

## Immunomodulatory Effects of Betulinic Acid Isolation from the Bark of *Melaleuca cajuputi*

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### ABSTRACT

Betulinic acid and its derivatives showed cytotoxicity against variety of tumour and cancer cell lines comparable to some clinically used drug. In the present study, the immunomodulatory effects of betulinic acid, isolated from the roots of *Melaleuca Cajuputi*, was studied. Immunomodulatory effect was evaluated by using lymphocytes proliferation assay on mice splenocytes, thymocytes and human peripheral blood mononuclear cells (PBMC), while the cell cycle progression of betulinic acid treated PBMC was also studied by using flow cytometer. The production of human interleukin-2 (IL-2) and human inteleukin-12 (IL-12) cytokines was also assessed using enzyme-linked immunosorbent assay (ELISA). The results showed that betulinic acid was able to stimulate the proliferation of mice thymocytes, splenocytes and human PBMC in a time and dose-dependent manner. Meanwhile, betulinic acid treated immune cells were proliferated well at lower concentration (7.5 µg /mL), but growth inhibition occurred at a higher concentration (30 µg /mL). The findings obtained from the cell cycle analysis exhibited the proliferation effect of betulinic acid on PBMC, whereby 43.66 ± 2.60% and 42.83 ± 2.40% of the cells entered G2/M phase after 24h and 48h, respectively. Moreover, betulinic acid also induced extracellular IL-2 and IL-12 production. This finding demonstrates that betulinic acid acts as an immunomodulatory agent that may be useful in enhancing immune system.

**Keywords:** Betulinic acid, cytokine, immunomodulation, *Melaleuca cajuputi*, PBMC

### INTRODUCTION

Betulinic acid [3β-hydroxy-lup-20(19) lupean-28-carbonic acid] is a lupine-type triterpene which was first isolated in 1948 from the bark of the London plane tree (*Platanus acerifolia*) (Jung & Duclos, 2006). It can also be found abundantly in the bark of white birch (*Betula alba*) or chemically derived from betulin

(Csuk *et al.*, 2006). Betulin is an abundant naturally occurring triterpene and it is found predominantly in bushes and trees. Both betulin and betulinic acids possess a wide spectrum of biological and pharmacological activities (Alakurtti *et al.*, 2006).

Jeremias *et al.* (2004) found that betulinic acid induced specific cell death of more than 75% in the primary glioblastoma multiforme cells *in*

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*vitro* at substantially higher rate than established cytotoxic drugs vincristine. When betulinic acid was compared for *in vitro* efficiency against several leukaemia cell lines with conventionally used cytotoxic drugs, the acid was found to be more active than 9 out of 10 standard therapeutics (Ehrhardt *et al.*, 2004). In fact, betulinic acid also has indicated selective apoptotic activity toward melanoma cells (Pisha *et al.*, 1995) as well as the tumour cells of neuroectodermal origin (Fulda *et al.*, 1998) and B16 (Liu *et al.*, 2004). Betulinic acid induced apoptosis is mediated by a decrease in mitochondrial permeability due to the release of mitochondrial cytochrome c into the cytosol (Fulda & Debatin, 2005), the formation of reactive oxidative species and the activation of crm-A-insensitive caspase activity (Wick *et al.*, 1999) rather than through a ligand/receptor system. When betulinic acid was used in combination with anticancer drug vincristine, it showed synergistic cytotoxic effect on the growth and metastases of melanoma cells (B16F10) *in vivo* (Sawada *et al.*, 2004). Moreover, betulinic acid has shown to be non-toxic up to 500 mg/kg body weight in mice (Pisha *et al.*, 1995), as well as also cytotoxic on medullablastoma (Daoy), glioblastoma (A172) and melanoma (Mel-Juso) cancer cells without effecting normal human fibroblast (Fulda & Debatin, 2005).

Besides, betulinic acid was also found to inhibit the replication of human immunodeficiency virus (HIV) (Zhou *et al.*, 2004; Hashimoto *et al.*, 1997). Several betulinic acid derivatives are also very potent and highly selective inhibitor of HIV-1 depending on their specific side-chain modification of the compounds. These compounds function by inhibiting HIV fusion, or as recently demonstrated, by interfering with a specific step in HIV-1 maturation (Aiken & Chen, 2005). Moreover, betulinic acid was able to activate macrophage to produce two groups of protein mediators for inflammation including interleukin-1 $\beta$  and tumour necrosis factor- $\alpha$  (Yun *et al.*, 2003). In addition, betulinic acid also possesses anti-malaria and anti-inflammatory activities. Furthermore, betulinic acid was found

to be active against chloroquine sensitive (T9-96 strain) and resistant (K1 strain) *Plasmodium falciparum* (Alakurtti *et al.*, 2006).

Undoubtedly, betulinic acid exhibited various pharmacological activities. Although comprehensive tests have been carried out to study the cytotoxic, anti-malaria, antiviral and even immunomodulatory effect (on macrophage) of this compound, not many studies have been addressed the immunomodulatory activity of betulinic acid towards mice and human lymphocyte *in vitro*. Thus, the present study was carried out to investigate the immunomodulatory effect of betulinic acid which had successfully been isolated from the bark of *Melaleuca cajuputi* on mice splenocyte, thymocytes proliferation, human peripheral blood mononuclear cells (PBMC) proliferation, as well as human PBMC cell cycle progression and cytokine (Interleukin 2 and 12) induction.

## MATERIALS AND METHODS

### Materials

Betulinic acid, extracted from the bark of *Melaleuca cajuputi*, was supplied by Prof. Dr. Faujan Ahmad, from Department of Chemistry, Faculty of Science, Universiti Putra Malaysia. The pure compound of betulinic acid appears as a white solid, with melting point of 295-297°C. It was isolated from *Melaleuca sp.* and was chromatographed on a silica gel column with increasing polarity of chloroform and methanol solvent system (Ahmad *et al.*, 1997). The concentrated compound was dissolved in dimethylsulphoxide (DMSO) (Sigma, USA) to obtain a stock solution of 10 mg/mL. Later, a sub-stock solution of 0.06 mg/mL was prepared by diluting 6  $\mu$ L of the stock solution into 994  $\mu$ L serum-free culture medium, RPMI 1640 (Sigma, USA) (note that the percentage of DMSO in the experiment should not exceed 0.5%). Then, the stock and sub-stock solutions were both stored at 4°C.

Concanavalin A (Con A) (Sigma, USA), lipopolysaccharide (LPS) (from *Salmonella enteritidis*) (Sigma, USA) and Pokeweed

mitogen (PWM) (1 mg/mL) were used as a positive control. This commercial immunomodulator was prepared by dissolving with RPMI 1640 (Sigma, USA). Meanwhile, the stock and sub-stock solutions were prepared as above. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), Bovine Serum Albumin (BSA), Tris, dimethyl sulphoxide (DMSO), triton X-100, EDTA, RNase A and propidium iodide (PI) were purchased from Sigma. Foetal bovine serum (FBS) was purchased from PAA, ammonium chloride and sodium citrate from Fisher (UK), Interleukin 2 and Interleukin 12 Instant Enzyme Link Immunosorbent Assay (ELISA) kit from Bender MedSystems, Austria, and Ficoll-Paque Plus from Amersham Biosciences.

#### *Preparation of Mice Splenocytes and Thymocytes Cell Suspensions*

Imprinting Control Region (ICR) mice, aged 5-8 weeks old, were used in all the experiments of this study. The animals were purchased from Animal House, the Faculty of Veterinary Medicine, Universiti Putra Malaysia. The animals were housed under standard conditions at  $25 \pm 2^\circ\text{C}$  and fed with standard pellets and tap water. The mice were avoided from stress or specific control. This work had earlier been approved by Animal Care and Use Committee, Universiti Putra Malaysia (UPM), (Ref: UPM/FPV/PS/3.2.1.551/AUP-R2).

The procedure for preparing mice thymus and spleen cell suspensions is quite similar. The mice were killed by cervical dislocation. The thymus (located above the heart) and the spleen (located right behind the liver) were removed and quickly washed with HANKS Balance Salt Solution (HBSS) (Sigma, USA). Then, these thymus and spleen were minced and pressed through a sterile wire mesh screen (80 $\mu\text{m}$ ) using a rubber syringe plunger. The cell suspension was washed with PBS (Sigma, USA), supplemented with 0.1% BSA (Sigma, USA) and 0.06% sodium citrate (Fisher, UK) (PBS-BSA-

SC) and spun down at 200 x g for ten minutes. The additional step in preparing the spleen cell suspension was the splenocytes which were spun down with 5 mL lysis buffer (7.56g ammonium chloride (Fisher, UK) with 2.42g of Tris (Sigma, USA) in 1L distilled water) to lyse the red blood cells. The supernatants were discarded and 4mL of Dulbecco's Modified Eagle Medium (DMEM) (Sigma, USA) with 10% heat inactivated FBS (PAA, Austria) were added. The spleen and thymus pellet was resuspended and cell counting was performed to determine the lymphocyte cell number in the suspensions.

#### *Isolation of Peripheral Blood Mononuclear Cells (PBMC)*

Venous blood (20-25mL) was aseptically collected from 20 healthy donors in preservative free heparin tubes. The blood was diluted with phosphate buffered saline (PBS), pH 7.4 and layered onto Ficoll plus at the ratio of diluted blood with Ficoll 2:1 (Amersham). After centrifugation at 400 x g for 50 min, the lymphocytes were collected at the interface and washed three times with the PBS. The cells were resuspended in DMEM with 10% foetal bovine serum and antibiotics.

#### *MTT Cell Viability Assay*

The effects of the compound on the cell proliferation of mice splenocytes, thymocytes and human PBMC were first determined using a colorimetric technique, which is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Yeap *et al.*, 2007). Briefly, 100  $\mu\text{L}$  of DMEM media with 10% of FBS was added into the all the wells, except row A in the 96-well plate (TPP, Switzerland). Then, 100  $\mu\text{L}$  of diluted compound at a concentration of 60  $\mu\text{g}/\text{mL}$  was added into row A and row B. A series of twofold serial dilution of compound was carried out down from row B until row G. Nonetheless, row H was left untouched and the excess solution (100  $\mu\text{L}$ ) was discarded. 100  $\mu\text{L}$  of the cells (mice thymocytes, human PBMC, HL60 or NIH3T3), with the cell

concentration at  $4 \times 10^5$  cells/mL, was then added into all the wells in the 96-well plate and incubated in 37°C, 5% CO<sub>2</sub> and 90% humidity incubator for selected periods of 24, 48 and 72 h. After the corresponding periods (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 4 h in 37°C, 5% CO<sub>2</sub> and 90% humidity incubator. The medium with MTT was removed from every well and 100 µL DMSO (Sigma, USA) was then added into each well to solubilise the formazan crystal and incubated for 20 min in 37°C, 5% CO<sub>2</sub> incubator. Finally, the plate was read at 570 nm using µ Quant ELISA Reader (Bio-Tek Instruments, USA). The results of the compound were compared with the result of ConA (1 µg/mL) and LPS (1 µg/mL) for immunomodulation as a control. Each compound and control was assayed in triplicate for three times. The percentage of proliferation was calculated by using the following equation:

$$\% \text{ Proliferation} = \frac{[\text{OD sample} - \text{OD control}]}{\text{OD control}} \times 100$$

#### *Flow Cytometer Analyses*

Flow cytometer was used to support the effect of betulinic acid on human PBMC cell cycle progression (Alitheen *et al.*, 2001). PBMC was chosen to evaluate the capability of betulinic acid to stimulate the proliferation of lymphocytes *in vitro*. In this study, PWM was used as a positive control. The active concentration chosen for the betulinic acid and PWM to stimulate the proliferation of PBMC was 30 µg/mL and 50 µg/mL, respectively. In this study, 1 mL of PBMC, with a density of  $4 \times 10^5$  cells/mL, was treated with 1 mL of betulinic acid and PWM, respectively, according to their active concentrations as mentioned above. The treatment was carried out in 6 well plates (Nunc, UK) with the total working volume of 2 mL for each well. The treated cells were then 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 -20°C for 2 h. Then, the cells

were spun down and washed twice with PBS pH7.5. The cell pellets were finally dissolved and stained in PBS buffer consisting 0.1% triton X-100 (Sigma, USA), 10 mM EDTA (Sigma, USA), 50 µg/mL RNase (Sigma, USA) and 3 µg/mL propidium iodide (PI) (Sigma, USA). This process was done in the dark because PI is sensitive to light. The cells were then incubated for 30 min in 4°C 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.

#### *Cytokine Production of Human Peripheral Blood Mononuclear Cells*

The expressions of extracellular IL-2 and IL-12 were performed by using Cytokine Instant Enzyme Link Immunosorbent Assay (ELISA) kit (Bender MedSystems, Austria) (Yeap *et al.*, 2007). Briefly, the extracted human PBMC, with a cell concentration at  $5 \times 10^5$  cells/mL, was treated with same volume of compound at 30 µg/mL in 6 well culture plates (TPP, Switzerland). The control cultures, without compound and positive control with Pokeweed Mitogen (50 µg/mL), were prepared simultaneously. The culture was then incubated for respective time periods of 24 hours, 48 hours, and 72 hours. After the corresponding period, the samples were washed and pelleted. 50 µL of the supernatant was added into the strip on the plate of the kit and incubated for three hours by shaking at 200 rpm. Then, the sample was washed and immediately added with 3,3',5,5'-tetramethylbenzidine (TMB) Peroxidase substrate in the dark. Finally, 1M Phosphoric acid stop solution was added and the plate was read at 450 nm and 620 nm as reference wavelength using µ Quant ELISA Reader (Bio-Tek Instruments, USA) at the Animal Tissue Culture Laboratory, FBBS, UPM. The result was compared to the control strip in the kit. Each compound and control was assayed in triplicates. Meanwhile, the data were expressed as pg/mL.

*Statistical Analysis*

All the experiments were performed in triplicates. The results were analysed by using SPSS version 13 and expressed as mean  $\pm$  standard error (SE). The differences between the means were evaluated using the ANOVA test (one way), followed by the Duncan test and  $P < 0.05$  was taken as statistically significant.

**RESULTS**

*Mitogenic Activity of Betulinic Acid on Mice Thymocytes*

Betulinic acid was demonstrated to stimulate the proliferation of mice thymocytes in a time and dose-dependent fashion (Fig. 1). The proliferation rate of mice thymocytes treated with betulinic acid was less than that of Con A after 24 and 48 h of incubation period. In fact, betulinic acid has also been shown to inhibit mice thymocytes at a concentration more than 15  $\mu\text{g}/\text{mL}$  after 72 h of treatment. These data revealed that betulinic acid was toxic towards

mice thymocytes at the concentrations higher than 15  $\mu\text{g}/\text{mL}$ . However, it has also been shown to stimulate better proliferation of mice thymocytes at the concentration of 7.5  $\mu\text{g}/\text{mL}$  throughout the treatment periods. In more specific, betulinic acid demonstrated a better proliferation of mice thymocytes compared to Con A after 72 h of treatment at the concentration of 7.5  $\mu\text{g}/\text{mL}$  with the value of 60.3% and 29.3%, respectively. This clearly indicates that betulinic acid has the capacity to stimulate mice thymocytes in a dose-dependent fashion at 24, 48 and 72 hours.

*Mitogenic Activity of Betulinic Acid on Mice Splenocytes*

As shown in Fig. 2, betulinic acid stimulated the proliferation of mice splenocytes in a time and dose-dependent fashion. At 24 h of treatment, the proliferation effects of betulinic acid increased significantly with a concentration up to 14.99% at 30  $\mu\text{g}/\text{mL}$ . After 48 h or treatment, however, betulinic acid was found to be toxic

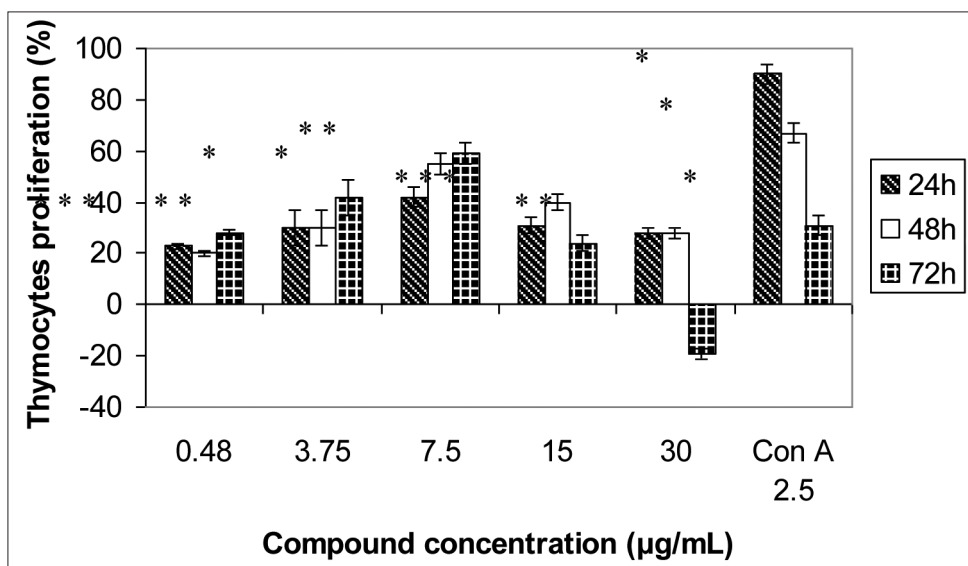


Fig. 1: The effects of betulinic acid on the proliferative response of mice thymocytes at 24, 48 and 72h of treatments. Data represent the means  $\pm$  SE of triplicate determinations from three independent experiments. The values were the means  $\pm$  SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA ( $P < 0.05$ ) and the significant data were marked with \*



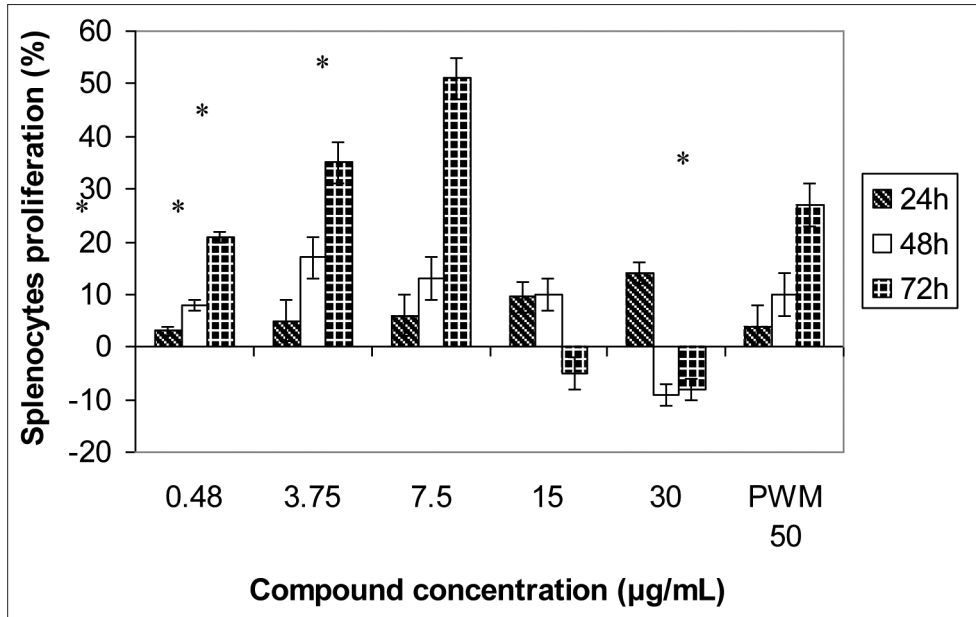


Fig. 2: The effects of betulinic acid on the proliferative response of mice splenocytes at 24, 48 and 72 h of treatment. Data represent the means  $\pm$  SE of triplicate determinations from three independent experiments. The values were the means  $\pm$  SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA ( $P < 0.05$ ) and the significant data were marked with \*

towards mice splenocytes at a concentration higher than 15  $\mu\text{g}/\text{mL}$ ; nonetheless, it induced proliferation at a concentration lower than that. On the other hand, betulinic acid inhibited mice splenocytes proliferation after 72 h of treatment at a concentration higher than 7.5  $\mu\text{g}/\text{mL}$ . Nevertheless, it showed the highest proliferation of mice splenocytes up to 50.77% at this concentration.

#### Mitogenic Activity of Betulinic Acid on Human Peripheral Blood Mononuclear Cells (PBMC)

Betulinic acid was shown to stimulate the proliferation of PBMC throughout the treatment periods (Fig. 3). It did not inhibit PBMC at all the concentrations tested. Meanwhile, the highest proliferation response of the PBMC treated with betulinic acid was 33.72%. Subsequently, the proliferation response of betulinic acid increased significantly with the increase of the

concentrations. At 24 h of treatment, betulinic acid exhibited better proliferation of PBMC when compared to the positive control, PWM. However, the proliferation response of the PBMC treated with betulinic acid was less than that of the PWM after 48 and 72 h of treatments. The data obtained showed that betulinic acid stimulated better proliferation of PBMC at 24 h of treatment and was able to sustain for longer incubation time. PWM exhibited a better proliferation of PBMC after 48 and 72 h of incubations.

#### Flow Cytometry Analysis of Cell Cycle Distributions on PBMC Based on Proliferation Effect of Betulinic acid and PWM at 24, 48 and 72 h of Incubation Time

As illustrated in Table 1, betulinic acid stimulated a higher percentage of cells proliferation at 24 h of treatment compared to the untreated cells that

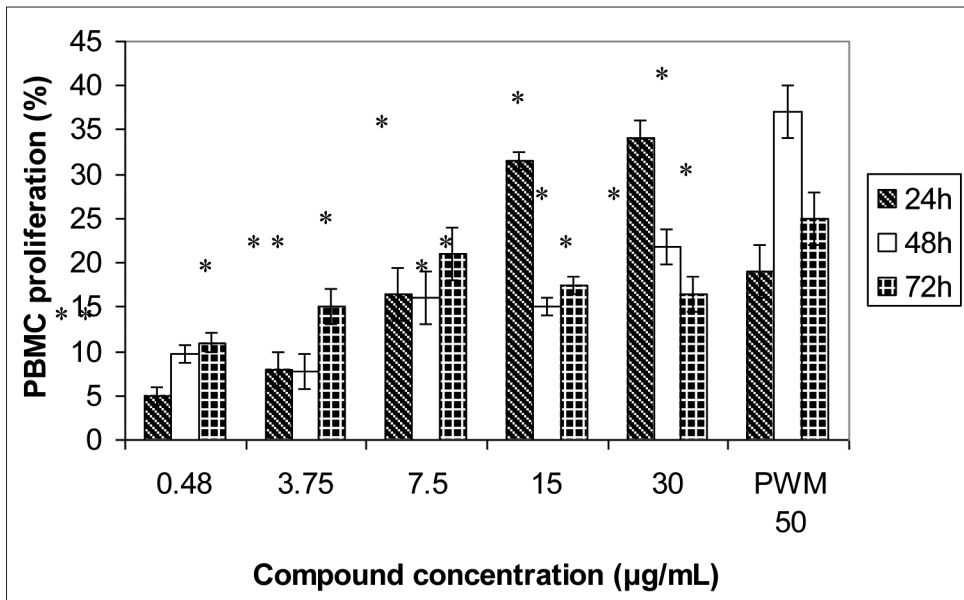


Fig. 3: The effects of betulinic acid on the proliferative response of human peripheral blood mononuclear cells (PBMC) at 24, 48 and 72 h of treatment. Data represent the means  $\pm$  SE of triplicate determinations from three independent experiments. The values were the means  $\pm$  SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA ( $P < 0.05$ ) and the significant data were marked with \*

entered G2/M with value of 45.28% and 4.63%, respectively. This result indicated that the treated cells were 10 folds higher in their cell number compared to the untreated ones. The cells treated with betulinic acid for 48 and 72 h also showed better proliferation with the value of 1.2 folds and 2.5 folds higher, respectively, as compared to the untreated ones. Nonetheless, the percentage of the cells stimulated to proliferate was found to be significantly reduced after 48 and 72 h of treatment with the value of 44.53% and 38.05%, respectively, as compared to those undergoing 24 h of treatment. The reduction of the cells that entered G2/ mitosis corresponded to an increase of cells entering the apoptosis Sub G1 phase. The data indicated that a longer exposure time to betulinic acid might cause apoptosis to PBMC up to 5.76% at 72 h treatment.

#### The Production of Human Interleukin-2 and Human Interleukin-12

To analyze whether betulinic acid enhanced or suppressed the production of cytokines, the modulatory effect in inducing both human IL-2 and human IL-12 upon stimulation of PBMC was evaluated by using ELISA.

Betulinic acid demonstrated a better induction of human IL-2 after 24 h with 9.6 fold higher compared to the control (Fig. 4). This figure showed that it decreased dramatically to 2.5 folds and 1.3 folds, respectively, after 48 and 72 h of the treatment. However, PWM showed a better induction of human IL-2 after 48 h treatment with 25.33 fold higher compared to the control. In addition, it was also demonstrated that there was a stable induction of human IL-2 throughout the treatment period. Betulinic acid was also shown to induce the

TABLE 1  
Flow cytometry analysis of cell cycle distribution on PBMCs based on the proliferation effects of betulinic acid and PWM

Compounds	Percentage of cell cycle distribution (%)	Incubation periods (h)	
		24	72
Untreated	Sub-G1 (apoptosis)	7.71 ± 0.69	1.80 ± 0.03
	G0/G1	5.82 ± 0.52	58.71 ± 1.12
	Synthesis	67.25 ± 6.02	27.58 ± 0.53
	G2/Mitosis	4.94 ± 0.44	15.79 ± 0.30
Betulinic acid	Sub-G1 (apoptosis)	1.43 ± 0.07	5.62 ± 0.19
	G0/G1	44.76 ± 1.44*	48.18 ± 1.62
	Synthesis	3.59 ± 0.25*	9.05 ± 0.13*
	G2/Mitosis	43.66 ± 2.60*	37.12 ± 1.30*
PWM	Sub-G1 (apoptosis)	17.10 ± 2.46*	0.65 ± 1.02
	G0/G1	7.70 ± 1.11	39.49 ± 1.11*
	Synthesis	45.11 ± 6.49*	24.02 ± 0.67
	G2/Mitosis	8.40 ± 1.20	41.09 ± 1.15*

The values were the means ± SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA (\* $P < 0.05$ ) and the significant data were marked with \*.



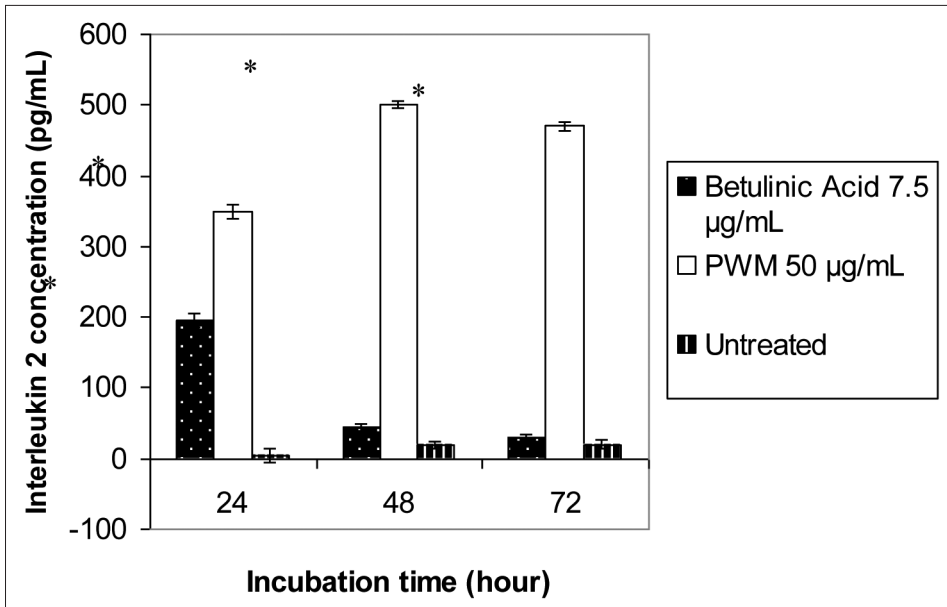


Fig. 4: The production of human IL-2 in culture supernatants upon the stimulation of PBMC by betulinic acid and PWM. PBMC were isolated and incubated at 24, 48 and 72 h with active concentrations (betulinic acid at 7.5 µg /mL; PWM at 50 µg /mL) and IL-2 induction was specifically determined by ELISA. The values obtained were the means ± SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA (\* $P < 0.05$ ) and the significant data were marked with \*

production of human IL-2 in a time-dependent fashion. The data obtained also indicated that those compounds had significantly decreased the induction of human IL-2 after 48 and 72 h of incubations. In contrast, the induction of human IL-2 was increased by PWM in a time-dependent manner, which was shown to induce higher production of human IL-2 at 48 h of incubation and decreased slightly after 72 h of the treatment, with the value of 506.66 pg /mL and 466.66 pg /mL, respectively.

In addition, betulinic acid also induced the production of human IL-12 in a time-dependent fashion. It significantly increased the production of human IL-12 from 24, 48 and 72 h with the value of 40 pg /mL, 56.66 pg /mL and 69 pg /mL, respectively, indicating 3.3 folds, 4.72 folds and 5.72 folds higher compared to the negative control (Fig. 5). However, the production of human IL-12 induced by LPS decreased sharply

after 48 and 72 h, with the value of 26.93 pg /mL or 2.24 folds and 22.06 pg /mL or 1.8 folds higher, respectively, compared to the negative control.

## DISCUSSION

The *in vitro* immunomodulatory study showed that betulinic acid was able to stimulate mice thymocytes, mice splenocytes and PBMC in a time and dose-dependent fashion. These results are similar to those of Yun *et al.* (2003), whereby a high concentration of betulinic acid (i.e. more than 10 µg/mL) caused the cytotoxic effect towards mice splenocytes after 72 h of the incubation period. Based on the proliferation effect from both the cells (thymocytes and splenocytes), it has been clearly shown that betulinic acid is more active towards mice thymocytes as compared to mice splenocytes and

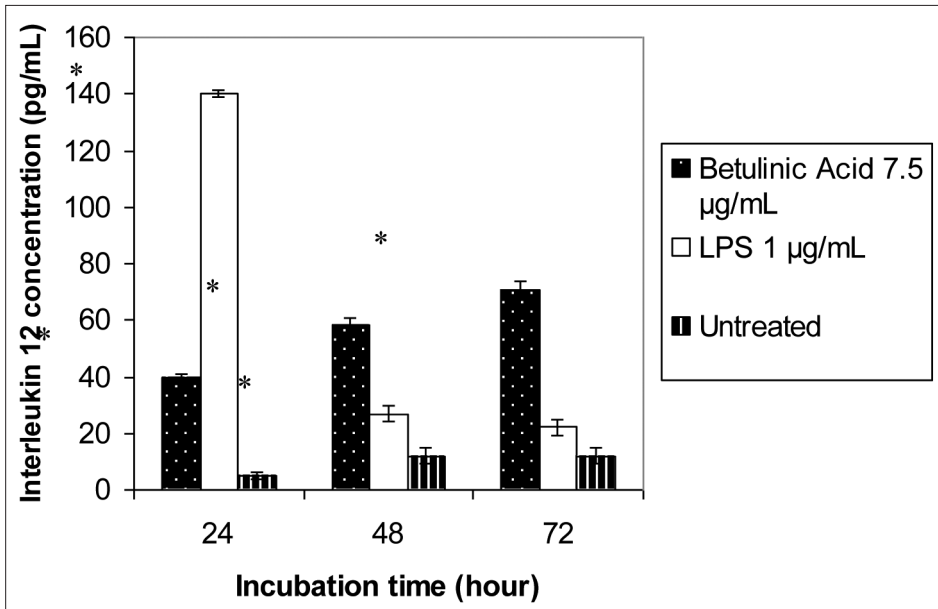


Fig. 5: The production of human interleukin-12 in culture supernatants upon stimulation of PBMC by betulinic acid and LPS. PBMC were isolated and incubated at 24, 48 and 72 h with active concentrations (betulinic acid at 7.5 µg/mL; LPS 1 µg/mL) and IL-12 induction was specifically determined by ELISA. The values were the means ± SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA (\* $P < 0.05$ ) and the significant data were marked with \*

a higher proliferation rate at the concentrations of 7.5 µg/mL and above has also been obtained.

Betulinic acid was able to stimulate the proliferation of PBMC without causing cytotoxicity at all the concentrations tested throughout the treatment periods. At 24 and 48 h of treatment, betulinic acid exhibited to be more active at a higher concentration (30 µg/mL) with the proliferation rate of more than 20%. The same pattern of lymphocytes proliferation with mice thymocytes and mice splenocytes could be clearly seen as betulinic acid exhibited to be more active at the concentration of 7.5 µg/mL after 72 h of treatment. This result clearly indicates that betulinic acid tends to induce T cells compared to B cells since the population of T cells in PBMC is about 90% compared to B cells which has only 10% of the PBMC population (Cerqueira *et al.*, 2004). This result is also supported by the capability of betulinic

acid to stimulate higher production of mice thymocytes (which produce only T cells) and thus supports the preferences of betulinic acid to stimulate T cells compared to B cells.

The cell cycle profile of PBMC treated with betulinic acid was then evaluated by using flow cytometer. In this cell cycle analysis, it was clearly indicated that half of the lymphocytes treated with betulinic acid entered G2 and Mitosis phase (G2/M) after 24 h treatment. However, the percentage of lymphocytes entering the G2/M phase was reduced slowly after 48 and 72 h of treatment, and this was due to the increase in lymphocytes entering the Sub G1 phase. Based on the combination results of the total population increase in the MTT assay and high population of cell under the G2/M phase, it was clearly demonstrated that betulinic acid was able to stimulate the proliferation of human lymphocytes (PBMC) with less toxicity

effect even for a longer incubation time. The result from the cell cycle analysis did support the result from MTT lymphocytes proliferation assay on PBMC. The less toxicity effect of betulinic acid towards normal lymphocytes is in agreement with the previous finding by Zuco *et al.* (2002) who showed that peripheral blood lymphoblasts were resistant against betulinic acid treatment *in vitro*.

Cytokines are soluble glycoproteins which are involved in the immune response activity. The functions of these proteins are diverse, and these include regulating the proliferation and differentiation of lymphocytes (Stanilove *et al.*, 2005). A study on cytokines demonstrated that betulinic acid induced the production of human IL-2 and the production of human IL-12 in a time-dependent fashion. Meanwhile, betulinic acid was showed to induce a higher production of human IL-2 after 24 h treatment and the production of IL-2 was reduced dramatically after 48 and 72 h of treatment. The pattern of betulinic acid in inducing the production of IL-2 is quite similar with the pattern of the MTT result of lymphocytes proliferation assay and cell cycle analysis on PBMC, which showed that betulinic acid was more active after 24 h of the treatment. Meanwhile, it could be speculated that the higher induction of IL-2 by betulinic acid was due to the capability of betulinic acid to stimulate the proliferation of T lymphocytes in both the experiments on MTT lymphocytes proliferation assay and cell cycle study. Furthermore, the induction of IL-2 by betulinic acid is relevant to this study since that IL-2 has been known as a central cytokine in the regulation of T cell responses (Ghosh *et al.*, 1999). In addition, the populations of T and B lymphocytes in the human peripheral blood lymphocytes, where 90% T cell and only 10% B cell (Cerqueira *et al.*, 2004) may contribute to a higher production of human IL-2 in culture supernatant treated with betulinic acid.

In contrast, betulinic acid was demonstrated to induce the production of human IL-12 in a time-dependent fashion. IL-12 is also known as pleiotropic cytokine with important proinflammatory cytokine and immunoregulatory

functions. It plays a key role in the modulation of the immune response by providing the stimuli for the differentiation of CD4+ T cells into Th1 and IFN- $\gamma$  secreting cells. IL-12 is released during the early stages of infections caused by a large variety of bacteria, intracellular pathogens, fungi, and certain viruses. The early production of IL-12 is T cell independent which is caused by direct interaction of pathogens or their products with phagocytic cells (Trinchieri *et al.*, 2003).

The capability of betulinic acid to induce the low concentration of human IL-12 is relevant since this compound has been reported to modulate the Th1/Th2 cells to produce IL-2 and IL-12 cytokines, which are the groups of Th1 as well as IL-10 anti-inflammatory cytokines, which is the group of Th2 (Zdzisińska *et al.*, 2003). In a similar experiment, Zdzisińska *et al.* (2003) demonstrated that betulinic acid did not influence TNF- $\alpha$  production, a proinflammatory cytokine. In addition, betulinic acid has also been known to be effective as an anti-inflammatory agent in various experiment systems (e.g. Mukherjee *et al.*, 1997; Bernard *et al.*, 2001). The capability of betulinic acid to induce small amount of IL-12 production is in agreement with the previous studies which have discovered that betulinic acid is an effective anti-inflammatory agent. Therefore, the evidence indicating that betulinic acid induces low concentrations of IL-12 and TNF- $\alpha$  suggests the involvement of NF- $\kappa$ B signal transduction pathway, which positively regulates proinflammatory cytokine gene expression.

## CONCLUSION

Although betulinic acid has shown encouraging results as an immunomodulator, further studies are still needed to elucidate the exact mechanism involved in betulinic acid. Once the mechanism of its action and comprehensive bioassay are elucidated, betulinic acid can be used as a lead molecule for the new generation of drugs in cancer treatment, particularly in boosting up the immune system.

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