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Cytotoxic effect of damnacanthal, nordamnacanthal, zerumbone and betulinic acid isolated from Malaysian plant sources

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Abstract: The present study was to evaluate the toxicity of damnacanthal, nordamnacanthal, betulinic acid and zerumbone isolated from local medicinal plants towards leukemia cell lines and immune cells by using MTT assay and flow cytometry cell cycle analysis. The results showed that damnacanthal significantly inhibited HL-60 cells, CEM-SS and WEHI-3B with the IC50 value of 4.0 µg/mL, 8.0 µg/mL and 3.3 µg/mL, respectively. Nordamnacanthal and betulinic acid showed stronger inhibition towards CEM-SS and HL-60 cells with the IC50 value of 5.7 µg/mL and 5.0 µg/mL, respectively. In contrast, Zerumbone was demonstrated to be more toxic towards those leukemia cells with the IC50 value less than 10 µg/mL. Damnacanthal, nordamnacanthal and betulinic acid were not toxic towards 3T3 and PBMC compared to doxorubicin which showed toxicity effects towards 3T3 and PBMC with the IC50 value of 3.0 µg/mL and 28.0 µg/mL, respectively. The cell cycle analysis exhibited that damnacanthal exerted its toxicity effect towards HL-60 cells by inducing apoptosis with value of 25% after 72 hours treatment. Thus, these compounds could be the potential anticancer drug with less toxic side effect.

Keywords: Betulinic acid, cytotoxic, damnacanthal, nordamnacanthal, zerumbone

Introduction

Malaysia is a tropical rainforest country and stores the most biologically diverse environment in the world (Lee and Houghton, 2005). The huge diversity of the Malaysian forest means that we can expect well diverse chemical structure from their secondary metabolite and chemical diversity for searching the new agent or novel drug which suite with the current demand particularly in curing various chronic diseases. It is believed that forest dwellers in Southeast Asia particularly in Malaysia use 6,500 different plants to treat illness. These same rain forests have supplied the western world with herbs and spices for centuries.

Damnacanthal and nordamnacanthal comprise a general class of athraquinone derivatives. Damnacanthal or 3-hydroxy-1-methoxyanthraquinone-2-carboxaldehyde ($C_{16}H_{10}O_5$), occurs as pale yellow crystals with a melting point of 210-211°C whereas nordamnacanthal or 2-formyl-1,3-dihydroxyanthraquinone ($C_{15}H_8O_5$), are orange yellow crystals with melting point of 214-218°C. Both these naturally occurring quinone are present widely in the *Morinda* species. Damnacanthal and nordamnacanthal have some unique chemical and biological properties. Ali *et al*. (2000) reported that damnacanthal and nordamnacanthal isolated from *M. elliptica* were cytotoxic towards the MCF-7 (breast carcinoma) and CEM-SS (T-lymphoblastic leukemia) cell lines.

Zerumbone is a monocyclic sesquiterpene can be found abundantly in rhizomes particularly from *Zingiber zerumbet* Smith and *Zingiber aromaticum* (Murakami *et al*., 2002). Its molecular formula is $C_{15}H_{22}O$. Zerumbone is a food phytochemical

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possessing great potential for use in chemoprevention and chemotherapy strategies against cancers. Based on the extensive research done previously, zerumbone has shown very potent anticancer and antitumor activity. Takada *et al.* (2005) found that zerumbone suppressed the activation NF-KappaB and NF-KappaB-regulated gene expression induced by carcinogens, and reported that this inhibition may provide molecular basis for the prevention and treatment of cancer. In the same year as well, Huang *et al*. (2005) identified that zerumbone inhibited the growth of P-388D cells and induced DNA fragmentation in culture and significantly prolonged the life of P-388D(1)-bearing CDF(1) mice. Furthermore, it also inhibited the growth of human leukemia cell line (HL-60 cell) and human colon cancer (HT-29) *in vitro* (Kirana *et al*., 2003).

Betulinic acid [3β-hydroxy-lup-20(19) lupean-28-carbonic acid] is a lupine-type triterpene which was first isolated in 1948 from bark of the London plane tree (*Platanus acerifolia*) (Jung and Duclos, 2006). It can be found abundantly in the bark of the white birch (*Betula alba*) or can also be chemically derived from betulin (Rene *et al*., 2006). Betulin is an abundant naturally occurring triterpene and it is found predominantly in bushes and trees. Both betulin and betulinic acid possess a wide spectrum of biological and pharmacological activities (Sami *et al*., 2006). Betulinic acid and its derivatives showed cytotoxicity against variety of tumor and cancer cell lines comparable to some clinically used drug. For instant, Jeremias *et al*. (2004) found that in vitro betulinic acid induced specific cell death of more than 75% in primary glioblastoma multiforme cells at substantially higher rate than established cytotoxic drugs vincristine. When betulinic acid was compared for *in vitro* efficiency against several leukemia cell lines with conventionally used cytotoxic drugs, betulinic acid was more active than 9 out of standard therapeutic such as L-asparaginase, dexamethasone, prednisolone and 6-thioguanine (Ehrhardt *et al*., 2004).

Although the cytotoxicity of these compounds which derived from local plant source has been studied intensively, they have not been tested and compared yet. Besides, the effect of the compounds on the cell cycle progression of HL60 also not been fully discovered yet. Thus, this study was carried out to investigate the cytotoxicity of these compounds and their effect towards the cell cycle progression of HL60.

Materials and Methods

Cells

The cancerous and non-cancerous cell lines (suspension and anchorage-dependent cells) were obtained from the American Type Culture Collection (ATCC), the National Cancer Institute (NCI) and the RIKEN Cell Bank (RCB). The cancerous cell lines used were CEM-SS (Human T-lymphoblastic leukemia) (RCB), HL-60 (Human acute promyelocytic leukemia) (ATCC) and WEHI-3B (Mouse myelomonocyte leukemia) (NCI). The non-cancerous cell line and normal cells were 3T3 (Mouse embryo fibroblast) (ATCC) and human peripheral blood mononuclear cells (PBMC). PBMC were kindly supplied by Mr. Yeap Swee Keong, Mr. Teo Guan Young, Ms. Haszalina Md. Isa and Ms. Rohaya Ibrahim from Institute of Biosciences and Faculty of Biotechnology and Biomolecular Sciences, UPM. Blood (20-25mL) was taken from four donors by using the 25 mL syringe. The blood sample was diluted with same volume of PBS. After that, the diluted blood sample was carefully layered on Ficoll-Paque Plus (Amersham Biosciences, USA) the ratio of 2:1. The mixture was centrifuged at 400 x g for 40 minutes at 18-20^{o}C. The undisturbed lymphocyte layer was carefully transferred out. The lymphocyte was washed and pelleted down with three volume of Phosphate buffer saline (PBS) for twice and resuspended in DMEM media (Flowlab, Australia) with 100 IU/mL of penicillin, 100 µg/mL of streptomycin (Flowlab, Australia), 10% v/v Fetal Bovine Serum (FBS) (PAA, Austria). Cell counting was performed to determine the PBMC cell number in equal volume of trypan blue. PBMC was used as a normal positive.

Compounds

The damnacanthal and nordamnacanthal which were kindly supplied by Prof. Nordin Haji Lajis from Institute of Bioscience were isolated from the roots of *Morinda elliptica* (Ismail *et al*., 1997). The zerumbone which was isolated from the rhizome of *Zingiber zerumbet* was obtained from Prof. Dr. Hasnah Siraj, Universiti Teknology Malaysia (UTM). Betulinic acid which was extracted from the bark of *Melaleuca cajuputi* was kindly supplied by Associate Prof. Dr. Faujan Ahmad, from Department of Chemistry, Faculty of Science, Universiti Putra Malaysia. All the compounds were isolated from the natural resources as stated above. The powdered-form compounds (damnacanthal, nordamnacanthal, zerumbone and betulinic acid) were dissolved in dimethylsulphoxide (DMSO) (Sigma, USA) to get a stock solution of 10

mg/mL. The substock solution of 0.06 mg/mL was prepared by diluting 6 µl of the stock solution into 994 µL serum-free culture medium, RPMI 1640 (the percentage of DMSO in the experiment should not exceed 0.5). The stock and substock solutions were both stored at 4^0C. Doxorubicin was used as a positive control. This commercial drug was prepared by dissolving it with RPMI medium. The stock and substock solutions were prepared as above.

Cytotoxicity Assay

Varying concentrations of 0.46, 0.93, 1.87, 3.75, 7.5, 15 and 30 µg/mL of damnacanthal, nordamnacanthal, zerumbone and betulinic acid were prepared from the substock solutions by serial dilution in RPMI-1640 to give a volume of 100 µL in each microtitre plate well. Each well was then added with 100 µL of cell lines in complete growth media (RPMI 1640) 5 X 10^5 cells/mL. Controls that contained only the cells were also prepared for each sample. The assay for each concentration of compounds was performed in triplicate. The plate was then incubated at 37^0C, 5% CO_2, 90% humidity for 72 h. Cytotoxicity of the tested compounds was measured using a modified MTT assay (Sigma, USA) (Mosmann, 1983). MTT was dissolved in phosphate buffer saline (PBS) (pH 7.5) at 5 mg/mL. The MTT stock solution was added directly to all appropriate microtitre-plate wells (20 µL for each well). The plate was then incubated for 2 to 4 h at 37^0C and 5% CO_2. After incubation, MTT were reduced to insoluble purple formazan crystals by metabolically active cells in the wells. Subsequently, the supernatant was aspirated and 100 µL of Dimethylsulfoxide (Sigma, USA) was added and mix thoroughly to dissolve the dark blue formazan crystals. The optical density (OD) was measured on an automated spectrophotometric EL 340 multiplate/microelisa reader (Bio-Tek instruments Inc) using test and reference wavelength of 570 nm. The cytotoxic dose that kills cells by 50% (IC_{50}) was determined from absorbance versus concentration curve.

Flow Cytometer Analyses

Flow cytometer was used to support the cytotoxicity results of the cancer cells and the proliferation of human PBMC in the MTT assay. For cytotoxicity study, HL-60 was chosen since that most of the compounds in this study exhibited stronger inhibition with the IC_{50} value less than 5 µg/mL. Nordamnacanthal however was not tested by cell cycle analysis due to the higher IC_{50} value (23.3 µg/mL) for cytotoxic effect as well as the source of nordamnacanthal was also limited. The HL-60 was treated for 24, 48 and 72 h with damnacanthal, betulinic acid, zerumbone and doxorubicin at their respective IC_{50} value.

In this study, 1 mL of HL-60 cells with a density 1 x 10^6 cells/mL, respectively were treated with 1 mL of damnacanthal, betulinic acid, zerumbone, doxorubicin and PWM according to their IC_{50} value (for cytotoxicity study). The treatment was carried out in the 6 well plates (Nunc) with the total working volume of 2 mL for each well. The treated cells were than incubated for 24, 48 and 72 h and harvested by centrifugation at 1000 rpm (200 x *g*) for 10 min. Subsequently, the treated cells were fixed with 80% ethanol at 4^oC for 2 h. Then, the cells were spun down and washed twice with PBS pH 7.5. The cell pellets were finally dissolved and stained in PBS buffer consist of 0.1% triton X-100, 10 mM EDTA, 50 µg/mL RNase and 3 µg/mL propidium iodide (PI). The cell was then incubated for 30 min in 4^oC and analyzed using the COULTER EPICS ALTRA flow cytometer (Beckman Coulter, USA) at the Laboratory of Biologic, Faculty of Veterinary Medicine, UPM within 24 h.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± S.E.

Results

Cytotoxicities of pure compounds

The results of cytotoxicities of compounds on leukaemia cell lines and normal cells are shown in Table 1. Damnacanthal was found to inhibit most leukemia cell lines tested with the IC_{50} value less than 10 µg/mL. Damnacanthal was showed stronger inhibition towards WEHI-3B and HL-60 with the IC_{50} value of 3.3 ± 0.5 µg/mL and 4.2 ± 0.1 µg/mL, respectively and it showed moderate effect towards CEM-SS with the IC_{50} value of 8.5 ± 0.5 µg/mL. More interestingly, it showed less effect towards 3T3 and PBMC with the IC_{50} value more than 30 µg/mL. Although nordamnacanthal share the same derivatives with damnacanthal, it exhibited variable effects towards leukemia cell lines. Nordamnacanthal only showed stronger inhibition towards CEM-SS with the IC_{50} value of 5.7 ± 0.4 µg/mL and exhibited less toxicity effect towards HL-60 cells with the IC_{50} value of 23.3 ± 0.7 µg/mL. In addition, it did not affect WEHI-3B, PBMC and 3T3 cells.

Betulinic acid, exhibited stronger inhibition towards HL-60 and WEHI-3B with the IC_{50} value of 4.5 ± 0.6 µg/mL and 10.0 ± 0.7 µg/mL, respectively. It also demonstrated less effect towards CEM-SS

Table 1. Effects of damnacanthal, nordamnacanthal, betulinic acid, zerumbone towards several leukemia cell lines and normal cell at their respective IC50. Data represent means ± SD of triplicate determinations from three independent experiments.

Cell Line / Compounds	IC50 value (µg/mL)				
	HL-60	CEM-SS	WEHI-3B	PBMC	3T3
Damnacanthal	4.2 ± 0.1	8.5 ± 0.5	3.3 ± 0.5	> 30	> 30
Nordamnacanthal	23.3 ± 0.7	5.7 ± 0.4	> 30	> 30	> 30
Zerumbone	7.4 ± 0.8	1.6 ± 0.8	4.8 ± 0.2	> 30	30.0
Betulinic acid	4.5 ± 0.6	20.5 ± 0.8	10.0 ± 0.7	> 30	> 30
Doxorubicin	1.2 ± 0.1	2.6 ± 0.2	1.0 ± 0.9	28.0 ± 0.1	3.0 ± 0.8

with the IC_{50} value of 20.5 ± 0.8 µg/mL. Similar to damnacanthal and nordamnacanthal, it also did not inhibit 3T3 and PBMC. In contrast, zerumbone was found to inhibit 3T3 cell at concentration of 30 µg/mL. Nonetheless, this toxicity effect is considered low as suggested by Shier (1991). According to Shier (1991), compounds which demonstrated the IC_{50} value of more than 10-25 µg/mL will be considered weak cytotoxicity while compounds with the IC_{50} value of less than 5.0 µg/mL were considered very active. Those compounds having intermediate value 5.0-10.0 µg/mL were classified as moderately active. Interestingly, zerumbone showed stronger inhibition towards the leukemia cell lines with the IC_{50} value less than 8 µg/mL. It was most toxic towards CEM-SS with the IC_{50} value of 1.6 ± 0.8 µg/mL, followed by WEHI-3B and HL-60 with the IC_{50} value of 4.4 ± 0.2 µg/mL and 7.4 ± 0.8 µg/mL, respectively.

As compared to all of the natural isolated compounds in this study, doxorubicin which is the drug for chemotherapy possessed strong cytotoxicity against HL-60, CEM-SS and Wehi-3B with IC_{50} lower than 5 µg/mL (Table 1). However, unlike all of the natural isolated compounds which did not show any IC_{50} at the range of the concentration in this study, doxorubicin exhibited strong cytotoxicity against mice fibroblast 3T3 (IC_{50} 3 µg/mL) and human PBMC (IC_{50} 28 µg/mL).

Cell Cycle Analysis of Damnacanthal, Betulinic Acid, Zerumbone and Doxorubicin on HL-60 Cell

The changes in the cell cycle distribution of HL-60 cells after been treated for 24, 48 and 72 h with damnacanthal, betulinic acid, zerumbone and doxorubicin at their respective IC_{50} value are illustrated in Table 2.

Analysis of the cell cycle profile showed there was an increase in Sub G1 cell population in HL-60 cells treated with damnacanthal after 24 h (Table 2). In addition, longer exposure time has lead to an additional increase in the percentage of Sub G1 cells with value of 1.73% after 24 h up to 25% after 72 h treatment, respectively. Data also showed that the percentage of cells at G2/M phases were decreased steadily from 32 % (control) down to 16.95% after 72 h treatment, suggesting that damnacanthal inhibited the progression of HL-60 cells after been treated for longer incubation periods. Among the three compounds tested, damnacanthal induced a higher percentage of apoptosis on HL-60 with value almost 25%.

On the other hand, HL-60 treated with 4.5 µg/mL of betulinic acid exhibited a slight increase in Sub G1 phase cells after been treated from 24 to 72 h with value of 1.07% up to 8.41%, respectively (Table 2). This data indicated that betulinic acid induced a low production of apoptosis rate if compared to damnacanthal. HL-60 treated with betulinic acid also showed a reduction in S phase cell population when induction time was persisted from 24, to 72 h with value of 21.7% reduced to 17.34%, respectively. Data also designated that betulinic acid arrested HL-60 cells at G0/G1 phase of the cell cycle with value of 63.18%, 67.14% and 64.45% after been treated for 24, 48 and 72 h. Apart from that, betulinic acid also showed to inhibit the progression of HL-60 cells by reducing the percentage of cells at G2/M phase from 32% (control) down to 6.33% after 72 h treatment,

Table 2. Cell cycle distribution of HL-60 cells after been treated at 24, 48 and 72 h with damnacanthal, betulinic acid, zerumbone and doxorubicin at their respective IC_{50} value. Data represent means ± SD of triplicate determinations from three independent experiments.

Compounds	Percentage of cell cycle distribution (%)	Incubation period (h)		
		24	48	72
Untreated cell	Sub-G1 (apoptosis)	1.06 ± 0.02	1.51 ± 0.42	1.14 ± 0.70
	G0/G1	47.35 ± 0.82	46.87 ± 1.35	16.63 ± 0.91
	Synthesis	17.74 ± 0.31	21.04 ± 0.60	55.64 ± 0.31
	G2/Mitosis	31.61 ± 0.55	27.90 ± 0.80	28.09 ± 0.16
Damnacanthal	Sub-G1 (apoptosis)	2.14 ± 0.72	6.24 ± 0.21	24.99 ± 0.56
	G0/G1	39.52 ± 0.69	13.96 ± 0.42	15.18 ± 0.35
	Synthesis	27.44 ± 0.70	67.32 ± 0.20	43.37 ± 0.91
	G2/Mitosis	30.58 ± 0.90	12.34 ± 0.20	16.95 ± 0.35
Betulinic acid	Sub-G1 (apoptosis)	2.14 ± 0.76	3.72 ± 0.89	8.33 ± 0.27
	G0/G1	61.97 ± 1.59	64.50 ± 3.48	61.55 ± 4.57
	Synthesis	23.56 ± 3.34	17.55 ± 1.96	16.24 ± 1.78
	G2/Mitosis	11.44 ± 1.80	10.35 ± 5.04	9.85 ± 3.82
Zerumbone	Sub-G1 (apoptosis)	2.43 ± 0.37	4.35 ± 0.06	4.81 ± 0.08
	G0/G1	63.75 ± 1.37	67.48 ± 0.82	68.51 ± 0.82
	Synthesis	19.22 ± 0.88	17.35 ± 0.85	16.71 ± 1.08
	G2/Mitosis	12.38 ± 2.92	15.68 ± 2.76	14.71 ± 1.32
Doxorubicin	Sub-G1 (apoptosis)	6.35 ± 0.01	17.2 ± 0.04	59.59 ± 0.77
	G0/G1	52.04 ± 0.12	24.81 ± 0.06	17.15 ± 0.47
	Synthesis	22.25 ± 0.09	37.86 ± 0.11	10.96 ± 13.86
	G2/Mitosis	20.95 ± 0.04	15.49 ± 0.04	6.53 ± 0.70

various leukemia cell lines. Damnacanthal and nordamnacanthal were also possessed cytotoxicity towards human small cell lung cancer (NCI-H187), human breast cancer (BC and MCF-7) and Epstein Barr Virus in Raji cell (Ali *et al.,* 2000; Jasril *et al.,* 2003; Kwanjai *et al.,* 2005) . These results suggested that damnacanthal and nordamnacanthal have broad cytotoxicity towards various cancer cell lines whilst damnacanthal was found to be more toxic towards HL-60, CEM-SS and WEHI-3B as compared to, nordamnacanthal in this study (Table 1).

Zerumbone is a natural cyclic sesquiterpene moiety that can be found abundantly in rhizome of the wild ginger, *Zingiber zerumbet Smith* (Lechat-Vahirua *et al*., 1993; Murakami *et al*., 1999). It is known to have a powerful tool in the implementation of green chemistry with latent reactivity which contains three double bonds, two conjugated and one isolated as well as a double conjugated carbonyl group in 11-membered ring structure (Kitayama *et al*., 2006). Zerumbone has been identified as a distinct suppressor of tumor promoter 12-*O*-tetradeconoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus (EBV) activation in Raji cells, inhibitor of free radical generation, iNOS expression and TNF-α release, inhibitor of various human colonic adenocarcinoma cell lines proliferation (L8174, LS180, COLO205 and COLO320DM) with little effects on the growth of normal dermal and colon fibroblast (Murakami et al, 1999; 2002; 2004) and suppressor of human cancer cells growth via the induction of apoptosis (Kirana *et al*., 2003).

In this study, zerumbone showed stronger cytotoxic effects towards CEM-SS, HL-60 and WEHI-3B. The variable cytotoxic effects of zerumbone towards those cells are due to its versatile chemical structure of α-β-unsaturated carbonyl group. This specific group in zerumbone had been shown to play the pivotal roles in its interaction with the most biological molecules (Murakami *et al.,* 2002). Murakami *et al*. (2002) discovered that α-humulene which lacked the functional group (α-β-unsaturated carbonyl group), was virtually and consistently inactive towards various pharmalogical activities.

indicating that it has anti-proliferative effect towards HL-60 cells.

HL-60 cultivated with 4.4 µg/mL zerumbone showed a slight increase in Sub G1 after been treated for 48 h with value of 4.36% (Table 2). However, this percentage remained constant until 72 h treatment indicating that zerumbone only induced low apoptosis rate in HL-60 cells throughout the treatment duration. A steady production of Sub G1 cell population after been treated for 48 h corresponded to an increase in cells population arrested at G0/G1 with almost 69.93% cells at this phase after 72 h treatment. On the other hand, data also demonstrated that the percentage of HL-60 cells treated with zerumbone reduced dramatically from 32% (control) down to 15.58% after been treated for 72 h, leading to inhibition of cell division. This data suggesting that zerumbone might have anti-proliferative effects towards HL-60 cells.

When compared to all of the isolated natural compounds, doxorubicin showed the best effect to induce apoptosis on HL-60 in the time dependent manner. The percentage of sub-G1 increased gradually from 24 h (6.35 ± 0.01%) to 72 h (59.59 ± 0.77%).

Discussion

Damnacanthal and nordamnacanthal are anthraquinones produced mainly by plant of the Rubiaceae family. Both compounds were commonly isolated from *Morinda citrofolia*, *Morinda elliptica*, *Morinda lucida* and *Prismatomeris fragrans* (Ismail *et al*., 1997; Tosa *et al*., 1998; Kwanjai *et al*., 2005). Damnacanthal and nordamnacanthal were known to possess some unique chemical and biological properties.

Apparently, both compounds showed toxicity effect towards leukemia cell lines which are categorized as anchorage-independent cell lines. Previously, Ali *et al*. (2000) reported that both compounds damnacanthal and nordamnacanthal exhibited to be more sensitive towards the anchorage-independent cell lines. This is due to the anchorage-dependent cell lines require attachment to a solid matrix which create cell to cell contact. This interaction generates transmembrane signal that affected the cell proliferation, migration, differentiation and survival of the cell from undergoing apoptosis. The importance of attachment of anchorage-dependent cell line was supported by Singh *et al*. (1996) which revealed that primary culture that require anchorage will undergo apoptosis in suspension culture. Thus, this has proven that anchorage-independent cell lines have undergone genetic changes through transformation that prevent expression of the apoptotic pathway in response to the lost of contact which caused them to be more sensitive towards cytotoxic drug and chemical.

Even though the chemical structure between damnacanthal and nordamnacanthal was closely related, there was a significant difference on the cytotoxicity effect towards several leukemia cell lines. This is due to the existence of methoxyl group ($-OCH_3$) of damnacanthal at position C-1 whilst nordamnacanthal had hydroxyl group (-OH) at the same position. The existence of this different group is predicted to be the contributing factor to the variable effects of damnacanthal and nordamnacanthal towards cancerous cell lines. According to Ali *et al*. (2000), the presence of hydroxyl group at C-1 and C-3 and/ or a formyl group at C-2 in the anthraquinone skeleton, such as nordamnacanthal and rubiacin may exert their cytotoxicity effects towards several cancerous cell lines. Nevertheless, Kamei *et al*. (1998) reported that anthraquinones with more hydroxyl groups were more effective than those with less or without the existence of the group, thus suggesting that nordamnacanthal was more active compared to damnacanthal in inhibiting cancer cells. However, current result demonstrated that damnacanthal was more toxic towards those leukemia cell lines with the IC_{50} value less than 10 µg/mL. This result is in accordance with previous finding by Konoshima *et al*. (1989). The group has discovered that not only the number but the position of hydroxyl groups would influence the activity of an anthraquinone, hence suporting that damnacanthal was more active compared to nordamnacanthal. Furthermore, Rajendran *et al*. (2004) showed that the existence of methoxyl group ($-OCH_3$) at C-1 position of damnacanthal is expected to show higher efficiencies for generation of singlet oxygen ($^1O_2$) and superoxide anion radical ($O_2^{\bullet-}$) than nordamnacanthal, thus supporting the capability of damnacanthal to be more toxic towards those leukemia cell lines compared to nordamnacanthal. Damnacanthal has also possesed high ability to generate reactive oxygen species compared to nordamnacanthal (Rajendran *et al*., 2004). Hence it has the capability to neutralize the toxicity of ROS which caused lipid peroxidation, protein oxidation and most importantly DNA damage (Flora and Ferguson, 2005). DNA damage can result in base mutation, single or double strand break, DNA cross-linking and chromosomal abnormalities (Issa *et al*., 2006). Irreversible DNA damage is recognized as an early step in the initiation of the carcinogenesis process. Therefore, damnacanthal which has methoxyl group ($-OCH_3$) at C-1 position might prevent carcinogenesis process and inhibiting

In addition, the chemical property of zerumbone to conjugate biological nucleophiles may be essential for exerting its activity. Nakamura *et al*. (2004) recently discovered that the α-β-unsaturated carbonyl in zerumbone has made it more electrophilic as compared to the non-electrophilicity of α-humulene and 8-OH-humulene. This electrophilic property enables zerumbone to react with biological nucleophiles, such as IκB protein by modifying the cysteine residue thus attenuating IκB phosphorylation to abolish the NF-κB activity (Murakami *et al*., 2002). This structure- activity relationship has also been found in some physiological activities, including suppressor of Epstein-Barr virus activation in Raji cells and inhibition of O_2^- generation of differentiated HL-60 cells (Murakami *et al*., 2002). Moreover, this property provides biological evidence of its role as an antioxidant in activating the detoxification enzyme system as well as neutralizing the lipid peroxidation in hepatocytes, which also could affect its cytotoxic towards cancer cells (Nakamura *et al*., 2004).

Betulinic acid, a plant-derived pentacyclic lupine type triterpene, has been recognized to possess potent pharmalogical properties. Current study demonstrated that betulinic acid had strong cytotoxic effects towards HL-60 and WEHI-3B but less toxicity towards CEM-SS. This result supported the report of it's cytotoxicity towards various leukemia cells. In previous reports, Ehrhardt *et al*. (2004) discovered that betulinic acid induced apoptosis in hematological malignancies where 65% of primary pediatric acute leukemia and all cell lines of these types were sensitive to betulinic acid *in vitro*. Poon *et al*. (2004) also reported that betulinic acid was cytotoxic and enhanced 1[alpha],25-dihydroxyvitamin D3-induced differentiation in human HL-60 promyelocytic leukemia cells. Sensitivity of betulinic acid towards cancerous cells was not only limited towards leukemia cells as it was known to be cytotoxic towards various cancerous cells. Research done by Pisha *et al*. (1995) exhibited that betulinic acid induced melanoma specific cytotoxic towards athymic mice and human melanoma xenografts by regressing more than 80% tumor growth. Fulda *et al*. (1998) reported betulinic acid induced apoptosis in neuroblastoma, medullablastoma and Ewing's sarcoma cell lines, which are the most common solid tumors in childhood. Subsequently, betulinic acid also showed to be cytotoxic towards primary tumor cell culture from medullablastoma and glioblastoma (Fulda *et al*., 1999), glioma cell lines as wells as head and neck squamous cellular carcinoma cell lines (Thurnher *et al*., 2003). Recently, betulinic acid was reported to be strongly cytotoxic and cytostatic towards most prevalent human cancer types such as lung, colorectal, breast, prostate and cervical cancer (Jan *et al*., 2007).

There are some possible reasons supporting the broad toxicity effect of betulinic acid towards various cancerous cells. One of them is due to its structure activity relationship or known as SAR. According to Sami *et al*. (2006) the hydroxylation at C-3 and C-28 position of betulinic acid is likely to enhance its cytotoxic effects towards various cancer cells. This finding was in an agreement with the finding by Liu *et al*. (2004) that showed the hydroxylation of C-3 position of betulinic acid is vulnerable to enhance the apoptotic activity of betulinic acid and its derivatives on murine melanoma B16 cells. In addition, betulinic acid which easily can be converted from its structurally related precursor, betuline was identified in chemical structure as a lipophilic and thus poorly soluble in water. Betuline was known to have three positions in its structure, namely secondary hydroxyl group at position C-3, primary hydroxyl group at position C-28 and alkene moiety at position C-20, where chemical modifications can be easily performed to yield derivatives such as betulinic acid. Some of betuline derivatives were commonly in a lipophilic structure. This may have a significant role or effects towards its cytotoxicity effects on cancer cells (Sami *et al*., 2006), thus supporting this present results on the cytoxic effects of betulinic acid towards HL-60, WEHI-3B and CEM-SS cells leukemia.

Cancers and many human diseases have been known as cell cycle diseases (Liu *et al*., 2004). This is due to common alteration and many regulatory factors of checkpoints are lost or arrested during the process of tumorigenesis. However, there are several chemopreventive phytochemicals such as curcumin and catechin could restore and alter regulatory checkpoints through inducing apoptosis and cell cycle arrest (Liu *et al*., 2004). Therefore, cell cycle analysis has been carried out to evaluate the effect of damnacanthal, betulinic acid, zerumbone and doxorubicin on HL-60 cells. Cell cycle analysis revealed HL-60 treated with betulinic acid has induced apoptosis and cell cycle arrest at G0/G1. After 72 h, approximately 8.41% of viable cells were in the sub G1, indicating the apoptotic phase. This finding was similar with finding by Poon *et al*. (2004). They reported that betulinic acid was cytotoxic towards HL-60 cells IC_{50} value = 5.7 μM) and induced apoptosis in cell cycle analysis with approximately 10% of viable cells were in the sub G1 phase after exposure of the cells to 12 μM of betulinic acid. However, the apoptosis rate of betulinic acid towards HL-60 is considered low when compared

to its derivatives 23-hydroxybetulinic acid. Ji *et al*. (2002) discovered that 23-hydroxybetulinic acid induced apoptosis in HL-60 cells with approximately 46.61% cells were in sub G1 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 the telomerase activity (Ji *et al*., 2002).

Conclusion

This current study has effectively demonstrated that those compounds have a good potential anti cancer drug that may lack toxic effects towards healthy tissue and immune system. Damnacanthal, nordamnacanthal and betulinic acid have successfully exhibited to be cytotoxic towards various leukemia cell lines without causing toxicity towards normal cell 3T3. Further *in vitro* study on the compounds involving their pharmacokinetics and toxicology effects should be carried out before undergoing with *in vivo* or clinical works.

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References

Ali, A.M., Ismail, N., Mackeen, M., Yazan, L., Mohamed, S., Ho, A. and Lajis, N. 2000. Antiviral, cytotoxic and antimicrobial activities of antharaquinones isolated from the roots of Morinda elliptica. Pharmaceutical Biology 38: 3791-3801.

Ehrhardt, H., Fulda, S., Fuhrer, M., Debatin, K. and Jeremias, I. 2004. Betulinic acid induced apoptosis in leukemia cells. Leukaemia 18: 1406-1412.

Flora, S.D. and Ferguson, L.R. 2005. Overview of mechanisms of cancer chemoprevention agents. Mutation Research 591: 8-15.

Fulda, S., Jeremias, I., Steiner, H.H., Pietsch, T. and Debatin, K. 1999. Betulinic acid: a new cytotoxic agent against malignant brain-tumor cells. International Journal of Cancer 82: 435-441.

Fulda, S., Susin, S.A., Kroemer, G. and Debatin, K.M. 1998. Molecular ordering of apoptosis induced by anticancer drugs in neuroblastoma cells. Cancer Research 58: 4453-4460.

Huang, G.C., Chien, T.Y., Chen, L.G. and Wang, C.C. 2005. Antitumor effects of Zerumbone from *Zingiber zerumbet* in P-388D cells in vitro and in vivo. Plant Medicine 71: 219-24

Ismail, N.H., Ali, A.M., Aimi, N., Kitajima, M., Takayama, H. and Lajis, N.H. 1997. Anthraquinones from *Morinda elliptica*. Phytochemistry 45: 1723-1725.

Issa, A.L., Volate, S.R and Wargovich, M.J. 2006. The role of phytochemicals in inhibition of cancer and inflammation: New directions and perspectives. Journal of Food Composition and Analysis 19: 405-419.

Jan, H.K., Franziska, B.M., Guido, M.D.R. and Jan, P.M. 2007. Broad in vitro efficacy of plant-derived betulinic acid against cell lines derived from the most prevalent human cancer types. Cancer Letters 251: 132-145.

Jasril., Nordin, H.L., Lim, Y.M., Abdullah, M.A., Sukari, M.A. and Ali, M.A. 2003. Antitumor promoting and antioxidant activities of anthraquinones isolated from the cell suspension culture of *Morinda elliptica*. Asia Pacific Journal of Molecular Biology and Biotechnology 11: 3-7.

Jeremias, I., Steiner, H.H., Benner, A., Debatin, K.M. and Henro, H.M. 2004. Cell death induction by betulinic acid, ceramide and TRAIL in primary glioblastoma multoforme cells. Acta Neurochir 146: 721-729.

Ji, Z.N., Ye, W.C., Liu, G.G. and Hsiao, W.W.L. 2002. 23-Hydroxybetulinic acid-mediated apoptosis is accompanied by decreases in bcl-2 expression and telomerase activity in HL-60 cells. Life Sciences 72: 1-9.

Jung, M.E. and Duclos, B.A. 2006. Synthetic approach to analogues of betulinic acid. Tetrahedron 62: 9321-9334.

Kamei, H., Koide, T., Kojima, T., Hashimoto, Y. And Hasegawa, M. 1998. Inhibition of cell growth in culture by quinones. Cancer Biotherapy and Radiopharmaceuticals 13: 185-188.

Kirana, C., McIntosh, G.H., Record, I.R. and Jones, **G.P.** 2003. Antitumor activity of extract of *Zingiber aromaticum* and its bioactive sesquiterpenoid zerumbone. Nutrition and Cancer 45: 218-225.

Kitayama, T., Furuya, A., Moriyama, C., Masuda, T., Fushimi, S., Yonekura, Y., Kubo, H. and Sawada, S. 2006. Elucidation of the sharpless epoxidation of zerumbol. Tetrehedron: Asymmetry 17: 2311-2316.

Konoshima, T., Kozuka, M., Koyama, J., Okatani, T. and Tagahara, K. 1989. Studies on inhibitors of skin tumor promotion, V1. Inhibitory effects of quinones of Epstein-Barr virus activation. Journal of Natural Products 52: 987-995.

Kwanjai, K., Somdej, K. and Ruchanee, P. 2005. Biological activity of Anthraquinones and triterpenoids from Prismatomeris fragrans. Journal of Ethnopharmacology 100: 284-288.

Lechat-Vahirua, I., Francois, P., Menut, C., Lamaty, G. and Bessiere, J.M. 1993. Aromatic Plants of French Polynesia. I. Constituents of the Essential Oils of Rhizomes of Three Zingiberaceae: *Zingiber zerumbet* Smith*, Hedychium coronarium* Koening *and Etlingera cevuga* Smithd. Journal of Essential Oil Research 5:55-59.

Lee, C.C. and Houghton, P. 2005. Cytotoxicity of Plants from Malaysia and Thailand used traditionally to treat cancer. Journal of Ethnopharmacology 100: 237-243.

Liu, J., Hu, W., He, L.F., Ye, M. and Li, Y. 2004. Effect of lycorine on HL-60 cells. Cancer Letters 17: 1189-1195.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assay. Journal of Immunology Methods 65: 55-63.

Murakami, A., Ohigashi, H., Hayashi, R., Takana, T., Kwon, K.H. and Safitri, R. 2004. Corrigendum to "Suppression of dextran sodium sulfate-induced colitis in mice by zerumbone, a subtropical sesquiterpene, and nimesulide: separately and in combination. Biochemical Pharmacology 67: 395.

Murakami, A., Takahashi, M., Jiwajinda, S., Koshimizu, K. and Ohigashi, H. 1999. Identification of zerumbone in *Zingiber zerumbet* Smith as a potent inhibitor of 12-O-tetradeconyolphorbol-13-acetate-induced Epstein - Barr virus activation. Bioscience Biotechnology and Biochemistry 63:1811-1812.

Murakami, A., Takahashita, D., Kinoshita, T., Koshimizu, K., Kim, H.W., Yoshihiro, A., Nakamura, Y., Jiwajinda, S., Tereo, J. and Ohigashi, H. 2002. Zerumbone, a Southeast Asian ginger sesquiterpene, markedly suppress free radical generation, proinflammatory protein production, and cancer cell proliferation accompanied by apoptosis. Carcinogenesis 23: 1961-1963.

Nakamura, Y., Yoshida, C., Murakami, A., Ohigashi, H., Osawa, T. and Uchida, K. 2004. Zerumbone, a tropical ginger sesquiterpene, activates phase II drug metabolizing enzymes. FEBS Letters 572: 245-250.

Pisha, E., Chai, H., Lee, I.S., Chagwedera, T.E., Farnsworth, N.R., Cordell, G.A., Beecher, C.W., Das Gupta, T.K. and Pezzuto, J.M. 1995. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nature Medicine 1: 1046-1051.

Poon, K., Zhang, J., Wang, C., Tse, A.W., Wan, C.K and Fong, W.F. 2004. Betulinic acid enhances 1[alpha],25-dihydroxyvitamin D3-induced differentiation in human HL-60 promyelocytic leukemia cells. Anti-Cancer Drug 15: 619-624.

Rajendran, M., Inbraj, J.J., Gandhidasan, R. and Murugesan, R. 2004. Photodynamic action of damnacanthal and nordamnacanthal. Journal of Phytochemistry and Photobiology 162: 615-623.

Rene, C., Kiang, S. and Renate, S. 2006. A practical synthesis of betulinic acid. Tetrahedron Letter 47: 8769-8770.

Sami, A., Taru, M., Salme, K. and Jari, Y.K. 2006. Pharmacological properties of the ubiquitous natural product betulin. European Journal Of Pharmaceutical Science 29: 1-13.

Shier, W.T. 1991. Mammalian Cell Culture on $5 a day: A lab manual of low cost methods. Nat. Inst. Of Biotech and Appl. Micro. (BIOTECH), pp 64-71. Philipines: Laguna.

Singh, R.P., Al-Rubeai, M. and Emery, A.N. 1996. Apoptosis: exploiting novel pathways to the improvement of cell culture processes. The Genetic Engineer and Biotechnologist 16: 227-251.

Takada, Y., Murakami, A. and Aggarwal, B.B. 2005. Zerumbone abolishes NF-kappaB and IkappaBalpha kinase activation leading to suppression of antiapoptotic and metastatic gene expression, upregulation of apoptosis and down regulation of invasion. Oncogene 17: 122-130.

Thurnher, D., Turhani, H., Pelzmann, M., Wannemacher, B., Wacheck, V. and Selzer, E. Betulinic acid: a new cytotoxic compound against malignant head and neck cancer cells. Head Neck 25: 732-740.

Tosa, H., Ilnuma, M., Asai, F., Tanaka., T., Nozaki, H. and Fujimori, S. 1998. Anthraquinones from Neonaucle calycina and their inhibitory activity against DNA topoisomerase II. Biological and Pharmaceutical Bulletin 21: 641-642.