by *Eurycoma longifolia* Jack Extracts

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**Abstract.** Extracts of the plant *Eurycoma longifolia* have been shown to possess cytotoxic, antimalarial, anti-ulcer, antipyretic and plant growth inhibition activities. The present study investigated the effects of extracts and their chromatographic fractions from the root of *E. longifolia* on the growth of a human breast cancer cell line, MCF-7. Our data indicated that *E. longifolia* extracts and fractions exert a direct antiproliferative activity on MCF-7. The bioassay-guided root fractionation resulted in the isolation of three active fractions, F5, F6 and F7, which displayed  $IC_{50}$  values of  $(6.17 \pm 0.38)$   $\mu\text{g/ml}$ ,  $(4.40 \pm 0.42)$   $\mu\text{g/ml}$  and  $(20.00 \pm 0.08)$   $\mu\text{g/ml}$ , respectively. The resultant from F7 purification, F16, exhibited a higher cytotoxic activity towards MCF-7, ( $IC_{50} = 15.23 \pm 0.66$   $\mu\text{g/ml}$ ) and a certain degree of selectivity against a normal breast cell line, MCF-10A ( $IC_{50} = 66.31 \pm 0.47$   $\mu\text{g/ml}$ ). F16 significantly increased apoptosis in MCF-7 cells, as evaluated by the Tdt-mediated dUTP nick end labelling assay and nuclear morphology. Western blotting revealed down-regulation of the anti-apoptotic Bcl-2 protein expression. F16, however, did not affect the expression of the pro-apoptotic protein, Bax. These results, therefore, suggest that F16 has antiproliferative effects on MCF-7 cells by inducing apoptosis through the modulation of Bcl-2 protein levels.

In a continuing collaborative search for naturally occurring medicinal agents, the roots of *Eurycoma longifolia* Jack were selected for investigation. *E. longifolia* is one of the popular folk medicines of South East Asia including Myanmar, Indochina, Thailand, Laos, Cambodia and Malaysia (1-4). *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 (2). *E.*

*longifolia* is identified locally as 'Tongkat Ali' in Malaysia, 'Pasakbumi' or 'Bidara Pahit' in Indonesia and 'Ian-don' in Thailand (2, 4, 5). In Vietnam, *E. longifolia* is named 'Cay ba binh', translated as 'a tree which cures hundreds of diseases' (6).

The roots of this plant are much sought after in herbal remedies and have been frequently prescribed either as a single ingredient or as a mixture with other herbs (7, 8) for the treatment of aches, persistent fever, tertian malaria, sexual insufficiency, dysentery, glandular swelling, antipyretic, complications after childbirth (2-4, 9, 10), or as health supplements (1, 8). *E. longifolia* is known to be a promising natural source of biologically-active compounds (2). From the roots, several classes of compounds have been identified and they include quassinoids (2, 4-6, 8, 11-21), canthin-6-one alkaloids (2, 5, 7, 11, 22, 23),  $\beta$ -carboline alkaloids (2, 5), squalene derivatives (18) and biphenylneolignans (24). Some of these constituents were shown to possess cytotoxic (2, 5, 11, 18, 19), antitumour (5, 12, 19), antimalarial (2, 5, 11, 12, 17), antischistosomal (12), anxiolytic (25) and plant growth inhibition activities (15).

The wide spectrum of pharmacological activities associated with the constituents and crude extracts of the title plant prompted us to undertake the biological investigation of the methanolic root extracts and their chromatographic fractions. Cytotoxicity screening models provide important preliminary data to help select plant extracts with potential antineoplastic properties for future work (26).

Previous studies on the methanolic extract from the root of *E. longifolia* suggest this plant has antiproliferative activity towards a panel of cancer cell lines (*i.e.*, human HT-1080 fibrosarcoma, human cervix HeLa adenocarcinoma, human lung A549 adenocarcinoma, murine colon 26-L5 carcinoma, murine Lewis lung carcinoma LLC, murine B16-BL6 melanoma cells and P-388 murine lymphocytic leukemia) (5, 27). In this study, we investigated the effects of the title plant extracts and their chromatographic fractions rather than a single compound. A study has showed that a multi-composition of herbal medicines was more effective than single compounds; the effects of the agents were thought to be related to the synergism of its

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*Key Words:* Antiproliferative effect, apoptosis, Bcl-2, *Eurycoma longifolia*, MCF-7.

contents (28). The mechanism of action of the methanolic extract, however, remains to be clarified.

Cancer cells acquire resistance to apoptosis by over-expression of anti-apoptotic proteins (Bcl-2, IAPs and FLIP) and/or by the down-regulation or mutation of pro-apoptotic proteins (Bax, Apaf-1, caspase 8 and death receptors) (29). Apoptosis is the ability of a cell to self-destruct by the activation of an intrinsic cellular suicide program when the cells are no longer needed or when they are seriously damaged (30). Apoptosis is an active form of physiological cell death, which is essential for development and for cellular and tissue homeostasis (29, 31-34). In cancer therapy, one approach that suppresses the tumor growth is by activating the apoptotic machinery in the cell (35-37). Moreover, the apoptotic process includes a mechanism that organizes both the packaging and disposal of dead cells, thereby preventing inflammation of the surrounding tissue (33). Evidence obtained during recent years is beginning to establish that a large majority of cancer chemotherapy agents affect tumor cell killing *in vivo* and *in vitro* through launching the apoptosis cascade (38). The apoptotic cell is characterized by loss of cell volume, plasma-membrane blebbing, nuclear condensation, chromatin aggregation and endonucleolytic degradation of DNA into nucleosomal fragments (31, 37, 39).

Bcl-2 and Bax are members of the Bcl-2 family of proteins that has been associated with apoptotic cell death both *in vitro* and *in vivo* (40). Bcl-2 is predominantly located in the outer mitochondrial membrane, and is found in the membranes of the endoplasmic reticulum and the nucleus (41). Bcl-2 molecules resemble bacterial toxins in their ability to control the influx and efflux of ions and small molecules as gatekeepers (42). Apoptosis is controlled by the ratio of various Bcl-2 family members (39). The switching 'on' and 'off' of apoptosis is determined by the ratio of anti-apoptotic and pro-apoptotic proteins; the Bcl-2/Bax ratio dictates the fate of the cell, *i.e.* the lower the ratio, the higher the probability of cell death (34, 43).

The main aim of the present study was to investigate the direct antiproliferative effects of some extracts and their chromatographic fractions from the roots of *E. longifolia* on the growth of MCF-7 cells, using a bioassay-guided fractionation method. We found that the methanolic extract has a higher antiproliferative activity towards MCF-7 cells compared to the aqueous extract from the roots of *E. longifolia*. Also, three active chromatographic fractions were found, *viz.* F5, F6 and F7. F7 was further purified to obtain F16. We found that F16 induced a higher percentage of MCF-7 cells to undergo apoptosis. In these cells, we observed that F16 was able to modulate the Bcl-2 expression by decreasing the level of this anti-apoptotic protein while having little effect on the expression of the pro-apoptotic Bax. The F16 showed a certain extent of cytoselectivity by sparing normal breast cells (MCF-10A).

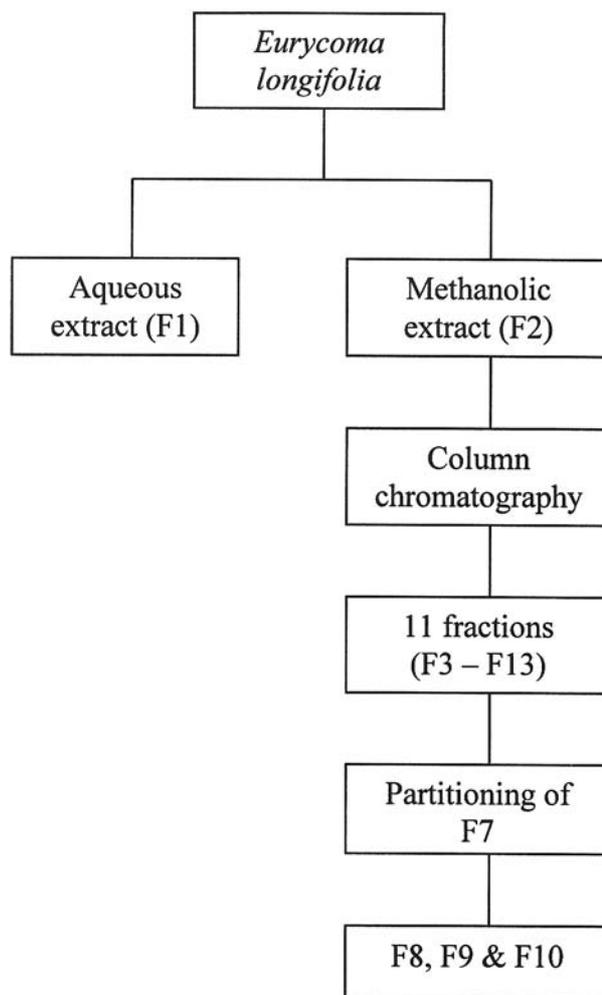


Figure 1. Extraction procedure for *E. longifolia* material. For further details see Materials and Methods section.

## Materials and Methods

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

**Plant extraction.** Figure 1 shows the extraction procedures for the root of *E. longifolia*. Three hundred g of air-dried and powdered root were extracted with deionized water for 30 minutes, to give 12 g lyophilized water extract (F1). Approximately 300 g of the starting powder was also extracted with methanol (5 X 500 ml) using a Soxhlet system under reflux for 48 hours to ascertain if a different extraction method could improve the quantity of the active principles. The methanol solution was evaporated under reduced pressure to give 4 g of methanol extract (F2). Two 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. fractions F5, F6 and F7, were further analyzed using thin layer chromatography in a  $\text{CHCl}_3$ :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 compounds present in the root of *E. longifolia* and is a potent cytotoxic agent towards a panel of cell lines including human epidermoid carcinoma of the nasopharynx KB, Vincristine-resistant KB, fibrosarcoma, melanoma, colon cancer, human lung cancer A-549 and MCF-7, with  $\text{IC}_{50}$  values of 0.40  $\mu\text{g/ml}$ , 0.8  $\mu\text{g/ml}$ , 0.2  $\mu\text{g/ml}$ , 8.2  $\mu\text{g/ml}$ , 1.2  $\mu\text{g/ml}$ , 8.1  $\mu\text{g/ml}$  and 1.1  $\mu\text{g/ml}$ , respectively (2, 5, 11, 19). Eurycomanone, when given orally, was found to be toxic to mice at  $\text{LD}_{50}$  value of 0.05 g/kg or 122.5  $\mu\text{mol/kg}$ , while the brine shrimp toxicity assay showed an  $\text{LD}_{50}$  of 3.5  $\mu\text{g/ml}$  (14). 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; a 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 the respective medium, supplemented with 5% fetal calf serum (FCS) and penicillin-streptomycin, in 125- $\text{cm}^2$  plastic flasks at 37°C in a humidified atmosphere with 5%  $\text{CO}_2$  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  $\text{IC}_{50}$  values for these cell lines, as previously described (44, 45). 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 density of 5,000 cells per well in the respective medium supplemented with 5% FCS. The cells were added to each well in a volume of 200  $\mu\text{l}$  and 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. The final DMSO concentration was 1% (v/v) or less. This concentration of DMSO does not affect cell viability (46). Each concentration of the compounds under study was assayed in triplicate. The cells were maintained for 3 days and the antiproliferative activity of each compound was determined by the procedure using Methylene blue staining. On the third day, glutaraldehyde was added to each well to a final concentration of 2.5% (v/v) and the surviving cells were fixed for 15 minutes. After washing with 0.15 M sodium chloride and removing the dead cells, the fixed cells were stained with 0.1 ml 0.05% (w/v) Methylene blue solution for 15 minutes. After washing off the excess dye with 0.15 M sodium chloride solution, dye elution was carried out with 0.33 M hydrochloric acid

Table I. Antiproliferative activity of aqueous extract, methanolic extract and fractions from the roots of *Eurycoma longifolia*. Results are presented as means  $\pm$  SD of 6 independent experiments.

Sample	$\text{IC}_{50}$ ( $\mu\text{g/ml}$ )	
	MCF-7	MCF-10A
F1	>99.99	-
F2	7.80 $\pm$ 0.45	-
F3	47.35 $\pm$ 0.87	-
F4	34.00 $\pm$ 0.71	-
F5	6.17 $\pm$ 0.38	-
F6	4.40 $\pm$ 0.42	-
F7	20.00 $\pm$ 0.08	-
F8	46.84 $\pm$ 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 $\pm$ 0.66	66.31 $\pm$ 0.47

(0.2 ml/well) and absorbance read at 660 nm. 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 non treated 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 labelling (TUNEL) with the Apoptosis Detection Kit, Fluorescein (Promega, USA), 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 (47). Briefly, the floating and trypsinized-adherent treated cells were collected and washed with PBS. The cells were then fixed with 4% paraformaldehyde for 30 minutes. After washing, the cells were incubated in Hoechst 33258 (Sigma Chemical Co., St. Louis, MO, USA) at a final concentration of 30  $\mu\text{g/ml}$  at room temperature for 30 minutes. Nuclear morphology was then examined with a Zeiss fluorescent microscope.

**Western blotting.** Equal amounts of protein (20  $\mu\text{g}$  per lane) from both the treated and untreated cells were loaded and electrophoresed on 12% SDS-polyacrylamide gels. After electrophoresis, the proteins were blotted onto polyvinyl-difluoride membranes (PolyScreen, NEN Life Sciences, USA). 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 or Bcl-2 (Pharmingen, USA) 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, USA) and

exposed to X-ray film. Densitometry analysis was performed with a GS 670 Imaging Densitometer with the software Molecular Analyst (Bio Rad, Hercules, USA). The membranes were reprobed with  $\beta$ -actin (Sigma Chemical Co., St. Louis, MO, USA) antibodies to ensure equal loading.

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

## Results

Table I shows the antiproliferative activity of aqueous (F1) and methanolic (F2) extracts from *E. longifolia* and their fractions. The methanolic extract was more effective than the aqueous extract with  $IC_{50}$  values of  $(7.80 \pm 0.45)$   $\mu\text{g/ml}$  and  $>99.99$   $\mu\text{g/ml}$ , respectively. 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  $(6.17 \pm 0.38)$   $\mu\text{g/ml}$ ,  $(4.40 \pm 0.42)$   $\mu\text{g/ml}$  and  $(20.00 \pm 0.08)$   $\mu\text{g/ml}$ , respectively. The rest of the fractions showed  $IC_{50}$  values of more than 30  $\mu\text{g/ml}$ . 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*, which has been well studied; hence, F5 and F6 needed no further elucidation.

Partitioning of F7 resulted in F16, which gave an  $IC_{50}$  value of  $(15.23 \pm 0.66)$   $\mu\text{g/ml}$  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  $\mu\text{g/ml}$ . 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, with  $IC_{50}$  value of  $(66.31 \pm 0.47)$   $\mu\text{g/ml}$ .

Cancer cells exposed to radiation and chemotherapy typically die through a process of programmed cell death or apoptosis (48). 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 induced a large increase in the percentage of cells with fragmented DNA; representative results are shown in Figure 2. The fragmented DNA generates 3'-OH DNA ends, which can be labelled 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 labelled the treated cells to visualize the extent of DNA fragmentation in a time-course manner. The labelled DNA was then visualized directly by fluorescence microscopy (Figure 3); the percentage of apoptotic cells

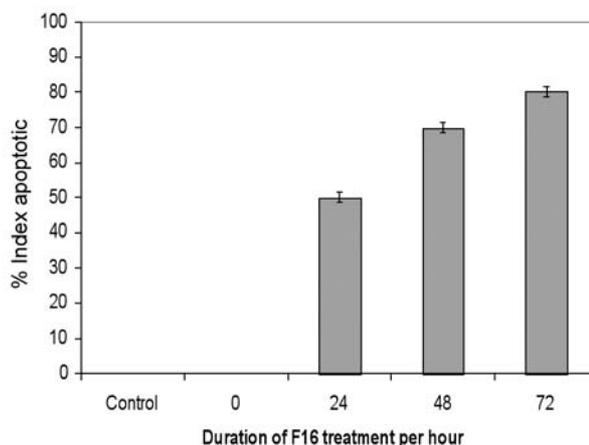


Figure 2. Apoptosis levels as determined by TUNEL assay. F16 treatment significantly increased the level of apoptosis in MCF-7 cells as compared to untreated controls. Augmented levels were observed for up to 72 hours of F16 treatment, as detected by Tdt-mediated dUTP nick end-labelling assay. Results are presented as means  $\pm$  SD of 6 independent experiments. \*\* $p < 0.005$  statistically significant values relative to time 0.

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 hours, 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 was seen as condensed into lumps, thus exhibiting the punctuated morphology typical of apoptotic cells (Figure 4), again confirming the mechanism of killing as apoptosis. In summary, MCF-7 cells exposed to F16 exhibited DNA fragmentation. These data confirmed that F16-mediated cell killing occurs *via* apoptosis.

One of the key elements conferring resistance to apoptosis is the anti-apoptotic protein Bcl-2. 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 desuppression of the anti-apoptotic protein Bcl-2. In some models, Bcl-2 down-regulation alone can induce the commitment of a cell to apoptosis (30, 49, 50). It is notable that Western blot is a highly specific method for measuring down-regulation of proteins (51). Here, we detected a significant decrease in the Bcl-2 expression following treatment with Western blotting (Figure 5), while the proapoptotic protein Bax levels were not altered and remained at the basal level throughout the treatment period (Figure 6). 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 anti-

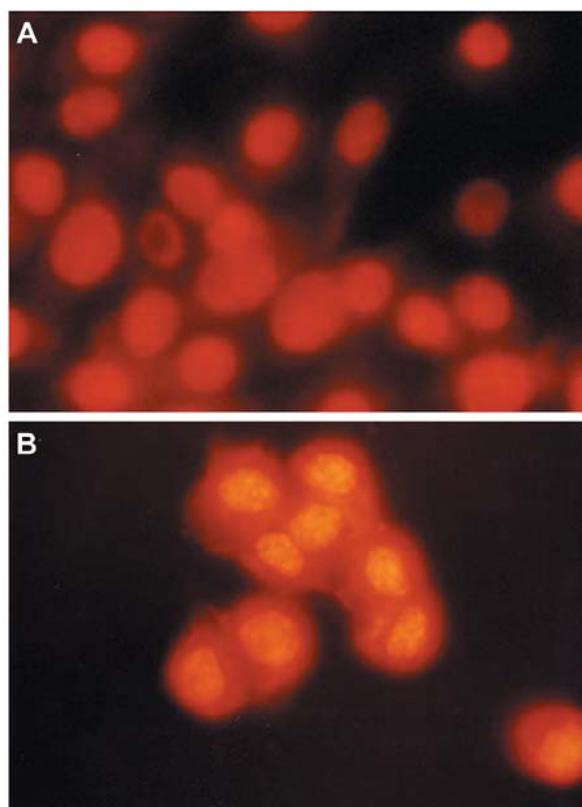


Figure 3. TUNEL labelling of MCF-7 cells. F16-treated and control cells were identified for DNA fragmentation by TUNEL assay, as described in Materials and Methods. (A) Untreated cells. No fluorescence was detected in the nucleus, as the cells were not apoptotic and did not exhibit DNA fragmentation. (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.

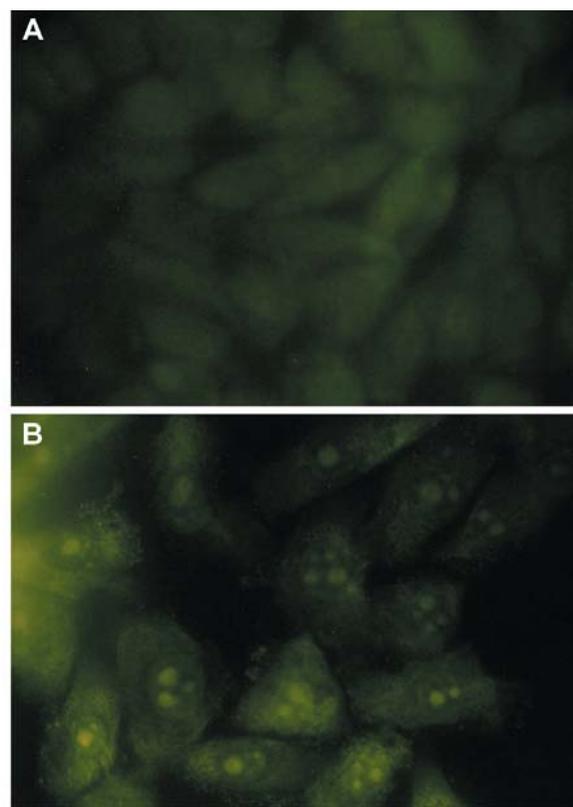


Figure 4. Nuclear staining of MCF-7 cells with Hoechst 33258. Cells were treated with F16 and compared to untreated controls. Nuclear morphology was observed by staining with the nuclear fluorochrome Hoechst 33258, as described in Materials and Methods. (A) Untreated control MCF-7 cells remained uniformly stained with round and unpunctured nucleus. (B) F16-treated MCF-7 cells showed apoptotic morphology; cell shrinkage, DNA condensation and nuclear fragmentation.

apoptotic Bcl-2 protein, suggests a Bcl-2-dependent apoptotic pathway by the active fraction F16. However, the mechanism whereby F16 down-regulates Bcl-2 expression is yet to be investigated.

## Discussion

Plants are a valuable source of new natural products. Despite the availability of different approaches for the discovery of therapeutics, natural products still remain one of the best reservoirs of new molecules (52). Natural plant products play an important role in chemotherapy, having contributed considerably to approximately 60 available cancer chemotherapeutic drugs (26). The need to develop more effective antitumor drugs has prompted investigators to explore new sources of pharmacologically-active compounds, especially from natural products (53).

Our data indicated that *E. longifolia* extracts and fractions 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  $(6.17 \pm 0.38) \mu\text{g/ml}$ ,  $(4.40 \pm 0.42) \mu\text{g/ml}$  and  $(20.00 \pm 0.08) \mu\text{g/ml}$ , respectively. Further purification of F7 resulted in F16, which displayed an  $IC_{50}$  value of  $(15.23 \pm 0.66) \mu\text{g/ml}$ . To our knowledge, this is the first time that the direct antiproliferative activity of this plant towards MCF-7 cells has been experimentally verified. In the light of the interesting data obtained, we will attempt to isolate the pure compounds responsible for such activity from F16, in order to study the possible mechanism(s) of action involved in the antiproliferative activity demonstrated in this study.

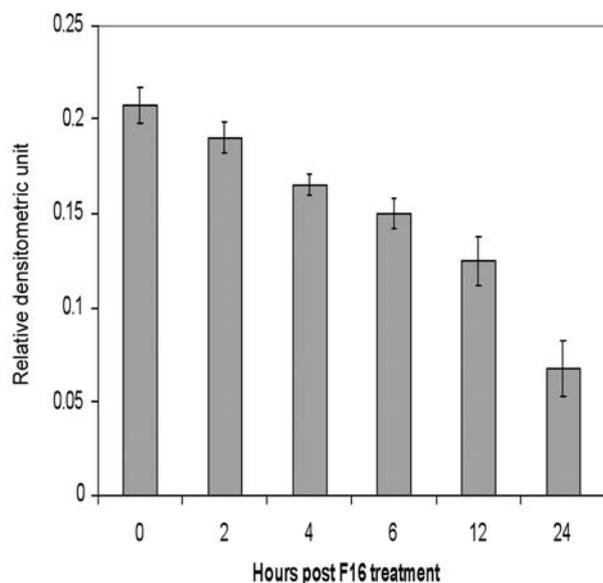
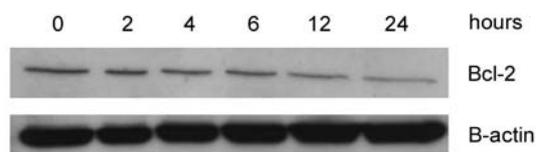


Figure 5. Western blot analysis of Bcl-2 protein in MCF-7 cells. MCF-7 cells treated with F16 for the indicated times were resolved on a 12% SDS-PAGE and submitted to Western blotting. Bcl-2 protein expression decreased as early as 2 hours following F16 treatment. Results are presented as means  $\pm$  SD of 6 independent experiments. \* $p < 0.05$ ; \*\* $p < 0.005$  statistically significant values relative to time 0.

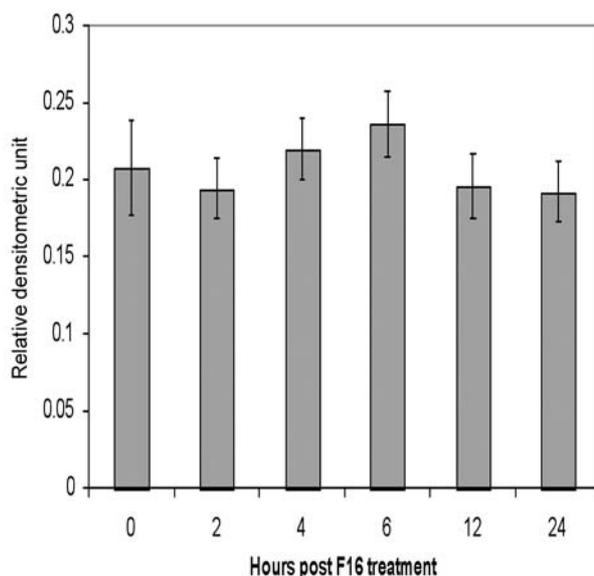
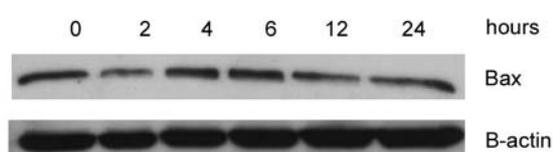


Figure 6. Western blot analysis of Bax protein in MCF-7 cells. MCF-7 cells treated with F16 for the indicated times were resolved on a 15% SDS-PAGE and submitted to Western blotting. Bax levels were not altered and remained at the basal level throughout the experiment. Results are presented as means  $\pm$  SD of 6 independent experiments.

Another purpose of the present study was to compare the antiproliferative effects of the aqueous extracts, traditionally used in folk medicine, and the methanolic extracts from the roots, in order to verify their biological activity. Our results indicate that the methanolic extracts show a higher antiproliferative activity than the corresponding aqueous extracts.

There is an increasing realization that chemotherapeutic agents act primarily by inducing cancer cell death through the mechanisms of apoptosis (36). However, many malignant cells develop defects in the regulation of genes that control apoptosis, rendering them resistant to the induction of apoptosis by a wide variety of stimuli, including chemotherapeutic drugs and radiotherapy, making it vital to develop novel drugs for combination chemotherapy (45, 54). 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.

Bcl-2 is the first of a rapidly expanding family of proteins, which are implicated in the response to chemotherapy (55). Indeed, a wide variety of human cancers, with a poor clinical response to chemotherapy, exhibit high levels of Bcl-2 expression (56). The *Bcl-2* proto-oncogene is involved in prolonging cell survival, by protecting cells against diverse cytotoxic insults, including  $\gamma$ - and UV-irradiation, cytokine withdrawal, dexamethasone, staurosporine and cytotoxic drugs. The over-expression of Bcl-2 can prevent or significantly reduce cell killing induced by a wide variety of physiological or chemical stimuli, while pro-apoptotic family members like Bax may act as tumor suppressors (43, 57). An important goal in chemotherapy is, therefore, to find new cytotoxic agents that are able to increase or restore the ability of tumor cells to undergo apoptosis through the activation of caspases and/or reduction of anti-apoptotic factors like Bcl-2.

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 hours following treatment with F16 and was maintained at a markedly lower level than the controls throughout the experiment. Before the 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 the breast, prostate, ovary, colon, lung and follicular B-cell lymphoma (58, 59). The Bcl-2 gene is over-expressed in 70% of cases of breast cancer and probably plays an important part in chemoresistance (51). However, when the Bcl-2 expression was down-regulated in cells with transfected Bcl-2 antisense oligonucleotide, sensitivity towards apoptosis strongly increased (51, 60). Thus, the effectiveness of chemotherapy might depend on the level of Bcl-2 expression in the tumor cells (33).

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 remained 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 the Bax expression level is conserved and the Bcl-2 expression level is low, homodimers of Bax will always be formed and apoptosis will be stimulated (34). Previous studies also found that the decreased expression of Bcl-2 sensitizes MCF-7 and non-breast-derived cells to undergo apoptosis (34, 50).

Interestingly, Bcl-2 expression correlated significantly with favorable tumor features such as DNA diploid status and expression of estrogen and progesterone receptors in stage I breast cancer (61). Estrogen stimulation is believed to be responsible for breast cancer promotion (62). Since anticancer agents can kill tumor cells *via* apoptosis, down-regulation of Bcl-2 protein expression may restore sensitivity to apoptotic stimuli in breast cancer cells. 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 pro-apoptotic protein. We will attempt to investigate other possible modes of action induced by F16. Investigations on the biological effects of these phytochemicals at the cellular level provides the molecular basis for their antiproliferative function and helps to establish the platform for generating more potent chemopreventive and even chemotherapeutic agents (63).

### Acknowledgements

This work was supported by a grant from the Massachusetts Institute of Technology (MAL-MIT 33-00-90173), USA. We thank Dr. N. Meenakshii for editing the manuscript.

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*Received November 3, 2004*

*Revised March 21, 2005*

*Accepted April 4, 2005*