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Inhibitory Effects of *Averrhoa bilimbi* Extract on Human Cervical Cancer Cells through Inducing G0/G1 Cell Cycle Arrest and p53-Mediated Mitochondrial Apoptosis Pathway

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Abstract

Decades of research have acknowledged that cancer is easier to prevent than to treat and that consumption of fruits and vegetables are linked to a reduce risk of cancer. One such promising approach is through cancer chemoprevention. In search for naturally occurring anticancer agents, a potential fruit extract was found to exert anticancer properties *in vitro* without any cytotoxic effect on normal cells. This study investigated the growth inhibitory and antiproliferative effect as well as the underlying cell death pathway induced by bilimbi (*Averrhoa bilimbi*) ethanolic extract on human cervical cancer cell line, HeLa. Anticancer potential of bilimbi extract was conducted by investigating the *in vitro* growth inhibitory effect, DNA fragmentation, cell cycle progression and anti-proliferation assay. Release of caspases, cytochrome c and apoptotic proteins were demonstrated to determine the mechanism of cell death pathway. Findings revealed that bilimbi significantly inhibited growth of HeLa cells *in vitro*. Bilimbi cytotoxic activity is likely due to the induction of apoptosis mediated by cell cycle arrest at G0/G1 checkpoint. Further evidences proved the induction of cell death as a consequence of apoptosis rather than necrosis. Released of cytochrome c coupled with upregulation of caspase-3/7, caspase-9, p53 and Bax pro-apoptotic proteins accompanied by downregulation of Bcl-2 anti-apoptotic proteins implied that bilimbi induced p53-mediated mitochondrial apoptosis pathway in HeLa. Collectively, this study suggests bilimbi ethanolic extract as a potential anticancer agent capable of inducing growth inhibitory effect and apoptosis on cervical cancer cell line.

Keywords: *Averrhoa bilimbi; HeLa; Cervical Cancer; Apoptosis*

Introduction

Over 6 million people around the world die from cancer each year. Despite improvement in chemotherapy treatment, cancer-associated morbidity and mortality remain unacceptably high while cancer survival remains relatively flat over the past several decades [1,2]. Chemotherapeutic drug, which is often administered together with surgery or radiation therapy, kills not only cancer cells but also highly proliferating normal cells, thereby resulting in adverse effects to patients. Since 1980's, more than 60% of cancer-approved drugs developed are of natural products origin [3] and 25% are directly derived from plants [4]. Hence, research on natural products continues to grow in an effort to discover new chemopreventive agents capable of blocking, reversing or preventing the development of invasive cancers [1]. Moreover, these natural products are regarded as important sources that could produce potential chemotherapeutics agents [5] since medicinal plants constitute the main source of new pharmaceuticals and healthcare products [6].

Anticancer drugs kill tumour cells through the induction of apoptosis [7,8]. Apoptosis or programmed cell death is a cellular suicide program in which individual cells are destroyed while the integrity and architecture of surrounding tissue is preserved [9]. Apoptosis is known to be triggered by anticancer drugs through two major pathways, the mitochondria-dependent intrinsic pathway and the death receptor-associated extrinsic pathway [10,11]. Cell death in intrinsic pathway occurs through the release of cytochrome c, which interacts with Apaf-1 and activates caspase-9. Consequently, Caspase-9 activates caspase-3 that cleaves substrates such as poly(ADP-ribose) polymerase [12]. On the other hand, extrinsic pathway involves Fas and other members of the tumour necrosis factor receptor family that activates caspase-8. Caspase-8 in turn activates caspas-3 or cleaves Bid, which eventually triggers the mitochondrial pathway [13].

Several reports in different human cell lines, animal models and epidemiological studies suggest that high intake of food rich in natural antioxidants reduce the risk and pathogenesis of chronic diseases associated to oxidative stress such as cancer [14-17]. Others reported that extracts from natural products such as fruits and vegetables have positive effects against cancer compared with chemotherapy or hormonal treatment [18]. These properties are attributed to a diversity of elements, including phenolic compounds and flavonoids, which are promising candidate for cancer prevention [19]. Flavonoids have been reported to exhibit a wide range of biological activities including anticarcinogenic, anti-inflammatory and antiviral actions [20]. The flavonoids exert these effects as antioxidants, chelators of divalent cations and free radical scavengers and thus may be involved in preventing free radical mediated cytotoxicity and lipid peroxidation which are associated with cell aging and chronic diseases [21]. Hence, much attention has been focused on the protective effects of natural antioxidants present in fruits and vegetables due to its capability in reducing oxidative stress and its role in defending the body against free radicals damage.

This study was undertaken to investigate the cytotoxic and apoptotic activities of bilimbi ethanolic extracts on human cervical cancer cell line and to identify the possible mechanism of cell death pathway involved in the anticancer activity. Previous studies have shown that bilimbi and carambola are good sources of natural antioxidant [22].

Materials and Methods

Materials

Ethanol and dimethyl sulfoxide (DMSO) were from Fisher Scientific (NH, USA). RNase A, acridine orange (AO), propidium iodide (PI), paraformaldehyde, Triton X-100, agarose powder and ethidium bromide were from Sigma-Aldrich (MO, USA). All antibodies for flow cytometry detection of apoptosis proteins (p53; Cat. No. MCA1710F, Bax; Cat. No. MCA2738, Bcl-2; Cat. No. MCA1550 and secondary antibody; Cat. No. STAR70) were from AbD Serotec (Kidlington, UK). All reagents used for cell culture were from Gibco (NY, USA); RPMI-1640 media, phosphate buffer saline (PBS), fetal bovine serum (FBS), penicillin, streptomycin and trypsin-EDTA.

Sample Preparation

Fresh bilimbi was purchased from Pasar Tani FAMA, Serdang, Selangor, Malaysia. Following purchase, samples were cleaned and washed with excess pipe water and pointed-ends were removed. Edible portions (100g) of the fruits were cut into small pieces and freeze-dried for 3 days. Then, samples were ground and fine powders were obtained using a fine mesh sieve. These were stored at –20°C prior to extraction.

Extraction

Fine powders (2.5g) were transferred into a 50 ml volumetric flask and 80% ethanol was added up to the mark. Mixture was shaken using shaking incubator at 200 rpm at 65°C for 24 hours. The mixture was then filtered through a 0.45 µm PES membrane vacuum filter (Nalgene, NY, USA). Filtrates were evaporated and freeze-dried for 3 days. The dry residue was dissolved in DMSO to obtain stock solution of 1 mg/ml and made up with the culture medium so that the final concentration of the vehicle was not > 1% DMSO. These final extracts were filtered through a 0.45 µm nylon membrane syringe filter (Milipore Corp., MA, USA) before use.

Cell Cultures

HeLa (human cervical cancer cell line; ATCC CCL-2) and Chang Liver (human non-malignant cell line; ATCC CCL-13) were obtained from American Type Culture Collection (VA, USA). The cells were cultured in RPMI-1640 media supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin and incubated in 5% CO_2 incubator at 37°C humidified atmosphere in 75 cm^2 flasks. Confluent monolayer cells were detached using 0.25% (w/v) trypsin-EDTA.

MTS Assay

Cytotoxicity study was performed as described according to instructions provided by the manufacturer of CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, WI, USA). Cell number and viability were determined using a haemocytometer after staining with trypan blue. Briefly, cell suspension was seeded into 96-well plates for 24 hours. Culture medium was discarded and cells were treated with extract in various concentrations performed through serial twofold dilutions. Cells treated with doxorubicin and culture medium (1% DMSO) served as positive control and negative control, respectively. After 72 hours, 20 µl per well of MTS reagent was added and incubated for 3 hours. Absorbance of coloured formazan produced, which was directly proportional to the number of living cells was recorded on an ELISA plate reader (Bio Rad Laboratories, CA, USA) at 490 nm. The concentration which gave 50% inhibition of cell growth (IC50) was determined from a plotted dose-response curve.

Morphological Observation

HeLa and Chang Liver cells were seeded into 6-well plates for 24 hours. Cells treated with doxorubicin (IC50) and culture medium (1% DMSO) served as positive control and negative control, respectively. Cells treated with bilimbi extract (70 µg/ml) were compared with that of controls. After 72 hours, cells were washed with 1x PBS. Changes in confluency and morphology features of both treated and untreated groups were viewed under inverted microscope (Olympus, Tokyo, Japan) with 100x magnification. Morphological changes to size, shape and cell volume were observed.

Acridine Orange/Propidium Iodide (AO/PI) Double Staining

HeLa cells were seeded into 6-well plate and incubated for 24 hours. Culture medium was discarded and cells were treated with 70 µg/ml bilimbi extract for 72 hours. Cells treated with culture medium (1% DMSO) served as negative control. After incubation, detached and adherent cells were collected by combining the spent medium and trypsin-EDTA-treated cells. The cells were harvested by centrifugation and washed twice with PBS. After the supernatant was discarded, 10 µl of cells were mixed with 5 µl of 10 µg/ml AO and 5 µl of 10 µg/ml PI. The mixture was put on glass slide, covered by cover slip and visualized immediately under fluorescence microscope (Olympus, Tokyo, Japan) with 200x magnification. Images were analyzed with cell^F fluorescence imaging software.

DNA Ladder Assay

Qualitative determination of DNA fragmentation was performed by DNA ladder assay as described according to instructions provided by the manufacturer of Apoptotic DNA Ladder Kit (Roche, Mannheim, Germany). HeLa cells were seeded into 6-well plate and incubated for 24 hours. Culture medium was discarded and cells were treated with bilimbi extract in different concentrations (0, 35, 70, 105 µg/ml) for 72 hours. After incubation, detached and adherent cells were collected by combining the spent medium and trypsin-EDTA-treated cells. The cells were harvested by centrifugation and washed twice with cold PBS. Cells were lysed and added with isopropanol. DNA was eluted and mixed with loading buffer before being electrophoresed on a 1% agarose gel pre-stained with ethidium bromide. The fragmented inter-nucleosomal DNA was visualized using FluorChem 5500 Chemiluminescent (Alpha Innotech, CA, USA).

TUNEL Assay

Quantitative determination of DNA fragmentation was performed as described according to instructions provided by the manufacturer of In Situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). HeLa cells were seeded into 6-well plate and incubated

for 24 hours. Culture medium was discarded and cells were treated with bilimbi extract in different concentrations (0, 35, 70, 105 µg/ml) over different incubation periods (24, 48, 72 hours). After incubation, detached and adherent cells were collected by combining the spent medium and trypsin-EDTA-treated cells. The cells were harvested by centrifugation and washed twice with cold PBS. Cells were fixed and permeabilized prior to incubation with TUNEL reaction mixture. Percentage of FITC positively stained cells was determined on FACSCalibur flow cytometer (BD Biosciences, NJ, USA) equipped with 488 nm argon laser light source and 525 nm band pass filter and analyzed using CellQuest Pro software (BD Biosciences, NJ, USA). Cells incubated without Label (Fluorescein-dUTP) or Enzyme (Terminal transferase) solution and cells incubated with Label (Fluorescein-dUTP) solution only were used as control for autofluorescence of cells. Percentage of FITC-positive stained cells was determined from the single-parameter histogram.

Cell Cycle Analysis

HeLa cells were seeded into 6-well plate and incubated for 24 hours. Culture medium was discarded and cells were treated with bilimbi extract in different concentrations (0, 35, 70, 105 µg/ml) over different incubation periods (24, 48, 72 hours). After incubation, detached and adherent cells were collected by combining the spent medium and trypsin-EDTA-treated cells. The cells were harvested by centrifugation and washed twice with cold PBS. Cells were fixed and incubated with RNase A and PI in the dark. Cell cycle profile was determined by using FACSCaliber flow cytometer equipped with 488 nm argon laser light source and 630 nm band pass filter and analyzed using CellQuest Pro software. Percentage of cells in each phase of the cell cycle was determined from the single-parameter histogram.

5-Bromo-2'-deoxyuridine (BrdU) Incorporation Cell Proliferation Assay

Number of proliferating cells was quantified by BrdU incorporation assay as described according to instructions provided by the manufacturer of Cell Proliferation ELISA, BrdU Colorimetric (Roche, Mannheim, Germany). HeLa cells were seeded into 96-well plates and incubated for 24 hours. Culture medium was discarded and cells were treated with 100 µl of bilimbi extract in different concentrations (0, 35, 70, 105 µg/ml) over different incubation periods (24, 48, 72 hours). Cells were labeled and fixed before the addition of antibody conjugate solution. Substrate solution was added and incubated until colour development was sufficient enough for photometric detection. Absorbance of coloured product, which was directly proportional to the amount of DNA synthesis and thereby the number of proliferating cells, was recorded on an ELISA plate reader at 370 nm and presented as O.D value.

Annexin V/Propidium Iodide (PI) Staining

The mode of cell death was performed by Annexin V/PI analysis as described according to instructions provided by the manufacturer of FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, NJ, USA). HeLa cells were seeded into 6-well plate and incubated for 24 hours. Culture medium was discarded and cells were treated with bilimbi extract in different concentrations (0, 35, 70, 105 µg/ml) over different incubation periods (0, 24, 48, 72 hours). After incubation, detached and adherent cells were collected by combining the spent medium and trypsin-EDTA-treated cells. The cells were harvested by centrifugation and washed twice with cold PBS. Pellet was resuspended in binding buffer and stained with Annexin V-FITC and PI in the dark. Induction of cell death was measured using flow cytometer. The settings for flow cytometer acquisition and analysis were performed as previously described in TUNEL assay. Unstained cells, cells stained with either Annexin V or PI alone were used for optimization and negative control. Percentage of cells in each quadrant was determined from the two-parameter histograms.

Caspase–8 and –9 Assay

Quantitative determination of human caspase-8 and -9 was performed as described according to instructions provided by the manufacturer of Human Caspase-8 Platinum ELISA and Human Caspase-9 Platinum ELISA (Bender MedSystems, Vienna, Austria). Both were of the same protocol. HeLa cells were seeded into 6-well plate and incubated for 24 hours. Culture medium was discarded and cells were treated with bilimbi extract in different concentrations (0, 35, 70, 105 µg/ml) over different incubation periods (24, 48, 72 hours). After incubation, detached and adherent cells were collected by combining the spent medium and trypsin-EDTA-treated cells. The cells were

harvested by centrifugation and washed with cold PBS. Cells were lysed and diluted before the addition of detection antibody and anti-rabbit-IgG-HRP. TMB substrate solution was added prior to addition of stop solution. Absorbance of coloured product was recorded on an ELISA plate reader at 450 nm. The concentration of human caspase-8 and -9 present was determined from a plotted dose-response standard curve.

Caspase–3/7 Assay

Quantitative determination of human caspase-3/7 was performed as described according to instructions provided by the manufacturer of Caspase-Glo® 3/7 Assay (Promega, WI, USA). HeLa cells were seeded into white-walled 96-well plates and incubated 24. Culture medium was discarded and cells were treated with bilimbi extract in different concentrations (0, 35, 70, 105 µg/ml) over different incubation periods (24, 48, 72 hours). Caspase-Glo® 3/7 reagent (100 µl per well) was added and gently mixed on a plate shaker for 30 seconds prior to incubation at room temperature (25ºC) for 1 hour. Luminescence was measured in a plate-reading GloMaxTM 96 Microplate Luminometer (Promega, WI, USA). The concentration of human caspase-3/7 present was recorded as percentage relative to control.

Cytochrome C Assay

Quantitative determination of human cytochrome c was performed as described according to instructions provided by the manufacturer of Human Cytochrome c Platinum ELISA (Bender MedSystems, Vienna, Austria). HeLa cells were seeded into 6-well plate and incubated for 24 hours. Culture medium was discarded and cells were treated with bilimbi extract in different concentrations (0, 35, 70, 105 µg/ml) over different incubation periods (24, 48, 72 hours). After incubation, detached and adherent cells were collected by combining the spent medium and trypsin-EDTA-treated cells. The cells were harvested by centrifugation and washed with cold PBS. Cells were lysed and diluted before the addition of Biotin-conjugate and Streptavidin-HRP. TMB substrate solution was added prior to addition of stop solution. Absorbance of coloured product was recorded on an ELISA plate reader at 450 nm. The concentration of human cytochrome c present was determined from a plotted dose-response standard curve.

p53, Bax and Bcl-2 Proteins Expression

HeLa cells were seeded into 6-well plate and incubated for 24 hours. Culture medium was discarded and cells were treated with 70 µg/ml of bilimbi extract over different incubation periods (0, 24, 48, 72 hours). After incubation, detached and adherent cells were collected by combining the spent medium and trypsin-EDTA-treated cells. The cells were harvested by centrifugation and washed twice with cold PBS. Cells were fixed and permeabilized. For direct staining, FITC-conjugated mouse anti-human p53 antibody (1:10 dilution) was added and incubated in the dark prior to flow cytometer analysis. For indirect staining, mouse anti-human Bax antibody (1:100 dilution) or mouse anti-human Bcl-2 antibody (1:100 dilution) was added and incubated prior to incubation with FITC-conjugated goat polyclonal anti-mouse secondary antibody (1:200 dilution). Percentage of FITC positively stained cells was measured using flow cytometer. The settings for flow cytometer acquisition and analysis were performed as previously described in TUNEL assay. Cells incubated with secondary antibody alone served as negative control. Percentage of FITC-positive stained cells was determined from the overlaid two single-parameter histograms.

Statistical Analysis

Data are presented as mean ± standard deviation (S.D) from three independent experiments. Statistical analysis was done using SPSS for Windows version 17.0. One-way analysis of variance (ANOVA) with Tukey's test was used to test for differences between multiple groups which were considered significant at $p < 0.05$.

Results

Cytotoxic effects of bilimbi extract on HeLa cells

Results showed that bilimbi was able to induce cytotoxicity in HeLa cells with estimated IC50 values of 70 µg/ml. On the other hand, results showed that viability of non-malignant Chang Liver was still above 50% even though concentration was increased up to 200 µg/ml. Comparably, doxorubicin, which is a highly toxic cancer chemotherapy drug, was used as positive control to assess test validity. Results proved that doxorubicin was capable to induce cytotoxicity in HeLa and non-malignant Chang Liver. Figure 1 shows the dose-response curve of bilimbi growth inhibition effect on HeLa.

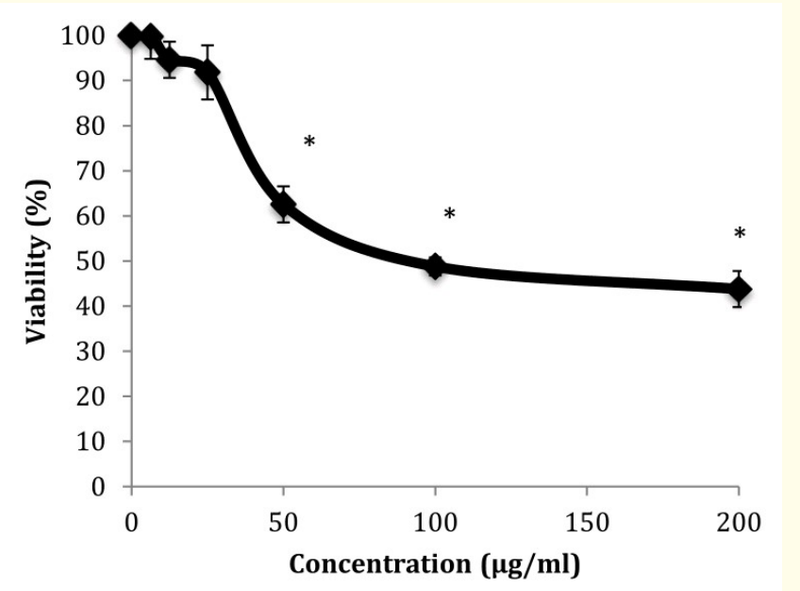

Figure 1: *Dose-response curve of bilimbi growth inhibition effects on HeLa after 72 hours treatment. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to negative control.*

Morphological changes in bilimbi-treated HeLa

Observations revealed several drug-induced morphological changes and typical morphological features of apoptosis in bilimbi-treated HeLa. The characterization of morphological changes observed was reduction in cell volume, which is one of the morphological hallmarks of apoptosis [23]. Apart from that, these cells appeared shrunken and detached as compared to untreated cells which demonstrated epithelial-like morphology. The untreated HeLa also showed high density of monolayer cells. Likewise, doxorubicin-treated HeLa showed typical morphological characteristic of apoptosis, comparable to bilimbi–treated cells (Figure 2A-2C). On the contrary, non-malignant Chang Liver showed little morphological changes after exposure to bilimbi for the same duration as in non-treated cell. Both appeared to have high confluency of monolayer cells with little difference in cell volume. In contrast, morphology of doxorubicin-treated Chang Liver appeared to possess similar characteristic features of apoptotic cells; being shrunken and reduced in cell volume (Figure 3A-3C).

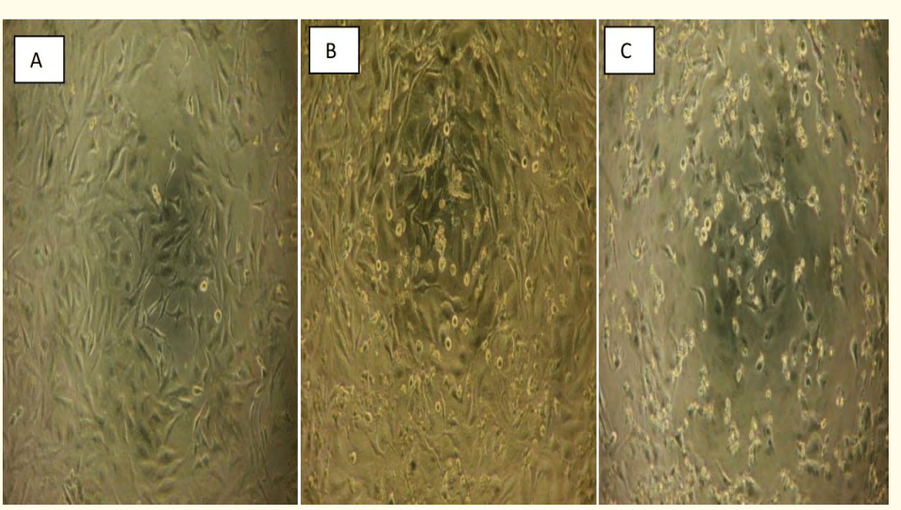

Figure 2: *Representative images to show morphological observation of HeLa with no treatment (A), with doxorubicin (B) and bilimbi (C) for 72 hours observed under inverted light microscopy (100x magnification). Similar cellular morphology was observed in three independent experiments (n = 3).*

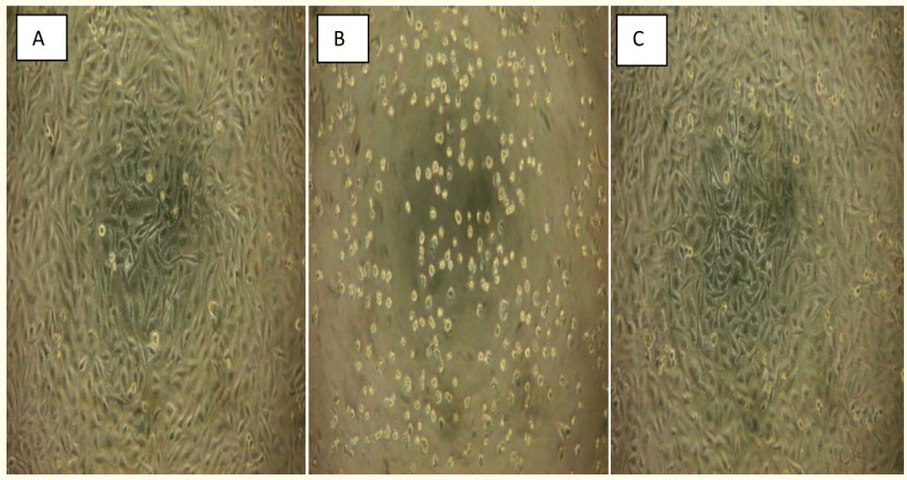

Figure 3: *Representative images to show morphological observation of Chang Liver with no treatment (A), with doxorubicin (B) and bilimbi (C) for 72 hours observed under inverted light microscopy (100x magnification). Similar cellular morphology was observed in three independent experiments (n = 3).*

Fluorescent-stained bilimbi-treated HeLa show characteristic of apoptosis

Based on IC50 values as determined by MTS assay, HeLa was treated with 70 µg/ml bilimbi for 72 hours and compared with that of untreated cells. Observations revealed that bilimbi induced apoptosis in HeLa after 72 hours incubation. Cells exhibited typical characteristics of apoptosis like chromatin condensation and membrane blebbing. As opposed to these early apoptotic cells, viable cells gave bright green fluorescence with intact nuclei. Cells treated with bilimbi also showed characteristic features of late apoptotic activity with the formation of apoptotic bodies, observed as red fluorescence (Figure 4A-4B).

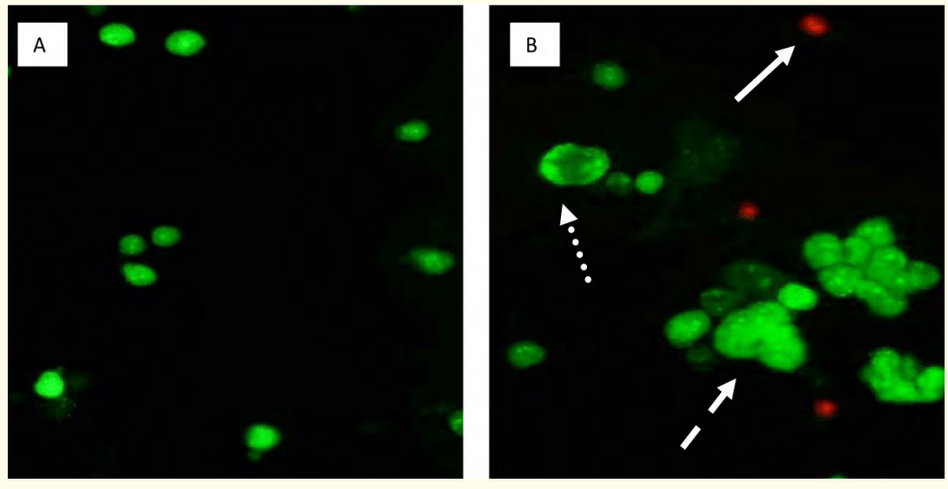

Figure 4: *Representative images to show fluorescent-stained HeLa with no treatment (A) and with bilimbi at 70 µg/ml (B) for 72 hours observed under fluorescence microscopy (200x magnification). Round dotted arrow indicates chromatin condensation; dash arrow indicates membrane blebbing and full arrow indicates apoptotic bodies. Similar cellular morphology was observed in three independent experiments (n = 3).*

DNA strands break in bilimbi-treated HeLa

DNA fragmentation is the characteristic feature of apoptosis [23]. To validate bilimbi-induced apoptosis in HeLa, chromosomal DNA from untreated cells and 72 hours bilimbi-treated HeLa of different concentrations were isolated, extracted and resolved using agarose gel electrophoresis. Images from the gel analyzed under chemiluminescent showed intact DNA band in untreated cells whereas bilimbi-treated DNA gel showed impaired DNA bands in the form of ladders (Figure 5). Similar gel pattern was observed for positive control with the presence of DNA laddering, indicating that bilimbi caused apoptosis in HeLa.

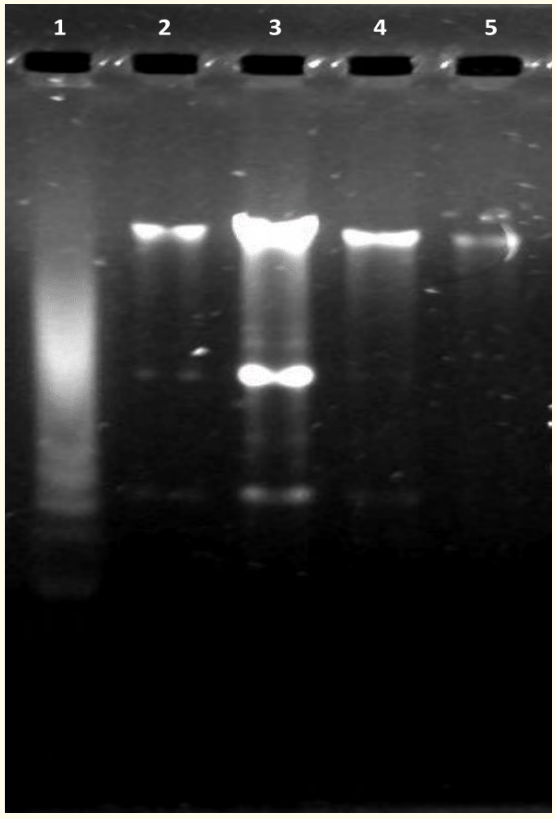

Figure 5: *Representative DNA gel in HeLa with no treatment and with bilimbi at 35, 70 and 105 µg/ml for 72 hours following agarose gel electrophoresis. Lane 1: DNA from positive control; Lane 2: DNA from 35 µg/ml bilimbi-treated HeLa; Lane 3: DNA from 70 µg/ml bilimbi-treated HeLa; Lane 4: DNA from 105 µg/ml bilimbi-treated HeLa; Lane 5: DNA from untreated HeLa. Similar gel pattern was observed in three independent experiments (n = 3).*

Effects of bilimbi extract on DNA fragmentation

Quantitative determination of DNA fragmentation was performed by TUNEL assay in which fluorescent nucleotides were enzymatically incorporated onto DNA fragments resulting from apoptosis [24]. When the percentage of HeLa stained with TUNEL was determined by flow cytometry, 7.46% of the cells showed positive staining in the untreated cells. After HeLa was treated with bilimbi for 24 hours,

the percentage of cells with positive staining increased to 11.59, 29.42 and 27.23% as concentrations increased to 35, 70 and 105 µg/ml, respectively. Positively-stained cells further increased with increasing concentrations over time as compared to untreated cells. After 48 hours treatment, the percentage of cells with positive staining increased significantly ($p < 0.05$) to 18.69, 59.11 and 36.87% as concentrations increased to 35, 70 and 105 µg/ml, respectively. At 72 hours, the percentages of cells with positive staining were 33.23, 49.26 and 39.4% at concentration of 35, 70 and 105 µg/ml, respectively (Figure 6). These results supported the observation on DNA gel electrophoresis on the presence of cleaved apoptotic nuclei visualized as ladders.

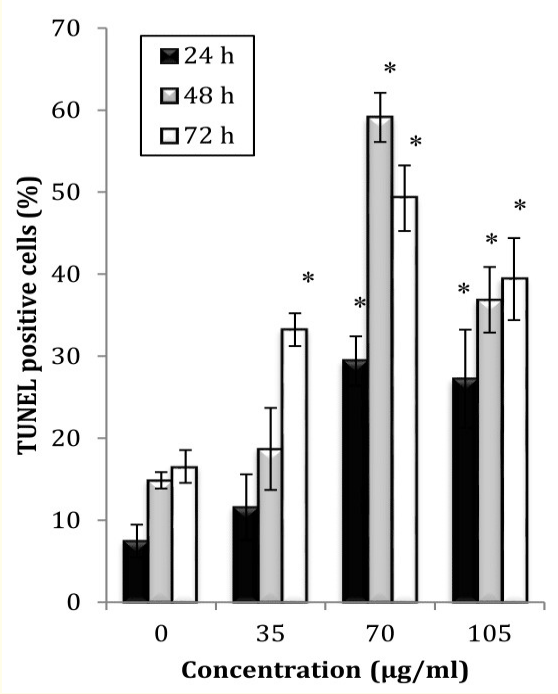

Figure 6: *Effect of bilimbi on DNA fragmentation in HeLa with no treatment and with bilimbi at 35, 70 and 105 µg/ml for 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to untreated cells.*

Effects of bilimbi extract on HeLa cell cycle progression

Flow cytometer analysis showed that compared to untreated cells, a significant ($p < 0.05$) increased of cell death was observed at 35 µg/ml after 48 hours treatment with bilimbi on HeLa. Increment was more pronounced at higher bilimbi concentrations (105 µg/ml) with cell death being recorded as early as 24 hours post-treatment ($p < 0.05$). The effect of bilimbi on the progression of HeLa through cell cycle is illustrated in Figure 7A-D. Exposure of cells to bilimbi at 70 µg/ml resulted in a relative increased (accumulation) in the number of cells with a 2N DNA content when compared to untreated cells, suggesting that bilimbi treatment inhibited the growth of HeLa through cell cycle arrest at G0/G1.

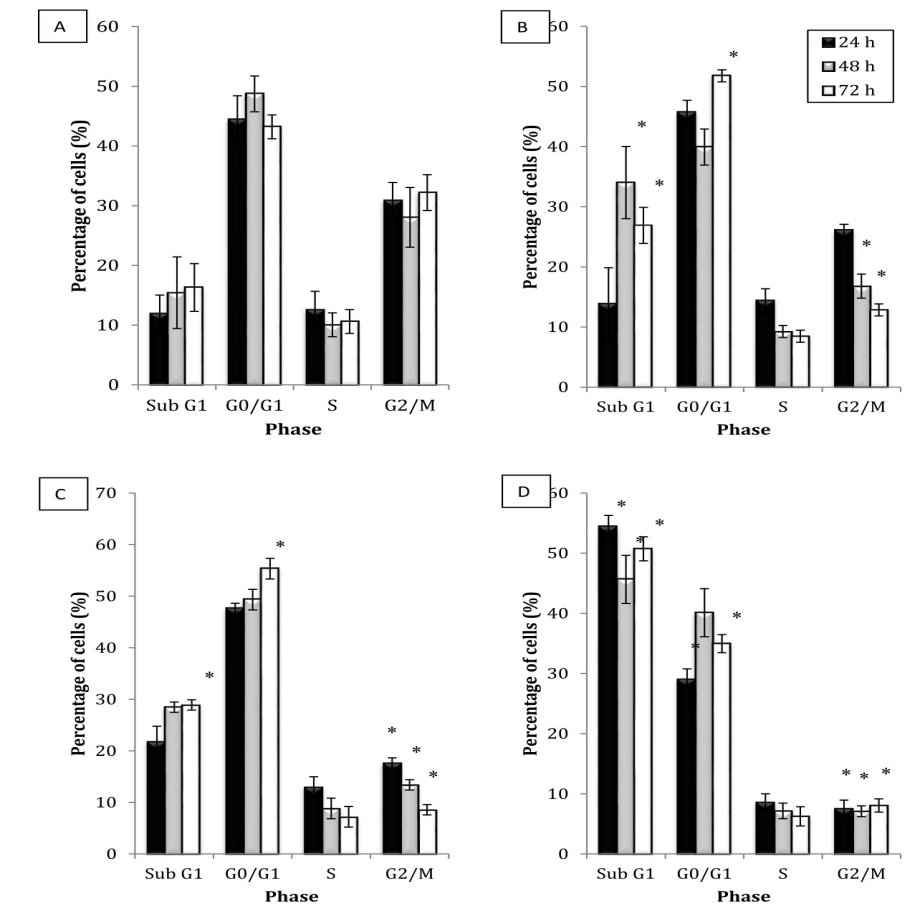

Figure 7: *The cell cycle profile of HeLa with no treatment (A) and with bilimbi at 35 µg/ml (B), 70 µg/ml (C) and 105 µg/ml (D) for 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to untreated cells.*

Effects of bilimbi extract on DNA synthesis in HeLa cells

Antiproliferative effect of bilimbi on HeLa is depicted in Figure 8. Over time, the numbers of proliferating cells decreased significantly ($p < 0.05$) as compared to negative cells. At 24 hours post-treatment, significant decreased of proliferating cells was obtained only at 105 µg/ml treatment. The same trend was observed at 48 hours post-treatment with the number of cells decreasing gradually as concentrations increased and was only significantly different with negative controls at 105 µg/ml. However, a significant decreased was observed 72 hours post-treatment as concentrations increased, indicating that growth inhibition effect of bilimbi on HeLa was due to inhibition of proliferation and cell cycle arrest at GO/G1 checkpoint.

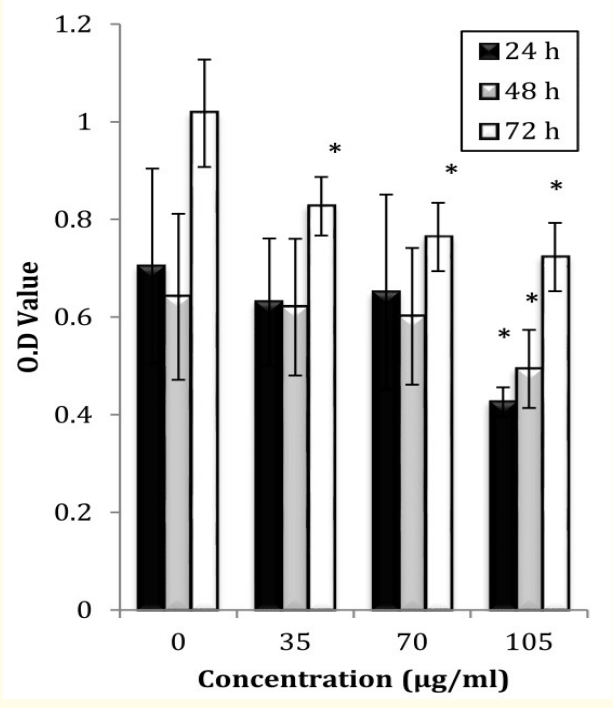

Figure 8: *Effect of bilimbi on cell proliferation of HeLa with no treatment and with bilimbi at 35, 70 and 105 µg/ml for 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to untreated cells.*

Bilimbi extract induces apoptotic death in HeLa cells

Data analysis revealed that compared to untreated cells, there were significant ($p < 0.05$) increased of cells entering into early apoptosis and late apoptosis stage after the treatment with bilimbi over time (Figure 9). After 24 hours, only 1.92% of untreated cells entered early apoptosis stage but 5.55% of 35 µg/ml, 2.9% of 70 µg/ml and 14.13% of 105 µg/ml of treated cells were in the early apoptosis stage. After 72 hours, the percentage of cells entering early apoptosis stage increased from 2.02% in untreated cells to 18.66%, 17.76% and 22.52% in 35, 70 and 105 µg/ml of treated cells, respectively. Besides, compared to 0.14% of untreated cells, 23.17%, 24.05% and 19.79% of 35, 70, and 105 µg/ml of treated cells entered into late apoptosis stage, respectively. Percentage of cells entering secondary necrosis was also significantly ($p < 0.05$) higher compared to untreated cells.

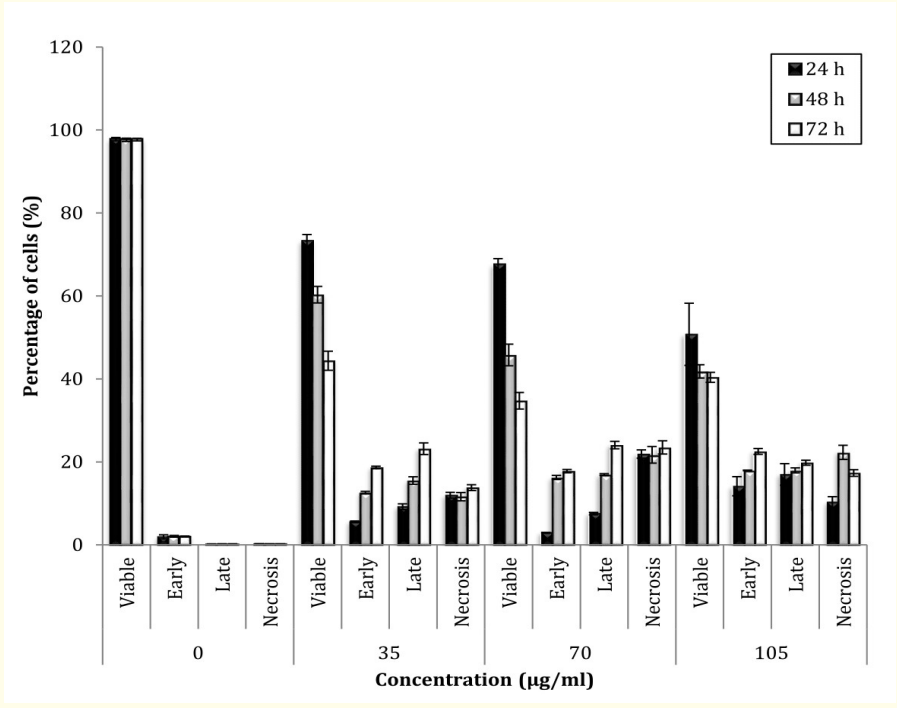

Figure 9: *Effect of bilimbi on the induction of apoptosis in HeLa with no treatment and with bilimbi at 35, 70 and 105 µg/ml for 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). Untreated cells were significantly different from all treated cells (p < 0.05).*

Effects of bilimbi extract on Caspase-8 and -9 expression in HeLa cells

Caspase-8 and -9 are major initiator caspases that regulate the initiation of apoptosis pathway. Caspase-8 is linked to the extrinsic pathway while caspase-9 is linked to the intrinsic pathway (or indirectly in extrinsic pathway in some cell systems). To investigate whether treatment with bilimbi on HeLa induced apoptosis via intrinsic or extrinsic pathway, these assays were performed. When HeLa was treated with bilimbi for 24 hours, no significant ($p > 0.05$) increased in caspase-8 activity was observed in all concentrations compared to untreated cells. Although there was very slight increased of caspase-8 activity over time, the increment was not significant ($p > 0.05$) enough relative to untreated cells (Figure 10). On the other hand, results revealed a significant ($p < 0.05$) upregulation of caspase-9 expression in bilimbi-treated HeLa at 35, 70 and 105 µg/ml after 48 hours treatment and at 105 µg/ml after 72 hours treatment compared to untreated cells (Figure 11), indicating that apoptosis induced by bilimbi on HeLa might be mediated through intrinsic pathway.

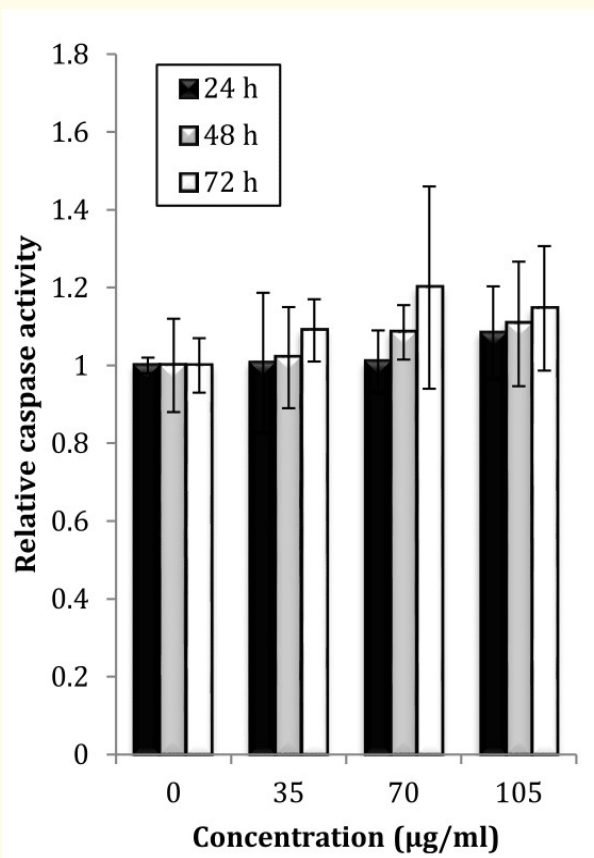

Figure 10: *Effect of bilimbi on caspase-8 expression in HeLa with no treatment and with bilimbi at 35, 70 and 105 µg/ml for 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to untreated cells.*

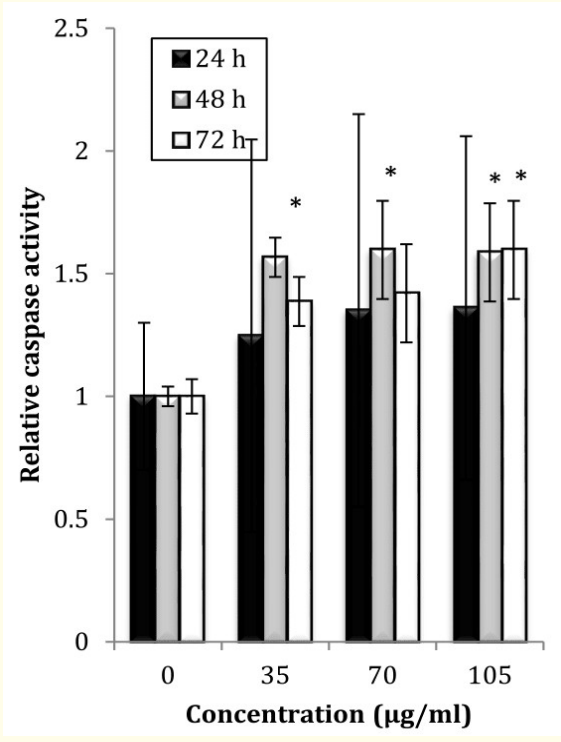

Figure 11: *Effect of bilimbi on caspase-9 expression in HeLa with no treatment and with bilimbi at 35, 70 and 105 µg/ml for 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to untreated cells.*

Effects of bilimbi extract on Caspase-3/7 expression in HeLa cells

Caspase-3 and -7 are effector caspases involves in the execution phase of apoptosis pathway. To observe whether caspase-9 activated downstream caspase-3 and -7, this assay was performed. When HeLa was treated with bilimbi for 24 hours, significant ($p < 0.05$) upregulation of caspase 3/7 expression was observed at 70 µg/ml compared to untreated cells. After 48 hours treatment, caspase-3/7 expression was further upregulated with increasing concentrations and upregulation was even more pronounced 72 hours post-treatment. Expression of caspase-3/7 was significantly ($p < 0.05$) upregulated in a concentration-dependent manner in bilimbi-treated HeLa compared to untreated cells (Figure 12).

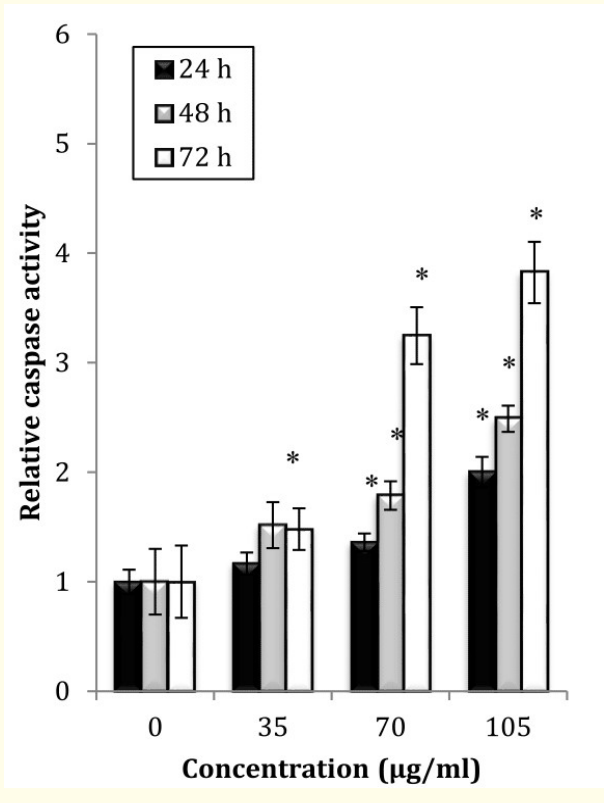

Figure 12: *Effect of bilimbi on caspase-3/7 expression in HeLa with no treatment and with bilimbi at 35, 70 and 105 µg/ml for 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to untreated cells.*

Effects of bilimbi extract on Cytochrome C expression in HeLa cells

Release of cytochrome c from mitochondria is a crucial step in apoptotic pathway to activate caspase cascades. When HeLa was treated with bilimbi for 24 hours, cytochrome c was significantly ($p < 0.05$) increased at 70 and 105 µg/ml treatments compared to untreated cells. Same trend was observed 48 hours post-treatment with higher cytochrome c released compared to 24 hours treatment. Cytochrome c levels remained elevated up to 72 hours post-treatment with significant ($p < 0.05$) level detectable at lower concentration (35 µg/ml) compared to untreated cells. Over time, released of cytochrome c increased considerably with increasing concentrations (Figure 13).

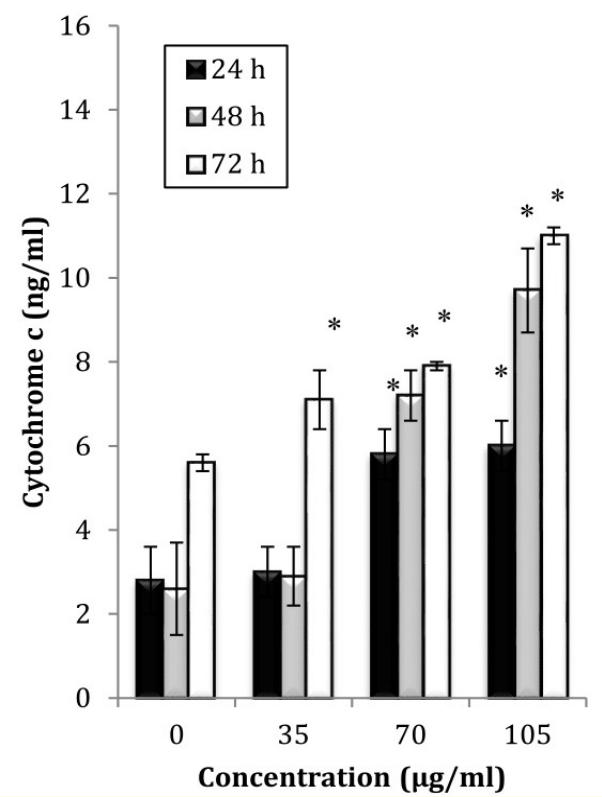

Figure 13: *Effect of bilimbi on cytochrome c release in HeLa with no treatment and with bilimbi at 35, 70 and 105 µg/ml for 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to untreated cells.*

Effects of bilimbi extract on pro- and anti-apoptotic proteins expression in HeLa cells

Effects of bilimbi on the expression of pro-apoptotic proteins (p53 and Bax) and anti-apoptotic proteins (Bcl-2) in HeLa were examined because of their important roles in the regulation of apoptosis. When the percentage of cells stained with p53 was determined by flow cytometry, 31.80% of the cells showed positive staining in the control groups. After HeLa was treated with bilimbi for 72 hours, the percentage of cells with positive staining increased significantly ($p < 0.05$) to 61.70%. When the percentage of positive cells staining for Bax was measured, 25.23% cells in the control groups showed positive staining. After bilimbi treatment, Bax level increased to 93.25% after 72 hours. On the other hand, expression levels of Bcl-2 reduced in a time-dependent manner after the induction of bilimbi, from

90.01% in control groups to 11.11% after 72 hours. The ratios of Bax to Bcl-2 protein expression revealed that cells treated with bilimbi showed at least one fold increased in Bax compared to Bcl-2 at 48 hours of treatment and eight fold at 72 hours. Figure 14 illustrates flow cytometry analysis on the effects of bilimbi on pro-apoptotic and anti-apoptotic proteins expression in HeLa.

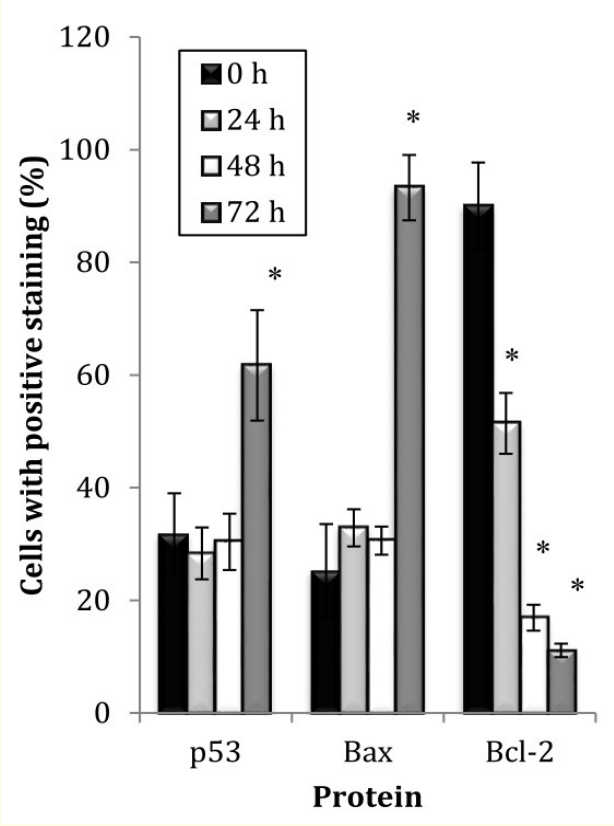

Figure 14: *Effect of bilimbi on pro-apoptotic and anti-apoptotic proteins expression in HeLa treated with bilimbi at 70 µg/ml for 0, 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to 0 hour.*

Discussion

In this study, cytotoxic effect of bilimbi on human cervical cancer cell line (HeLa) was investigated by MTS colorimetric assay. Results revealed that bilimbi was capable of inducing growth inhibitory effect on HeLa with IC50 values of 70 µg/ml without any cytotoxic effect on non-malignant Chang Liver cell line. Although IC50 value was much higher compared to doxorubicin, the fact that bilimbi selectively inhibit growth of cancer cells without affecting normal cells made bilimbi potential as a choice of cancer chemoprevention agent towards targeting cervical cancer. This selectivity feature of bilimbi is very important in terms of considering certain natural products as potential cancer chemotherapy drugs so as not to cause harmful side effect against normal dividing cell [25,26]. Many cancer patients fail chemotherapy primarily due to side effects or multi-drug resistance [27]; therefore, induction of programmed cell death specifically in cancer cells without affecting the immune cells might be of substantial advantage for cancer chemoprevention or even chemotherapy. Since a crude ethanolic extract was used in this study, it is not practical to attribute the growth inhibitory effect of bilimbi to a specific compound.

Moreover, it is possible that the cytotoxic effect were contributed by a synergistic effect of few compounds in the extract as shown in a study by Yang and Liu [28] or might be the effect of the crude extract itself. Hence, future work involving separation and purification of compounds may be done to uncover its potency.

The growth inhibitory and cytotoxic effect of bilimbi was also confirmed in morphological study of bilimbi-treated HeLa. Observations revealed morphological changes in bilimbi-treated HeLa as compared to untreated cells. Cells appeared shrunken, detached and reduced in volume. These are among the typical features of apoptotically dying cells [23] which are not observable in cells dying from necrosis. Rather, necrotic cells swell, round up, and then suddenly collapse like a punctured balloon, spilling their contents in the medium [29]. On the other hand, little or no morphological alteration was observed in Chang Liver treated with bilimbi compared with untreated cells, indicating the absence of cytotoxic activity. Hence, morphological observation suggested that bilimbi induced apoptosis in HeLa. According to Dartsch., *et al*. [30], apoptosis is distinguishable from necrosis by an early occurrence of nuclear and DNA fragmentation concomitant with membrane integrity, showing apoptotic bodies, phosphatidylserine externalization, reduction of nuclear volume and chromatin condensation. Consequently, AO/PI staining was performed in order to observe nuclei morphology of bilimbi-treated HeLa as well as to ascertain its mode of cell death.

Fluorescence microscopic images showed cells stained yellowish-green in bilimbi-treated HeLa compared with untreated cells when stained with acridine orange (AO) and propidium iodide (PI). The untreated cells showed bright green fluorescence as viable cell with intact membrane excluded PI and only AO could enter to bind to its DNA. On the contrary, bilimbi-treated HeLa showed reduced green fluorescence because AO was absorbed by PI when both bound to its DNA. Dead cells with damaged plasma membrane allowed the entrance of both AO and PI. Therefore, observations indicated that treatment with bilimbi induced apoptosis in HeLa cells.

Another biochemical hallmark of apoptosis is DNA fragmentation observable from the gel pattern of agarose gel electrophoresis. DNA laddering is an absolute evidence of whether cells are undergoing apoptosis or necrosis [31]. Bilimbi-treated HeLa showed degraded DNA bands in the form of ladders as opposed to untreated cells that showed intact DNA bands when observed under chemiluminescent. DNA band was not so apparent in cells treated with highest concentration probably due to cells undergoing necrosis. Cleavage of genomic DNA during apoptosis also marks a defining moment for a dying cell because DNA fragmentation is an irreversible event that executes cell to die [32]. Previous studies also reported the formation of DNA ladder in HeLa upon induction of apoptosis [33,34].

Further evidence in support of DNA fragmentation was obtained from TUNEL assay to identify and quantify apoptotic cells using flow cytometry by labeling DNA strand breaks with fluorochromes. The fluorescent nucleotides were enzymatically incorporated onto 3'-OH ends of DNA fragments resulting from apoptotic event [24]. Results showed that treatment with bilimbi increased the percentage of TUNEL positively stained cells, indicating apoptosis as the mode of cell death. TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis, allowing the discrimination of apoptosis from necrosis [35,36]. However, percentage of TUNEL positively stained cells was higher at 70 µg/ml compared to 105 µg/ml although both were significantly higher than untreated cells. This could be due to the possibility that cells at different stages of apoptosis might co-exist at any one time. Since TUNEL assay detects DNA breakage that occurs during late stage of apoptosis, percentage of TUNEL positively stained cells at highest concentration was not highest as expected.

Induction of apoptosis by bilimbi on HeLa might be mediated by cell cycle arrest. Thereupon, effects of bilimbi on disruption of cell cycle progression were examined by flow cytometry since cell cycle arrest has become an appreciable goal for cancer management [37]. Analysis showed an increased of sub G1 area in bilimbi-treated HeLa, indicating cell death. There was report suggesting that these sub G1 populations as apoptotic cells [38]. Based on the results of TUNEL assay, percentage of TUNEL positively stained cells was highest at 70 µg/ml instead of 105 µg/ml whereas, in cell cycle analysis, sub G1 populations was highest at 105 µg/ml. This might be due to the fact that TUNEL assay only detects cells at the late stage of apoptosis while sub G1 populations represent apoptotic cells with hypodiploid DNA content. Therefore, higher number of apoptotic cells was obtained in cell cycle analysis compared with TUNEL assay at the same

concentration of 105 µg/ml. Besides, bilimbi exerted growth inhibitory effect on HeLa by perturbation in G0/G1 phase of the cell cycle. Numerous recent studies reported a G0/G1 cell cycle arrest in human cervical cancer cells [39-41] upon induction of apoptosis as well as G2/M arrest [42,43]. Hence, cell cycle arrest might be related to the type of cervical cancer cells and extracts used.

Cell proliferation assay measured the incorporation of BrdU during DNA synthesis in proliferating cells. It is a basic parameter in cancer studies and an accurate method to measure cell proliferation because it is a direct assay of DNA synthesis [44]. It is a more precise way to analyze the ability of bilimbi in inducing cell cycle arrest at G0/G1 checkpoint of HeLa because the changes in DNA content may not be significant enough in cell cycle analysis [45]. Findings revealed that percentage of proliferating cells decreased in a concentration-dependent manner compared to untreated cells. However, proliferating cells at 72 hours post-treatment was more than those at 24 and 48 hours post-treatment at all concentrations probably because many cellular processes can result in the synthesis of new DNA without the cell going through mitosis [46]. Hence, it was possible that the increased in BrdU labeling following bilimbi treatment was due to other cellular processes rather than cell proliferation since BrdU can be incorporated into DNA during these cellular processes. Results were comparable to cell cycle analysis because the percentage of proliferating cells was also decreasing over time. In order to determine the mode of cell death induced by bilimbi, Annexin V-FITC/PI staining was performed.

Cell death could be a consequence of apoptosis or necrosis. However, apoptosis is more preferable because cells die in a controlled manner and each play an active role in their own death. In contrast, necrotic cells die in an uncontrolled manner leading to lysis of cells, inflammatory responses and potentially serious health problems. Analysis showed that percentage of viable cells decreased over time with increasing concentrations. In comparison, early apoptosis, late apoptosis, and necrotic cells gradually increased over time with increasing concentrations. These necrotic cells were derived from apoptotic bodies as well as remaining cell fragments which were ultimately swelled and finally lysed due to the absence of phagocytes *in vitro*. Therefore, bilimbi-treated HeLa underwent early apoptosis, late apoptosis and secondary necrosis rather than normal necrosis.

There are two major apoptotic pathways known to date, initiated by either the mitochondria (intrinsic pathway) or the cell surface receptors (extrinsic pathway). Both apoptotic pathways are regulated in an orderly way by a series of caspase-cascade which play vital roles either as initiators or effectors in the execution of apoptotic cell death [47]. Mitochondria (intrinsic) pathway occurs in response to a wide range of death stimuli, including activation of tumor suppressor proteins (such as p53) and oncogenes, DNA damage, chemotherapeutic agents, serum starvation, and ultraviolet radiation [48]. It is activated by the release of proapoptotic factors from mitochondria, such as cytochrome c [49]. Released cytochrome c interacts with Apaf-1 and activates caspase-9 that in turn proteolytically activates downstream caspase-3, one of the principle protease participating in the execution phase of apoptosis. Activated caspase-3 cleaves a lot of substrates, including poly(ADP-ribose)polymerase (PARP), a DNA repair enzyme, and leads to inevitable cell death [50].

On the contrary, cell surface receptors (extrinsic) pathway involves Fas and other members of the tumor necrosis factor receptor family that activate caspase-8 [13]. Caspase-8 can activate downstream events either by directly activating caspase-3 or by cleaving Bid, which then in turn trigger the mitochondrial pathway [50]. To elucidate the molecular pathway of apoptosis induced by bilimbi, activation of caspase-3/7, -8, -9 and cytochrome c was measured. Results revealed that cytochrome c increased considerably with increasing concentrations upon treatment with bilimbi on HeLa. Caspase-3/7 and caspase-9 assays showed concentration-dependent upregulation of caspase-3/7 and caspase-9 expression, respectively in bilimbi-treated HeLa compared to untreated cells. However, there was no significant ($p > 0.05$) increased in caspase-8 activity in all concentrations compared to untreated cells, indicating that treatment with bilimbi induced apoptosis via mitochondria-mediated intrinsic pathway in HeLa.

There was report that the response of mitochondria-mediated apoptosis pathway may be dependent or independent of p53 protein [51]. Tumour suppressor protein p53 plays a role in cellular responses to a variety of genotoxic stresses resulting in cell cycle arrest or apoptosis [52]. To have better insight of apoptosis induction of bilimbi towards HeLa at molecular level, p53 expression was assayed. Results revealed that treatment of bilimbi on HeLa increased the expression of p53 protein. While some p53 protein remains in the nucleus,

a fraction also accumulates in the mitochondria, where it directly initiates an apoptotic program. At the mitochondria, p53 initiates permeabilization of the outer mitochondrial membrane via activation of pro-apoptotic Bcl-2 family members such as Bax [53]. Mitochondrial p53 shifts the balance between anti-apoptotic and pro-apoptotic Bcl-2 family members, leading to release of cytochrome c into the cytoplasm, and initiation of the caspase cascade, eventually resulting in cell death. Findings in the present study so far indicated that bilimbi induced p53-mediated mitochondrial apoptosis pathway in HeLa.

Another important regulator of apoptosis is the Bcl-2 family proteins. Bcl-2 family includes pro-apoptotic (such as Bax, Bak, Bad, Bid) and anti-apoptotic (such as Bcl-2, Bcl-xL) proteins. Pro-apoptotic proteins trigger the release of caspases from death antagonists via heterodimerization and also by inducing the release of mitochondrial apoptogenic factors into the cytoplasm via acting on mitochondrial permeability transition pore, thereby leading to caspase activation. Contradictory, anti-apoptotic proteins prevent apoptosis either by withdrawing proforms of death-driving caspases (apoptosome) or by preventing the release of mitochondrial apoptogenic factors such as cytochrome c and AIF (apoptosis-inducing factor) into the cytoplasm [54]. To investigate the involvement of pro-apoptotic and anti-apoptotic proteins in p53-mediated mitochondrial apoptosis pathway in HeLa, expression of Bax and Bcl-2 were investigated. Data indicated that upon treatment with bilimbi, level of Bax was upregulated while level of Bcl-2 was downregulated in HeLa, supporting the antagonist functions of these two members of the Bcl-2 family. Increment in the ratios of Bax to Bcl-2 suggests that upregulation of Bax and downregulation of Bcl-2 may be one of the molecular mechanism through which bilimbi induces apoptosis. It was in agreement with previous studies reporting that apoptosis induction on HeLa was dependent of p53 and was accompanied by an increased in the ratio of Bax to Bcl-2 [55,56]. Likewise, Tsujimoto [54] described that enforced expression of pro-apoptotic proteins Bax can result in increased mitochondrial potential and release of cytochrome c, thereby leading to apoptosis. Hence, induction of apoptosis by bilimbi on HeLa involved the upregulation of Bax and downregulation Bcl-2 in a p53-dependent pathway.

Conclusion

In summary, the present results provided scientific evidences for the potential use of bilimbi in the prevention or even its therapeutic value on human cervical cancer. Findings indicated that bilimbi significantly inhibited growth of HeLa cells *in vitro* without cytotoxic effect on non-malignant Chang Liver cells. Bilimbi cytotoxic activity is likely due to the induction of apoptosis mediated by cell cycle arrest at G0/G1 checkpoint. Further investigation revealed that apoptosis in bilimbi-treated HeLa was initiated by mitochondria in a p53-dependent pathway. As a natural food, bilimbi offers a much safer approach to cancer chemoprevention for not being cytotoxic to normal cells and therefore, may be a good candidate worth further evaluation as a cancer chemotherapeutic agent.

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