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Full Length Research Paper

Cytotoxic effect of betulinic acid and betulinic acid acetate isolated from *Melaleuca cajuput* on human myeloid leukemia (HL-60) cell line

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The cytotoxic effect of betulinic acid (BA), isolated from *Melaleuca cajuput* a Malaysian plant and its four synthetic derivatives were tested for their cytotoxicity in various cell line or peripheral blood mononuclear cells (PBMC) by 3-[4,5-dimethylthizol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Betulinic acid acetate (BAAC) was most effective than other betulinic acid derivatives. It had most active cytotoxic activity against human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B) and human cervical epithelial carcinoma (HeLa) but not on normal human lymphocytes (PBMC), suggesting its action is specific for tumor cells. BA and BAAC inhibit HL-60 cell line at low concentration after 72 h with IC_{50} values at 2.60 and 1.38 µg/mL, respectively. DNA fragmentation analysis showed ladder formation in the 100 - 1500 bp region in HL-60 cell lines after 24 h of treatment with IC_{50} values. The induction of apoptosis was also confirmed by flow cytometric analysis of cell cycle. BA and BAAC have been shown to induce a time dependant increase in the sub G_1 peak indicating apoptotic phenomenon as obtained from the DNA content histogram analysis. Thus, betulinic acid isolated from Malaysia plant showed good potential as an anti-cancer compound with less toxicity to human normal cells.

Key words: Betulinic acid, HL 60, cytotoxicity, MTT assay, DNA laddering, Cell cycle PI

INTRODUCTION

Betulinic acid (3β-hydroxy-lup-20(29)-ene-28-oic acid), an example of a pentacyclic triterpene is widely distributed in plant kingdom (Maurya et al., 1989). This compound can be chemically derived from betulin, a substance found in the outer bark of white birch tree *Betula alba* (Pisha et al., 1995). Some biological activities have been ascribed to betulinic acid, includes anti-inflammatory, anti-tumor (Mukherjee et al., 1997; Liu et al., 2004), anti-angiogenesis (Mukherjee et al., 2004), anti-viral (De Clercq, 1995; Baltina et al., 2003; Parlova et al., 2003), anti-HIV (Hashimoto et al., 1997; Huang et al., 2006; Qian et al., 2007), anti-neoplastic (Fulda et al., 1999) and anti-plasmodial (Ziegler et al., 2004).

Betulinic acid exerts a selective anti-tumor activity on cultured human melanoma (Pisha et al., 1995), neuroblastoma (Schmidt et al., 1997), malignant brain tumor

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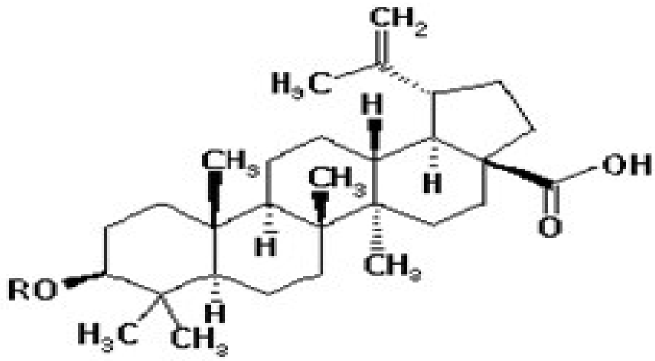

Compound	R
BA	H
BAAC	$COCH_3$
BAES	$COC(CH_3)_2CH_2COOH$
BASUC	$CO(CH_2)_2COOH$
BCL	COC_6H_5

Figure 1. Chemical structure of betulinic acid and its derivatives.

(Fulda et al., 1999) and leukemia cells (Hata et al., 2003). This compound showed inhibitory effect on leukemia (HL60, U937 and K562) and neuroblastoma (GOTO and NB-I) cell growth (Hata et al., 2003). It was reported selective against neuroblastoma cells and lacked side-effects. Moreover, its activity against neuroectodermal has been reported and related to the induction of apoptosis via direct mitochondrial alterations (Fulda et al., 1998). The anti-tumor activity of betulinic acid has been reported to other human neoplastic cell lines including lung, ovarian, cervical, colorectal, breast, and prostate cancer (Zuco et al., 2002; Kessler et al., 2007). Besides, Raghuvar Gopal et al. (2005) reported that betulinic acid had cytotoxic effect on human chronic myelogenous leukemia (CML) cells. Currently, betulinic acid is undergoing phase II clinical trials to treat melanoma (Gauthier et al., 2009). Betulinic acid was known to induce apoptosis selectively in tumor cells lines, but not on normal cell lines (Zuco et al., 2002). The favorable therapeutic index from the lack of toxicity towards normal cells suggested betulinic acid to be an attractive and promising anti-tumor agent (Pisha et al., 1995). This feature makes betulinic acid unique in comparison to compounds that are currently used in cancer therapy, such as taxol, camptothecin, elipticine, etoposide, vinblastine and vincristine. All these anti-tumor compounds are very toxic and inhibit replication of both cancer and normal cells (Zuco et al., 2002). In this project, the potential of betulinic acid isolated from Malaysian plant as anti-cancer agent on human myeloid leukemia will be investigated. Here, the *in vitro* cytotoxic activity of betulinic acid and its potent derivatives will be assessed using leukemic cell lines and compared with conventional anti-neoplastic drug (doxorubicin).

MATERIALS AND METHODS

Cell lines

The cancerous cell lines (anchorage-dependent and suspensions cells) were obtained from the American Type Culture Collection (ATCC), the National Cancer Institute (NCI) and the RIKEN Cell Bank (RCB). The suspensions cell lines were human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS) and BALB/c murine myelomonocytic leukemia (WEHI-3B) whereas the anchorage-dependent cell lines were human cervical epithelial carcinoma (HeLa), human breast adenocarcinoma (MCF-7), human glioblastoma (DBTRG0.5MG) and mouse skin melanoma (B16).

The human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B), human cervical epithelial carcinoma (HeLa), human breast adenocarcinoma (MCF-7) and mouse skin melanoma (B16) were maintained in RPMI 1640 medium (Sigma, USA) supplemented with 10% foetal calf serum (GIBCO, UK) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) (GIBCO, UK). Human glioblastoma (DBTRG0.5MG) were maintained in RPMI 1640 medium (Sigma, Germany) supplement with 1% HT (Flowlab, Australia), 1% L-glutamine 200 mM (Flowlab, Australia), 0.5% sodium pyruvate 100 mM (Flowlab, Australia), 10% foetal calf serum (GIBCO, UK) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) (GIBCO, UK). All type of cells was incubated under 37°C, 5% CO_2.

Isolation of peripheral blood mononuclear cells (PBMC) from human peripheral blood

Blood samples were collected by venepuncture in 5 ml lithium heparin coated Vacutainers. Blood 20 to 25 mL was diluted 1:1 with phosphate buffered saline (PBS) and layered onto Ficoll-paque plus (Amersham) by using Pasteur pipette and centrifuged at 400 x g (1500 rpm) for 40 min at 18 to 20°C. The lymphocyte layer was transfered using a clean Pasteur pipette to a clean centrifuge tube and washed three times in balanced salt solution. The lymphocytes suspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (GIBCO, UK) and antibiotics (100 units/ml penicillin and 100 µg/mL streptomycin) (GIBCO, UK). The lymphocytes were diluted to 1.0×10^6 cells/ml in DMEM and used immediately for MTT assay experiment.

Betulinic acid and its derivatives

Betulinic acid (BA), betulinic acid acetate (BAAC), 3-*O*-(2',2'-dimethylsuccinyl)-betulinic acid (BAES) and 3-*O*-succinyl-betulinic acid (BASUC) were kindly donated by Dr. Faujan B.H. Ahmad, while betulinic acid benzoate (BCL) was donated by Dr Yamin B. Yassin, both from the Department of Chemistry, Faculty of Science, University Putra Malaysia. Doxorubicin (DOX) was obtained from Sigma Aldrich, USA. These compounds (Figure 1) were used for the screening experiments however only betulinic acid, betulinic acid acetate and doxorubicin were used for further detail in cytotoxicity studies.

Betulinic acid

The pure compound of betulinic acid appears as a white solid, malted at 295 - 297°C. It was isolated from *Melaleuca cajuput* and was chromatographed on a silica gel column using chloroform as eluent (Ahmad et al., 1997).

Betulinic acid acetate

Betulinic acid acetate was prepared by refluxing betulinic acid (1.0 g) with acetic anhydride and pyridine in dichloromethane for several hours. After cooling to room temperature, water was then added and the white crystal was filtered off. Betulinic acid acetate appears as a pale yellow solid crystal. It also can be prepared by enzymatic reaction of betulinic acid and acetic anhydride in the present of lipase (Ahmad et al., 2005).

The compounds were dissolved in dimethylsulphoxide (DMSO) (Sigma, USA) at concentration of 10 mg/ml as a stock solution. It was then diluted further to a concentration of 60.0 µg/ml in RPMI 1640 by diluting 18.0 µl of the stock solution into 2982.0 µl RPMI 1640 as a working stock. The stock solution and working stock were both stored at 4°C.

MTT cytotoxic assay

MTT colorimetric assay was developed for measuring cell survival and proliferation. The principle behind this assay is that the tetrazolium ring in MTT is cleaved by dehydrogenases present in active mitochondria, resulting in the formation of an insoluble MTT formazan product (Mosmann, 1983). Briefly, 100 µl of RPMI-1640 or DMEM media with 10% of FBS was added into all the wells except row A in the 96 well plate (TPP, Switzerland). Then, 100 µl of diluted compound at 60 µg/ml was added into row A and row B. A series of two fold dilution of extract was carried out down from row B until row G. The row H was left untouched and the excess solution (100 µl) was discarded and 100 µl of cell line or PBMC with cell concentration at 1 X 10^5 cells/ml was added into all wells in the 96 well plate and incubated in 37°C, 5% CO_2 and 90% humidity incubator for selected period (24, 48, 72 h). After the corresponding period (either 24, 48 or 72 h), 20 µl of MTT (Sigma, USA) at 5 mg/ml was added into each well in the 96 well plate and incubated for four hours in 37°C, 5% CO_2 and 90% humidity incubator. Then, the plate was centrifuged at 200 x g for 5 min and 170 µl of medium with MTT was removed from every well. 100 µl DMSO (Fisher Scientific, UK) was added to each well to extract and solubilize the formazan crystal by incubating for 20 min in 37°C, 5% CO_2 incubator. Finally, the plate was read at 570 nm wavelength by using µ Quant ELISA Reader (Bio-Tek Instruments, USA). The results of the compounds were compared with the result of Doxorubicin and without drug by using the same method. Each compound and control was assayed in triplicate for three times. The percentage of proliferation was calculated by the following formula: Proliferation (%) = [(OD sample – OD control) / OD control] X 100.

DNA fragmentation assay

The HL-60 cells at concentration 1 x 10^5 cells/ml were treated with doxorubicin, betulinic acid and betulinic acid acetate with IC_{50} and 30 µg/ml concentration in 6 well plates. The triplicate samples were incubated for 24, 48 and 72 h at 37°C, 5% CO_2, 90% humidity. The treated cells were collected by centrifugation at 300 x g for 10 second and the DNA was isolated from cells for each treatment using The Wizard® Genomic DNA Purification Kit (Promega, USA). After harvesting, the cells were washed with 200 µl phosphate buffer saline (PBS). The cell pellet was then resuspended in 600 µl Nuclei Lysis Solution in the presence of 3 µl RNase and incubated for 30 min at 37°C. Then, the sample was allowed at room temperatures for 5 min. After 5 min, 200 µl of Protein Precipitation Solution was added to sample and vortex for 20 s. The sample chilled on ice for 5 min and centrifuged at 13000 x g for 4 min. The supernatant was removed to clean microcentrifuge tube contain 600 µl isopropanol and centrifuged at 13000 x g for 1 min. After decanting the supernatant, 600 µl 70% ethanol was added and centrifuged at 13000 x g for 1 min. Then, the ethanol was aspirated using pipette and dried the pellet for 15 min. 100 µl of DNA Rehydration Solution and incubated for 1 h at 65°C. The DNA was stored at 4°C.

Agarose gel (1%) was prepared. The gel was then submerged into running buffer (TBE buffer). Subsequently, 8 µl of DNA mixed with 2 µl loading dye (80% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol) was loaded on 1% agarose gel. Samples were loaded into the wells and were run at 40 V for 12 h. The agarose gel was then stained with ethidium bromide (Sigma, USA) (0.5 µg/ml in running buffer) for 30 min and destained with distilled water for an hour. DNA was visualized by UV illuminator (245 nm).

Flow cytometric propidium iodide cell cycle analysis

The HL-60 cells at concentration 1 x 10^6 cells/ml were treated with doxorubicin, betulinic acid and betulinic acid acetate with IC_{50} values in 6 well plates. The triplicate samples were incubated for 24, 48 and 72 h at 37°C, 5% CO_2, 90% humidity. The treated cells were collected by centrifugation at 2000 rpm for 10 min and the pellet was fixed in 500 µl ice-cold ethanol 70% at -20°C for 2 h. Then, the cell suspension was centrifuged, washed twice with 1 ml of PBS solution containing 0.06% sodium azide and resuspended in 1 ml of PBS solution containing 0.1% triton X-100, 100 mM EDTA, 50 µg/ml RNase (Sigma, USA) and 2 µg/ml propodium iodide (PI) (Sigma, USA). The tubes were placed on ice in the dark until the red fluorescence of DNA-bound PI in individual cells was measured by Beckman Epics Ultra flow cytometer and the results were analyzed using Expo32 software (Beckman Coulter, USA).

RESULTS

MTT cytotoxic effect of betulinic acid, betulinic acid derivatives and doxorubicin on various cancer cell lines

The cytotoxic effect of betulinic acid (BA), betulinic acid acetate (BAAC), 3-*O*-(2',2'-dimethylsuccinyl)-betulinic acid (BAES), 3-*O*-succinyl-betulinic acid (BASUC), betulinic acid benzoate (BCL) and doxorubicin (DOX) obtained against human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), murine myelomonocytic leukemia (WEHI-3B), human cervical epithelial carcinoma (HeLa), human breast adenocarcinoma (MCF-7), human glioblastoma (DBTRG0.5MG) and mouse skin melanoma (B16) cell lines was determined by a rapid colorimetric assay, using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT).

The cytotoxic activity of BA and its derivatives were tested using different cell lines and the cytotoxicity data of these compounds on HL-60 (human myeloid leukemia), CEM-SS (human T4-lymphoblastoid), WEHI-3B (BALB/c murine myelomonocytic leukemia), HeLa (human cervical epithelial carcinoma), MCF-7 (human breast adenocarcinoma), DBTRG0.5MG (human glioblastoma) and B16 (mouse skin melanoma) were summarized in Table 1.

The cytotoxic effects of betulinic acid and its derivatives were examined in HL-60 leukemia cell line after 72 h treatment. The IC_{50} values for betulinic acid (BA),

Table 1. The cytotoxicity data of betulinic acid (BA) and betulinic acid derivatives (BAAC, BAES, BASUC and BCL) against various cell lines. The inhibition concentration of 50% (IC_{50}) value was measured by MTT assay after 24, 48 and 72 h treatment.

Cell line	Time (h)	IC_{50} value (µg/ml)				
		BA	BAAC	BAES	BASUC	BCL
HL-60	24	20.70 ± 5.39	15.10 ± 2.57	>30.0	>30.0	>30.0
	48	14.60 ± 2.31	4.20 ± 1.67	18.70 ± 6.47	2.00 ± 1.00	>30.0
	72	2.60 ± 1.50	3.50 ± 1.03	1.38 ± 0.50	1.45 ± 1.06	2.10 ± 1.01
CEM-SS	24	5.40 ± 1.06	9.20 ± 2.19	>30.0	>30.0	>30.0
	48	2.30 ± 1.14	8.90 ± 3.55	>30.0	>30.0	>30.0
	72	2.10 ± 0.52	3.60 ± 0.53	>30.0	1.60 ± 0.81	>30.0
WEHI-3B	24	18.90 ± 1.65	13.70 ± 1.21	>30.0	>30.0	>30.0
	48	15.50 ± 6.87	16.60 ± 3.65	>30.0	>30.0	>30.0
	72	2.10 ± 1.03	3.90 ± 2.45	22.0 ± 2.16	>30.0	>30.0
HeLa	24	>30.0	>30.0	>30.0	>30.0	>30.0
	48	16.8 ± 0.60	3.0 ± 1.10	22.7 ± 0.42	27.4 ± 1.83	>30.0
	72	2.5 ± 3.70	3.1 ± 0.92	10.5 ± 2.53	22.8 ± 3.55	>30.0
MCF-7	24	>30.0	>30.0	>30.0	>30.0	>30.0
	48	>30.0	>30.0	>30.0	>30.0	>30.0
	72	20.4 ± 2.91	10.5 ± 1.03	20.3 ± 2.14	>30.0	10.4 ± 1.05
DBTRG0.5MG	24	>30.0	>30.0	>30.0	>30.0	>30.0
	48	>30.0	>30.0	25.9 ± 2.97	>30.0	>30.0
	72	>30.0	>30.0	10.1 ± 1.87	>30.0	>30.0
B16	24	>30.0	>30.0	>30.0	>30.0	>30.0
	48	>30.0	>30.0	>30.0	>30.0	>30.0
	72	>30.0	>30.0	>30.0	>30.0	>30.0

betulinic acid acetate (BAAC), 3-*O*-(2',2'-dimethyl-succinyl)-betulinic acid (BAES), 3-*O*-succinyl-betulinic acid (BASUC) and betulinic acid benzoate (BCL) against HL-60 cell lines were 2.60 ± 1.50, 3.50 ± 1.03, 1.38 ± 0.50, 1.45 ± 1.06 and 2.10 ± 1.01 µg/ml, respectively.

Amongst the leukemia cell lines, BAAC was the most toxic than other betulinic acid derivatives after 24 h treatment on HL-60, CEM-SS and WEHI-3B cell lines with the IC_{50} values of 15.10 ± 2.57, 9.20 ± 2.19 and 13.70 ± 1.21 µg/ml, respectively. Higher IC_{50} values were observed when BAES, BASUC and BCL were tested against HL-60, CEM-SS and WEHI-3B after 24 hours with IC_{50} values more than 30.00 µg/mL for all the cell lines. The sensitivity of HL-60, CEM-SS and WEHI-3B cell lines treated with BAAC at 72 h were almost similar resulting in the IC_{50} value of 3.50 ± 1.03, 3.60 ± 0.53 and 3.90 ± 2.45 µg/ml, respectively.

The results indicate that both BA and BAAC showed cytotoxic activity against HL-60, CEM-SS and WEHI-3B cell lines after 24 h treatment. The IC_{50} values of BA against HL-60, CEM-SS and WEHI-3B were 20.70 ± 5.39, 5.40 ± 1.06 and 18.90 ± 1.65 while the IC_{50} values for BAAC were 15.10 ± 2.57, 9.20 ± 2.19 and 13.70 ±1.21 µg/ml, respectively. BA showed strong cytotoxicity against HL-60, CEM-SS and WEHI-3B after 72 h treatment with IC_{50} value of 2.60 ± 1.50, 2.10 ± 0.52 and 2.10 ± 1.03 µg/ml, respectively.

In HeLa cancer cell line, BA was the best compound for cytotoxic activity followed by BAAC, BAES and BASUC. The results showed that both BA and BAAC exhibited the most cytotoxic to HeLa cell line after 72 h treatment with the IC_{50} values of 2.50 ± 3.70 and 3.10 ± 0.92 µg/ml, respectively. BAES showed the low cytotoxic effect against HeLa and DBTRG0.5MG after 72 h treatment with the IC_{50} value of 10.5 ± 2.53 and 10.1 ± 1.87 µg/ml, respectively.

In MCF-7 cancer cell line, BA and its derivatives showed less cytotoxic activity with IC_{50} values more than 10.00 µg/mL, each. The IC_{50} values of HL-60 after treatment with BA, BAAC, BAES and BCL for 72 h were 20.4 ± 2.91, 10.5 ± 1.03, 20.3 ± 2.14 and 10.4 ± 1.05 µg/ml, respectively. Betulinic acid and all its derivatives did not show a significant cytotoxicity against DBTRG0.5MG and B16 cell lines with their IC_{50} values were more than 30.00 µg/ml. The screening results for cytotoxic activity showed that HL-60 was the most sensitive cell line towards betulinic acid and its derivatives after 72 h treatment.

Betulinic acid and its derivatives were also screened for cytotoxicity on other cancer cell lines such as the

Table 2. The cytotoxicity data of doxorubicin (DOX) betulinic acid (BA) and betulinic acid acetate (BAAC) against human myeloid leukemia (HL-60) and normal human lymphocytes (PBMCs). The inhibition concentration of 50% (IC_{50}) value was measured by MTT assay after 24, 48 and 72 h.

Cell line	Time (h)	IC_{50} value (µg/ml)		
		DOX	BA	BAAC
HL-60	24	0.56 ± 0.25	20.7 ± 5.39	15.1 ± 2.57
	48	0.43 ± 0.06	14.6 ± 2.31	4.2 ± 1.67
	72	0.21 ± 0.03	2.6 ± 1.50	3.5 ± 1.03
PBMC	24	>30.0	>30.0	>30.0
	48	>30.0	>30.0	>30.0
	72	>30.0	>30.0	>30.0

cytotoxicity data of BA and its derivatives on HeLa, MCF-7, DBTRG0.5MG and B16 cell lines were summarized in Table 1.

Since BAAC exhibited strong cytotoxicity against all leukemia cells, it was selected as the test compound in further study. Table 2 showed the cytotoxicity data of DOX, BA and BAAC against human myeloid leukemia (HL-60) and normal human lymphocytes (PBMC) after 24, 48 and 72 h treatment. DOX a known commercial drug was used as a positive control in this study.

In HL-60 cell line, DOX appeared to be the most toxic drug with the IC_{50} values of 0.56 ± 0.25, 0.43 ± 0.06 and 0.21 ± 0.03 µg/ml at 24, 48 and 72 h of treatment, respectively. BAAC was more cytotoxic at 24 and 48 h treatment compared with BA with IC_{50} values of 15.10 ± 2.57, 4.20 ± 1.67 and 20.70 ± 5.39, 14.60 ± 2.31 µg/ml, respectively. In the 72 h treatment, BAAC was less cytotoxic than BA with IC_{50} values of 3.5 ± 1.03 and 2.60 ± 1.50 µg/ml, respectively.

The IC_{50} values of DOX, BA and BAAC on HL-60 were also compared with PBMC cell lines. Table 2 showed the results that DOX, BA and BAAC were not toxic to normal human lymphocytes (PBMC) with IC_{50} values more than 30.00 µg/ml, each.

DNA fragmentation effect of doxorubicin, betulinic acid and betulinic acid acetate on HL-60 cell lines

The DNA fragmentation, a biochemical hallmark of apoptosis was detected by DNA fragmentation assay. Detection of DNA fragmentation is currently one of the most frequently used techniques in the study of cell death. Internucleosomal DNA fragmentation can be visualized by gel electrophoresis as the characteristic DNA ladder pattern. DNA from apoptotic cells display a ladder formation by 1% agarose gel electrophoresis analysis of DNA extracted from HL-60 cells treated with IC_{50} values of doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC).

DNA fragmentation was observed at 24, 48 and 72 h after exposure to 0.2 µg/ml doxorubicin (Figure 2a). Cells treated with 2.6 µg/ml betulinic acid (Figure 2b) and 3.5 µg/ml betulinic acid acetate (Figure 2c), demonstrated a ladder pattern of DNA fragments were slightly detectable after 24 h and became visible after 48 and 72 h exposured. The patterns could not be detected in untreated HL-60 cells as the negative control (lane 1). The internucleosomal DNA fragmentation was confirmed by the pattern of DNA laddering into fragments with multiples of 180 - 220 base pairs detected in agarose gel electrophoresis of extracts obtained at 24 h from HL-60 treated with DOX, BA and BAAC.

Flow cytometry analysis of doxorubicin, betulinic acid and betulinic acid acetate on HL-60 cell lines

The cells with hypodiploid DNA were analyzed by Beckman Epics Ultra flow cytometer after PI staining at 24, 48 and 72 h treatment with IC_{50} values of doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC) to confirm the state of apoptosis. The percentage of cells with hypodiploid (sub G_1), which represent the fraction undergoing apoptotic DNA degradation that appeared in the cell distribution with DNA content less than G_1 was measured.

The cell cycle distribution of treated HL-60 cell lines in both BA and BAAC was almost similar with a sub G_1 of apoptosis population. The number of apoptotic cells in HL-60 cells increased slightly at this stage after 24, 48 and 72 h of treatment with BA and BAAC. When exposed to 2.6 µg/ml BA for 24, 48 and 72 h the apoptotic cells was demonstrated 8.75, 9.90 and 12.21% (Table 3) respectively. Approximately 6.20, 8.37 and 13.35% (Table 3) of apoptotic cells after 24, 48 and 72 h of treatment with 3.5 µg/ml of BAAC.

The DNA content of HL-60 cells treated with IC_{50} values of doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC) was determined using flow cytometer. The distribution of DNA content was expressed as sub G_1, G_1/G_0, S and G_2/M phase, inclusively to see if there was any arrest of the growth of the treated cells. The IC_{50} concentration of DOX, BA and BAAC was used to assess the extent of the arrest of cell growth after 72 h of treatment. Table 3 shows the cell cycle

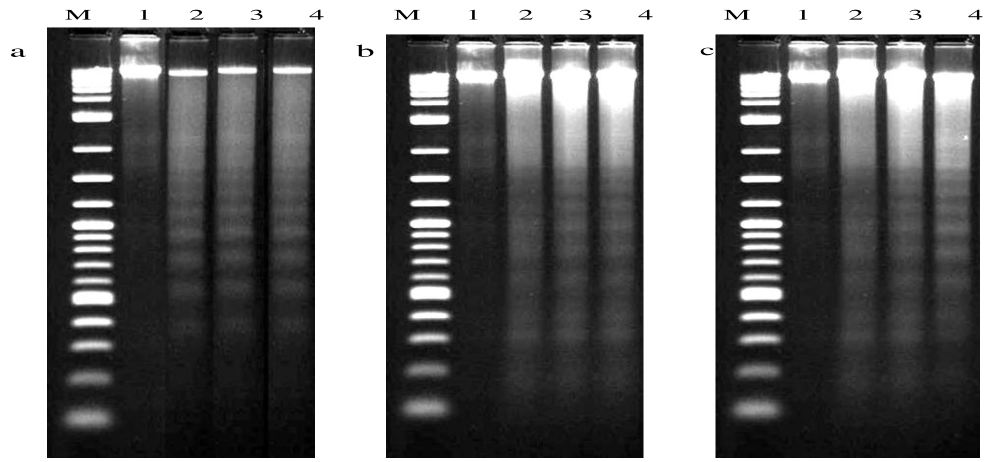

Figure 2. DNA ladder formation following exposure of HL-60 cells to doxorubicin, betulinic acid and betulinic acid acetate. (a) DNA fragmentation induced by untreated HL-60 cells (lane 1), HL-60 cells treated with 0.2 µg/ml doxorubicin for 24, 48 and 72 h (lane 2 - 4), (b) DNA fragmentation induced by untreated HL-60 cells (lane 1), HL-60 cells treated with 2.6 µg/ml betulinic acid for 24, 48 and 72 h (lane 2 - 4) and (c) DNA fragmentation induced by untreated HL-60 cells (lane 1), HL-60 cells treated with 3.5 µg/ml betulinic acid acetate for 24, 48 and 72 h (lane 2 - 4). M is 1 kb ladder.

Table 3. The cell cycle distribution of doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC) in HL-60 cell lines. HL-60 cells were treated with 0.2 µg/ml of DOX, 2.6 µg/ml of BA and 3.5 µg/ml of BAAC at 24, 48 and 72 h.

Treatment		**Cell cycle (% of total cells)**			
		Sub G_1	**G_1/G_0**	**S**	**G_2/M**
DOX (0.2 µg/ml)	Untreated	1.08	48.30	18.29	32.33
	24 h	5.92	51.68	21.85	20.55
	48 h	16.85	24.47	43.55	15.13
	72 h	60.11	7.62	27.28	4.99
BA (2.6 µg/ml)	Untreated	1.08	48.30	18.29	32.33
	24 h	8.75	21.78	42.54	26.93
	48 h	9.90	32.77	38.35	18.98
	72 h	12.21	43.67	23.15	20.97
BAAC (3.5 µg/ml)	Untreated	1.08	48.30	18.29	32.33
	24 h	6.20	39.99	37.92	15.89
	48 h	8.37	27.78	46.36	17.49
	72 ho	13.35	33.56	40.49	12.60

distribution of HL-60 cells following exposure to 0.2 µg/ml of DOX, 2.6 µg/ml of BA and 3.5 µg/ml of BAAC in the sub G_1, G_1/G_0, S and G_2/M phase. The cell cycle distribution after 24, 48 and 72 h of treatment were compared with untreated control cells. The distribution of untreated HL-60 cells represented 1.08% in sub G_1 population, 48.30% in G_1/G_0 population, 18.29% in S population and 32.33% in G_2/M population. The majority of cells were in G_1/G_0 phase in untreated HL-60 cells with 48.30% of cell population. The sub G_1 cell population was increased with an accompanying significant decrease in the G_2/M population after 24, 48 and 72 h of treatment with DOX, BA and BAAC.

The cell cycle profile in Table 3 showed an increase in the proportion of cells in S phase with 42.54% of cells and decrease in the proportion of cells in G_1/G_0 and G_2/M with 21.78 and 26.93% of cells after 24 h of treatment with 2.6 µg/ml of BA. The HL-60 cells have undergone arrest at S phase with 42.54 and 38.35% of cells after 24 and 48 h followed by arrest at G_1/G_0 phase with 43.67% of cells after 72 h. As a result, cells were unable to enter the subsequent G_2/M phase with 20.97% cells compared to 26.93% at 24 h. The percentage of cells population in G_2/M phase was reduced to 20.97% compared to 32.33% of untreated HL-60 after 72 h showed that BA also inhibit the proliferation of HL-60 cells.

On the other hand, BAAC at 3.5 µg/ml also induce G_1/G_0 arrest after 24 h of treatment. The cell cycle distributions in G_1/G_0 phase were 39.99, 27.78 and 33.56% after 24, 48 and 72 h of treatment, respectively. BAAC induced a significant increase in the proportion of cells in S phase after 48 h. The percent of cells in this phase became depreciate from 46.36% at 48 h to 40.49% at 72 h after treatment. At 72 h of treatment with BAAC the S phase arrested cells appeared to be capable of entering the following G_2/M phase, accounting for 12.60% cells compared to 17.49% at 48 h. The cell cycle blockage progress from G_1/G_0 to S and G_2/M phase with increasing the incubation time. Collectively, the data show that in HL-60 cells, the IC_{50} value of BAAC produced a cell population in which some cells were undergoing S phase cell cycle arrest while others were undergoing DNA degradation.

DISCUSSION

Cytotoxic has been defined as the cell killing property of a chemical compound independent from the mechanism of death (Graham-Evans et al., 2003). Assessment of a compound's toxicity to various cell types can be made using *in vitro* cytotoxicity tests, which are available and widely used. The inclusion of an *in vitro* cytotoxicity assay in early discovery efforts provides an important advantage in identifying potentially cytotoxic compounds (Hamid et al., 2004). One effect of reactive chemicals potentially encountered at subtoxic concentrations is the direct interaction with DNA that will result in various types of damage, including promutagenic lesions (Eisenbrand et al., 2002).

Cytotoxicity data are of their own intrinsic value in defining toxic effects (e.g. as an indicator of acute toxic effects *in vivo*) and are also important for designing more in-depth *in vitro* studies (Eisenbrand et al., 2002). The effective dose for a 50% reduction in cell number for plants products to be considered cytotoxic should be less than 20 µg/ml (Geran et al., 1972). The IC_{50} which is the drug concentration that kill 50% of the cells was determined graphically after 24, 48 and 72 h of treatment with doxorubicin (DOX), betulinic acid (BA) and betulinic acid derivatives on human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B), human glioblastoma (DBTRG0.5MG), mouse skin melanoma (B16) and human peripheral blood mononuclear cells (PBMC) and used as a measure of cytotoxic effect.

The cytotoxic effects of four betulinic acid derivatives compounds (BAAC, BAES, BASUC and BCL) were examined in seven cancer cell lines. These BA and its derivatives are the lupane triterpene with a carbonyl group at C-17 that has been modified at C-3 hydroxy group. In screening studies, several derivatives have shown better cytotoxicity than BA. Betulinic acid acetate (BAAC) had shown broad spectrum cytotoxicity than other betulinic acid derivatives. It had most active cytotoxic activity against human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B) and human cervical epithelial carcinoma (HeLa) but not on normal human lymphocytes (PBMC).

It is known that the cytotoxicity of isoprenoid carboxylic acid derivatives is often related to the presence of a free carboxyl group in the molecule (Mutai et al., 2004). In general, C-28 carboxylic acid group in betulinic acid and its derivatives was found essential for providing cytotoxic activity (Muherjee et al., 2004). Furthermore the position of the hydroxyl on C-3 is more important than on C-28, the presence of the conjugated carbonyl influences the activity very slightly and finally the presence of two hydroxyls (on C-3 and C-28) results in a reduction of activity (Mutai et al., 2004).

The study of the relationships between structure and activity of lupane triterpenes demonstrated that the carbonyl group at C-17 played an important role on the induction of melanoma cell apoptosis (Hata et al., 2003) Lup-28-al-20(29)-en-3-one, betulinic acid (BA) and other lupane triterpene with a carbonyl group at C-17 showed marked cytotoxic effects on leukemia, neuroblastoma and melanoma cells, but not on normal lung fibroblast cells (W138). It seemed that the carbonyl group at C-17 might be essential for the induction of cancer cell apoptosis by these triterpenes (Hata et al., 2003).

The modification structure at C-3 hydroxy group of BA produced potentially BAAC which may develop as antitumor drugs. The relationships between the structure and activities of BA and BAAC on the induction of HL-60 cell differentiation and apoptosis were studies. This study focused on HL-60 cells, a human leukemia cell line that readily undergoes apoptosis in response to a variety of chemotherapeutic agents (Kaufmann, 1989). HL-60 cell has been widely used as a model for studying premyelocytic cell differentiation and the identification of differentiation-inducing agents (Poon et al., 2004).

In this study, BA isolated from *M. cajuput* a Malaysian plant and all its derivatives have indicated significant growth inhibition in HL-60 human leukemia cancer cell line at low concentration of IC_{50} values. BA from this *M. cajuput* marked the IC_{50} values at 2.60 µg/ml (5.70 µM). This result is same as the experiment that was done using BA from Aldrich (St Louis, MO) at the concentration range of 1 to 12 µM (0.47 to 5.47 µg/ml) that showed toxicity to HL-60 cells with IC_{50} value at 5.7 µM and induced apoptosis in about 10% of surviving cells. Part of the toxic effect may be due to its inhibitory effects on topoisomerase I and II in some cell types and therefore may affect DNA replication (Chowdhury et al., 2003). In other study, it was reported that the IC_{50} values of BA against the cell growth of HL-60 was 6.6 µM (Hata et al., 2003).

One of the interesting findings was that DOX, BA and BAAC showed very high IC_{50} values towards normal PBMC cell lines. The IC_{50} values of DOX, BA and BAAC were up to 30 µg/ml. It has been reported that BA from Sigma Chemical, St. Louis, MO, USA also showed the IC_{50} values up to 50 µg/ml on the peripheral blood lymphoblast (PBL) whereas the IC_{50} of DOX was 0.020 ± 0.002 µg/ml. Thus on normal PBL BA was at least 1000 fold less toxic than DOX (Zuco et al., 2002). This differential cytotoxicity of BA and these compounds towards the normal and cancerous cell, could be taken advantage of in therapeutics.

It has been reported that the low concentrations of several different drugs in HL-60 cells induced cell death by apoptosis while higher concentration caused necrotic cell death when cells were assessed morphologically and by DNA gel electrophoresis (Lennon et al., 1991). Low concentration of BA and BAAC took a longer time for the degradation of large fragments of DNA to smallest fragments of approximately 200 bp. This kind of ladder formation was because DNA fragmentation during apoptosis proceeds through an ordered series of stages beginning with the production of DNA fragments of 300 kbp, which are then degraded to fragments of 50 kbp. Fragments of this size are further degraded to smaller fragments of 10 to 40 kbp and finally to small oligonucleosome fragments of 180 to 200 bp that are recognized as the characteristic DNA ladder. These phenomena could be further confirmed using pulsed-field gel electrophoresis (PGFE), which can detect the initial stage of DNA fragmentation to fragments of 300 and 50 kbp (Walker et al., 1993).

The biochemical hallmark of apoptosis is the orderly 200 base pairs DNA ladder fragmentations, which have recently been further characterized as the signature of apoptosis from apoptosis specific endonucleolytic cleavages (Zhang and Xu, 2002). The apoptosis signature cleavages are also suggested as the cause of 50 kilobase (kbp) high molecular weight fragmentations that mark early or stage 1 apoptosis (Zhang et al., 2000), but the association appears to be not an imperative correlation (Samejima et al., 2001). 50 kbp fragmentations are known to be expressed in necrosis which has not been shown expressing neither 200 bp ladder fragmentations nor specific megabase level fragmentations (Bicknell and Cohen, 1995).

In many systems, DNA fragmentation has been shown to results from activation of an endogenous Ca^{2+} and Mg^{2+} dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units, a linker DNA generating mononucleosomal and oligonucleosomal DNA fragments. These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 base pairs subunit. This is the biochemical hallmark of apoptosis with the fragmentation of the genomic DNA, an irreversible event that commits the cell to die (Cohen, 1993).

Flow cytometry allows a simultaneous estimation of cell cycle parameters and apoptosis. There is compelling evidence that apoptotic death induced by chemopreventive or chemotherapeutic agents is closely linked to perturbation of a specific phase of the cell cycle. The effect of a given antiproliferative agent on cell cycle progression appears to depend on the concentration of the compound and also on the duration of the treatment (Surh et al., 1999). The induction of apoptosis was also confirmed by flow cytometric analysis of cell cycle. Doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC) have been shown to induce a time dependant increase in the sub G_1 peak with the decrease of cells in diploid regions (G_1, G_2 and S-phase) indicating apoptotic phenomenon as obtained from the DNA content histogram analysis.

Cell cycle analysis revealed that BA induced apoptosis and cell cycle arrest at G_0/G_1 phase in HL-60 cell. Approximately 8.75% of viable cell were in the sub G_1 indicating the apoptotic phase was found after treatment with 2.6 µg/ml of BA for 72 h. The G_0/G_1 arrest shown by the above compounds therefore, suggest that these agents may slow down the growth of cancer cells by artificially imposing the cell cycle checkpoint. Among these checkpoints, p53 is the most vital G1 checkpoint protein that can either lead to growth arrest in G1 or apoptosis (Dou et al., 1995).

This finding was similar with the previous report that apoptosis towards HL-60 cell with the IC_{50} value of 5.7 µM induced apoptosis in cell cycle analysis with approximately 10% of viable cells in the sub G_1 phase after exposure of the cell to 12 µM of BA for 72 h treatment (Poon et al., 2004). However, the apoptosis rate of BA towards HL-60 is considered low if compared to its derivatives 23-hydroxybetulinic acid. 23-hydroxybetulinic acid induced apoptosis in HL-60 cells with approximately 46.61% cells were in sub G_1 after exposure of cells to 10 µM for 24 h. Subsequently, the apoptotic events in their experiment were associated with concurrent down-regulation of Bcl-2 and telomerase activity (Ji et al., 2002).

It has been widely reported that DHD_3 initially increases cell proliferation, which is followed by cell differentiation and maturation (Brown et al., 1999). DHD_3 at 1 µM altered cell cycle distribution with an increased G_1 population after 72 h of incubation. The addition of various concentrations of BA (3 to 6 µM) to 1 µM DHD_3 resulted in a does-dependent and statistically significant increase in the G_1 population with a concomitant reduction in the S phase population. Less than 2% of cells were apoptotic under these conditions (Poon et al., 2004). Arrest in S and G2/M phase as strongly evident in etoposide-treated population may be contributed by extensive chromosome damage (Arita et al., 1997). Principally, it acts by inhibiting topoisomerase II that entangles excessive twists or knots in the DNA helix which would otherwise arise during replication. This enzyme makes a transient double strand break in the first duplex to create DNA 'gate' for the nearby second duplex to pass through and then reseals the break (Berger et al., 1996).

Differentiated cells are normally inactive in cell division and arrested in G_1 phase of the cell cycle. Significant G_1 arrest was observed when HL-60 cells were treated with DHD_3. This G_1 arresting action of DHD_3 was also enhanced with the addition of BA, but cell number was not affected under the same conditions (Poon et al., 2004). DHD_3 initially accelerates cell proliferation, which is followed by cell differentiation and maturation (Brown et al., 1999). A single DHD_3-treated HL-60 cell would give rise to 10 or more matured monocytes. The action of BA in enhancing DHD_3 induced NBT reduction, membrane marker expression and G_1 cell cycle arrest provide corroborative evidence that BA and DHD_3 act synergistically in inducing differentiation in HL-60 cells (Poon et al., 2004).

Cell cycle arrest is one of the targets of many anticancer drugs, such as doxorubicin, cisplatin, 5-fluorouracil and paclitaxel. It has been shown that the ability of cells to arrest cell cycle in G_2/M or S phase was related to their drug sensitivity and increased with cell resistance (Dubrez et al., 1995). Induction of apoptosis and/or cell proliferation inhibition is highly correlated with the activation of a variety of intracellular signaling pathways to arrest the cell cycle in the G1, S, or G2 phase. In malignant tumors cell, population in G_1 phase appear less frequent (< 70%) than in normal tissue (> 90%). The damages that cause G_1-check point arrest are believed to be irreversible process and the cells ultimately undergo apoptosis (Roy et al., 2004).

In summary, betulinic acid (BA) and betulinic acid acetate (BAAC) showed selective cytotoxic towards all HL-60, CEM-SS and WEHI-3B leukemic cell line and are not toxic to PBMC. This study demonstrated that those compounds are potentially good anti cancer drug since they are non-toxic towards healthy cell.

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