Anti-proliferation of MDA-MB-231 Cells by *Averrhoa bilimbi* Extract is Associated with G0/G1 Perturbation and Mitochondria-mediated Apoptosis Independent of p53

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Abstract

Practiced as folk medicine since ancient times, bilimbi *(Averrhoa bilimbi)* is commonly consumed and widely cultivated in Malaysia. 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 anti-proliferative effect and underlying cell death pathway induced by bilimbi ethanolic extract on human non-hormone-dependent breast cancer cell line, MDA-MB-231. 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 inhibited growth of MDA-MB-231 cells through the induction of apoptosis mediated by cell cycle arrest at G0/G1 checkpoint. Released of cytochrome c coupled with up-regulation of caspase-3/7, caspase-9 and Bax pro-apoptotic proteins in addition to down-regulation of dysfunctional p53 and Bcl-2 anti-apoptotic proteins implied that bilimbi induced a p53-independent mitochondrial apoptosis pathway in MDA-MB-231. These results suggest that bilimbi could induce tumor cell anti-proliferation through apoptosis. As a natural product, bilimbi could be a promising anticancer agent and an inexpensive approach to cancer chemoprevention strategy.

Introduction

Searching for natural products that have therapeutic potential for the treatment of cancer is the aim of numerous studies. Many naturally occurring agents and extracts have shown antioxidant and chemopreventive potential in a diversity of bioassay systems and animal models, having relevance to human diseases (Aziz *et al.*, 2003). Hence, much attention has been focused on the use of natural antioxidants because of the worldwide trend to minimize the use of synthetic antioxidants which has been restricted due to their carcinogenicity (Ito *et al.*, 1983). Bilimbi *(Averrhoa bilimbi)* is among the underutilized fruits commonly consumed and widely cultivated throughout the tropics in Malaysia. To date, there are limited scientific evidences and scarcity of data concerning the impact of bilimbi on cancer. Hence, it is worthwhile to investigate its anticancer activity. This study was undertaken to investigate the cytotoxic and apoptotic activities of bilimbi ethanolic extract on human non-hormone-dependent breast cancer cell line, MDA-MB-231 and to identify the possible mechanism of cell death pathway involved in the anticancer activity.

Materials and Methods

Materials

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).

Sample preparation and extraction

Fresh bilimbi fruit was freeze-dried to obtain fine powders and extracted with 80% ethanol at 65°C for 24 hours (Extraction conditions were determined though an optimization study to extract phenolic compounds using single-factor experiments). The extract was filtered, evaporated and freeze-dried. The dry residue was dissolved in DMSO to obtain stock

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solution 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 before use.

Cell cultures

MDA-MB-231 (human non-hormone-dependent breast cancer cell line; ATCC HTB-26) 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 37ºC humidified atmosphere in 75 cm^2 flasks. Confluent monolayer cells were detached using 0.25% (w/v) trypsin-EDTA.

MTS assay

Cytotoxic study was performed as described according to instructions (CellTiter 96® AQueous One Solution Cell Proliferation Assay) (Promega, WI, USA). Cells were seeded into 96-well plate and treated with extract in various concentrations for 72 hours, performed through serial twofold dilutions. Cells treated with doxorubicin and culture medium (1% DMSO) served as positive control and negative control, respectively. Absorbance of colored formazan produced was recorded on an ELISA plate reader at 490 nm. The concentration which gave 50% inhibition of cell growth (IC_{50}) was determined from a plotted dose-response curve.

TUNEL assay

Quantitative determination of DNA fragmentation was performed as described according to instructions (In Situ Cell Death Detection Kit, Fluorescein) (Roche, Mannheim, Germany). MDA-MB-231 cells were seeded into 6-well plate and treated with bilimbi extract in different concentrations over different incubation periods. After incubation, cells were fixed and permeabilized prior to incubation with TUNEL reaction mixture. Percentage of FITC positively stained cells was determined on FACSCaliber flow cytometer equipped with 488 nm argon laser light source and 525 nm band pass filter and analyzed using CellQuest Pro software. Percentage of FITC-positive stained cells was determined from the single-parameter histogram.

Cell cycle analysis

Cell cycle distribution was determined according to a method as described by Mothanna *et al.* (2011) with slight modification. MDA-MB-231 cells were seeded into 6-well plate and treated with bilimbi extract in different concentrations over different incubation period. After incubation, 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 (Cell Proliferation ELISA, BrdU Colorimetric) (Roche, Mannheim, Germany). MDA-MB-231 cells were seeded into 96-well plate and treated with bilimbi extract in different concentrations over different incubation periods. Cells were labeled and fixed before the addition of antibody conjugate solution. Substrate solution was added and incubated until color development was sufficient enough for photometric detection. Absorbance of colored product was recorded on an ELISA plate reader at 370 nm and presented as O.D value.

Caspase–8 and –9 assays

Quantitative determination of human caspase-8 and -9 was performed as described according to instructions (Human Caspase-8 Platinum ELISA and Human Caspase-9 Platinum ELISA) (Bender MedSystems, Vienna, Austria). Both were of the same protocol. MDA-MB-231 cells were seeded into 6-well plate and treated with bilimbi extract in different concentrations over different incubation periods. After incubation, 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 colored 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 (Caspase-Glo® 3/7 Assay) (Promega, WI, USA). MDA-MB-231 cells were seeded into white-walled 96-well plate and treated with bilimbi extract in different concentrations over different incubation periods. Caspase-Glo® 3/7 reagent was added and gently mixed. Luminescence was measured in a plate-

reading GloMaxTM 96 Microplate Luminometer. 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 (Human Cytochrome c Platinum ELISA) (Bender MedSystems, Vienna, Austria). MDA-MB-231 cells were seeded into 6-well plate and treated with bilimbi extract in different concentrations over different incubation periods. After incubation, 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 colored 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

MDA-MB-231 cells were seeded into 6-well plate treated with 90 µg/ml of bilimbi extract over different incubation periods. After incubation, cells were fixed and permeabilized with 1% Triton X-100 for 10 minutes prior to incubation with antibody. 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 and Discussion

Growth inhibition

This study evaluated the cytotoxic effect of bilimbi on human non-hormone-dependent breast cancer cell line, MDA-MB-231 as well as non-malignant Chang Liver cell line. Results showed that bilimbi was able to induce cytotoxicity in MDA-MB-231 cells with an IC50 values of 90 ± 3.46 µg/ml. On the other hand, viability of non-malignant Chang Liver was still
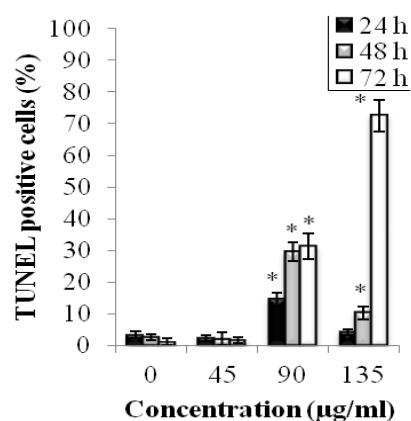

Figure 1. Effect of bilimbi on DNA fragmentation in MDA-MB-231. TUNEL positive-stained MDA-MB-231 with no treatment and treated with bilimbi at 45, 90 and 135 µg/ml for 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to untreated cells

above 50% even though concentration was increased up to 200 µg/ml. Literature has shown that antioxidant properties of fruits might be associated with anticancer activities (Son et al., 2003). The presence of high antioxidant vitamins (vitamin A, C and E) in bilimbi could be related to its growth inhibition effect (Yan et al., 2013). 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 MDA-MB-231 cells (0.4 ± 0.14 µg/ml) including non-malignant Chang Liver (0.65 ± 0.13 µg/ml). The fact that bilimbi displayed only selective inhibitory effect against cancer cells without affecting normal cells made bilimbi potential as a choice for development of cancer chemoprevention agent towards targeting breast cancers. The selective preference of bilimbi is fundamental as cancer drug targeting has been nowadays considered a promising strategy in cancer chemotherapy as well. Specific targeting of tumor cells serves to enhance therapeutic efficacy of cytotoxic drug while at the same time reducing organ toxicities (Arafa, 2009).

DNA fragmentation

Identification of apoptotic cells in vitro could be affirmed by TUNEL assay. This assay is capable of detecting DNA strand breaks that occur prior to the nucleus fragmenting (Brien et al., 1997) and may serve as a marker of apoptosis rather than necrosis (Gorczyca, 1999). Results showed that over time and with increasing concentrations, percentage of TUNEL positively stained cells increased compared with untreated cells; supporting the hypothesis that bilimbi induced apoptosis in MDA-MB-231 (Figure
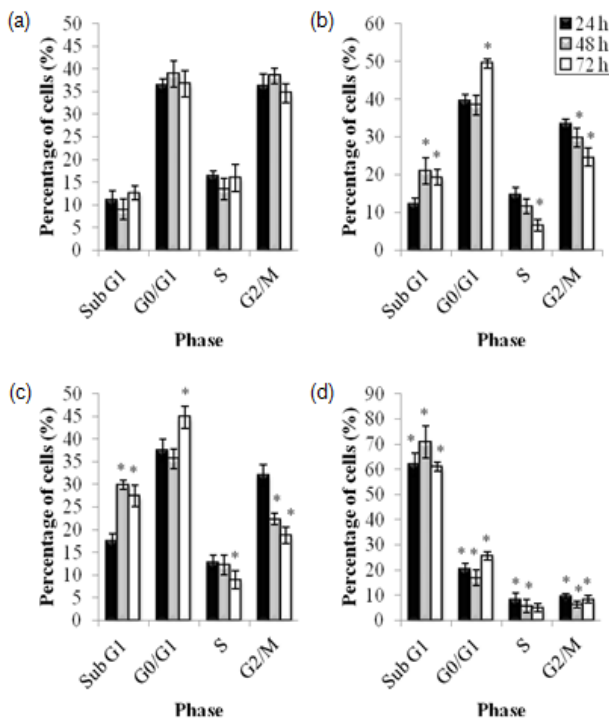
Figure 2. Effect of bilimbi on cell cycle progression of MDA-MB-231. (a) Cell cycle profile of MDA-MB-231 with no treatment for 24, 48 and 72 hours. (b) Cell cycle profile of MDA-MB-231 treated with bilimbi at 45 µg/ml for 24, 48 and 72 hours. (c) Cell cycle profile of MDA-MB-231 treated with bilimbi at 90 µg/ml for 24, 48 and 72 hours. (d) Cell cycle profile of MDA-MB-231 treated with bilimbi at 135 µg/ml for 24, 48 and 72 hours. Data

1). Percentage of TUNEL positively stained cells was higher at 90 µg/ml compared to 135 µg/ml at 24 and 48 hours 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 (Su *et al*., 2015). 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.

Cell cycle analysis

Induction of apoptosis by bilimbi on MDA-MB-231 was further evidenced in cell cycle analysis. Endonucleolytic DNA degradation of apoptotic cells that results in the extraction of low molecular weight DNA are recognized by their fractional DNA content (sub G1 peak, sub-diploid peak) through flow cytometry analysis (Gorczyca, 1999). Results showed that there were increased of sub G1 area in bilimbi-treated MDA-MB-231 with increasing concentrations over time (Figure 2a-d). Analysis also revealed bilimbi exerted growth inhibitory effects on MDA-MB-231 by perturbation at G0/G1 phase of the cell cycle. There were reduction of cells observed
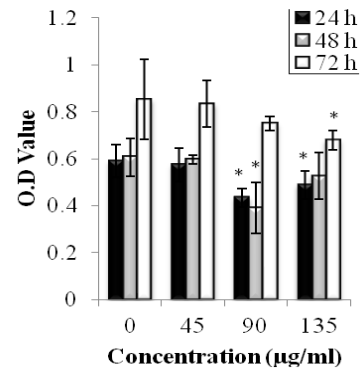

Figure 3. Effect of bilimbi on cell proliferation of MDA-MB-231 with no treatment and treated with bilimbi at 45, 90 and 135 µg/ml for 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to untreated cells

in the S and G2/M phases and accumulation of cells at G0/G1 phase upon treatment with bilimbi. Few studies also reported that upon induction of apoptosis in MDA-MB-231, cells were arrested at G0/G1 phase of the cell cycle (El-Sherbiny *et al*., 2001; Nigam *et al.*, 2008; Zhong *et al.*, 2011)

Cell proliferation

Through cell cycle analysis, results showed that cells in the S phase decreased gradually as concentrations increased over time. S phase (synthesis phase) is the part of the cell cycle in which DNA is replicated. Hence, a decreased in S phase indicates a decrease in DNA replication. To confirm anti-proliferative activity of bilimbi on MDA-MB-231, cell proliferation assay was performed. This assay measures cell proliferation by quantitating BrdU incorporated into newly synthesized DNA of replicating cells only during S phase of the cell cycle. Findings revealed that percentage of proliferating cells decreased drastically upon induction of apoptosis for 72 hours compared to untreated cells (Figure 3). However, proliferating cells slightly increased as concentration increased to 135 µg/ml at 24 and 48 hours post-treatment. These DNA syntheses could be due to DNA repair process (McDevitt et al., 1995) as BrdU may also be incorporated during DNA synthesis not related to cell proliferation, such as normal DNA turnover or DNA repair (Nowakowski and Hayes, 2000; Cooper-Kuhn and Kuhn, 2002). Proliferating cells at 72 hours post-treatment was also 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 (Landgren and Curtis, 2010).
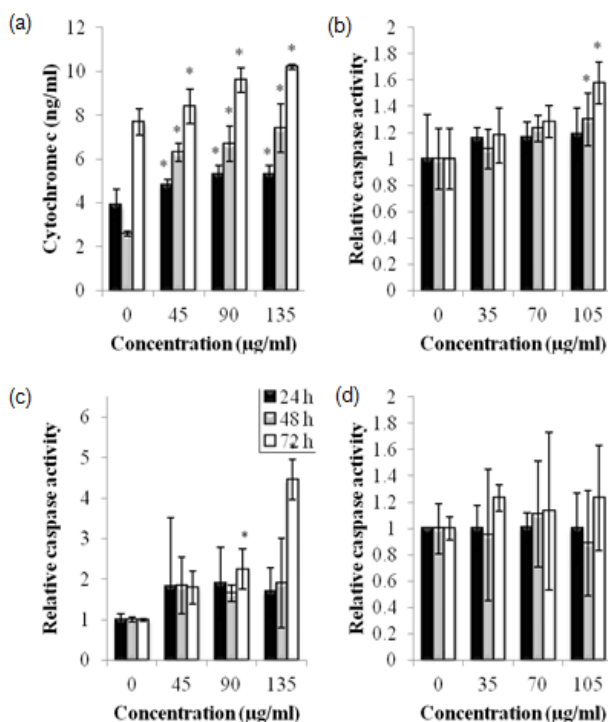
Figure 4. Effect of bilimbi on caspases and cytochrome c expression in MDA-MB-231. (a) Cytochrome c release in MDA-MB-231 with no treatment and with bilimbi at 45, 90 and 135 µg/ml for 24, 48 and 72 hours. (b) Caspase-3/7 expression in MDA-MB-231 with no treatment and with bilimbi at 45, 90 and 135 µg/ml for 24, 48 and 72 hours. (c) Caspase-9 expression in MDA-MB-231 with no treatment and with bilimbi at 45, 90 and 135 µg/ml for 24, 48 and 72 hours. (d) Caspase-8 expression in MDA-MB-231 with no treatment and treated with bilimbi at 45, 90 and 135 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

Caspase and cytochrome c expression

To elucidate the apoptotic pathway 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 MDA-B-231 (Figure 4a). Intrinsic pathway is mediated by the release of mitochondrial cytochrome c. In some cases, cytochrome c may be released in extrinsic pathway as well (Kiechle and Zhang, 2002). Consequently, activation of caspases was investigated in order to determine the apoptotic pathway induced by bilimbi on MDA-MB-231. Results showed a concentration-dependent up-regulation of caspase-3/7 (Figure 4b) and caspase-9 (Figure 4c) expression, respectively in bilimbi-treated MDA-MB-231 compared to untreated cells. However, there was no significant (p > 0.05) increased in caspase-8 (Figure 4d) activity in all concentrations compared to untreated cells, indicating that treatment with bilimbi

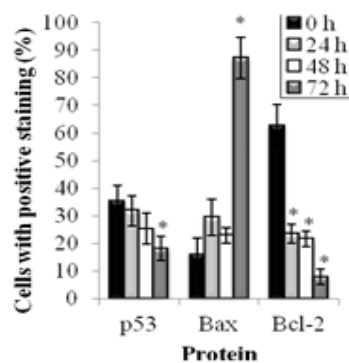Figure 5. Effect of bilimbi on pro-apoptotic and anti-apoptotic proteins expression in MDA-MB-231 treated with bilimbi at 90 µg/ml for 0, 24, 48 and 72 hours. Data are presented as mean ± standard deviation of three independent experiments (n = 3). *p < 0.05 compared to 0 hour

induced apoptosis via mitochondria-mediated intrinsic pathway in MDA-MB-231.

Expression of pro- and anti-apoptotic proteins

Data showed that treatment of bilimbi on MDA-MB-231 slightly down-regulated the expression of p53 protein (Figure 5). This could be due to the fact that MDA-MB-231 possesses mutant p53. Accordingly, p53 is the most widely mutated gene in human tumorigenesis (Wang and Harris, 1997; Noa et al., 2011) and it is mutated in over 50% of all human cancers (Hollstein *et al*., 1991; Chiarugi *et al*., 1994; Toshinori and Akira, 2011). Induction of apoptosis is also partly mediated, intracellularly by Bax and Bcl-2 (Chiarugi *et al.*, 1994). These two proteins play a major role in determining whether cells will undergo apoptosis that promote cell death. Results showed that upon treatment with bilimbi, level of Bax was up-regulated while level of Bcl-2 was slightly down-regulated in MDA-MB-231. It was in agreement with studies reporting that the increased expression of Bax can induce apoptosis (Findley *et al.*, 1997; Salomons *et al*., 1997; Wu and Liu, 2015) while Bcl-2 prevents cells from apoptosis (Kane *et al.*, 1993; Sano *et al*., 1995; Niture and Jaiswal, 2012). Hence, results suggested that induction of apoptosis by bilimbi on MDA-MB-231 involved the up-regulation of Bax and down-regulation Bcl-2 in a p53-independent pathway.

Conclusion

In summary, the present results provided scientific rationale for using bilimbi as a chemopreventive or even as therapeutic agent against human breast cancer. Findings revealed that bilimbi significantly

inhibited growth of MDA-MB-231 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 evidences revealed that apoptosis in bilimbi-treated MDA-MB-231 was initiated by mitochondria independent of p53. As a natural product, bilimbi may be a promising candidate in search for natural anticancer agent.

Acknowledgement

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