

IMMUNOMODULATORY EFFECTS OF ZERUMBONE ISOLATED FROM ROOTS OF *ZINGIBER ZERUMBET*

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ABSTRACT

In this study, the immunomodulatory effects of zerumbone isolated from *Zingiber zerumbet* were investigated by evaluating the effects of this compound towards the lymphocytes proliferation (mice thymocytes, mice splenocytes and human peripheral blood mononuclear cells, PBMC), cell cycle progression and cytokine (interleukin 2 and 12) induction. Lymphocyte proliferation assay showed that zerumbone was able to activate mice thymocytes, splenocytes and PBMC at dosage dependent pattern where the best concentration was 7.5 µg/mL. Flow cytometry analysis showed the highest population of PBMC entered into G2/M phase after treatment for 72 h with 7.5 µg/mL zerumbone. The production of human interleukin-2 and human interleukin-12 cytokines in culture supernatant from zerumbone activated lymphocytes was prominently upregulated at 24 hour and decreased from 48 h to 72 h. The above results indicate that zerumbone can be used as immunomodulatory agent which can react toward the immune cell cytokine production in dosage dependent pattern.

Keywords: *Zingiber zerumbet*, zerumbone, immunomodulation, interleukin 2.

INTRODUCTION

Zerumbone is a monocyclic sesquiterpene that can be found abundantly in rhizomes particularly from *Zingiber zerumbet* Smith and *Zingiber aromaticum* (Murakami *et al.*, 2002). Its molecular formula is C₁₅H₂₂O. Zerumbone is a food phytochemical 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. In 2005, Takada and his groups 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 (Takada *et al.*, 2005). 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). Zerumbone acted as a very potent chemoprevention agents against colon and skin cancer. It was shown to inhibit both azoxymethane-induced rat aberrant crypt foci and phorbol ester-induced papilloma formation in mouse skin (Tanaka *et al.*, 2001). It also activated phase two drug metabolizing enzymes, such as GST

(Gluthathione S-transferase), epoxide hydrolase and hemeoxygenase via the transcription factor Nrf2-dependent pathway (Nakamura *et al.*, 2004) and was able to inhibit HIV (Dai *et al.*, 1997).

In 1987, Elliot and Brimacombe (1987) reported that zerumbone also possessed an anti-inflammatory property especially in treating UC (ulcerative colitis), which is an inflammation bowel disease (Murakami *et al.*, 2003a). Zerumbone which is used as anti-inflammatory folk medicine in Indonesia was a distinct potent inhibitor of 12-O-tetradecanoyl-13-acetate-induced Epstein-Barr virus (Murakami *et al.*, 1999; Vimala *et al.*, 1999). Besides that, previous study has indicated that zerumbone was able to suppress free radicals (superoxide anion) generation from NADPH oxidase xanthine oxidase, expression of iNOS (inducible nitric oxide synthase) and COX (cyclooxygenase)-2 as well as release of TNF-α (Murakami *et al.*, 2002).

Zerumbone possesses α, β-unsaturated carbonyl group. A-humulene, a zerumbone analog lacking this group has been found to be inactive and is not able to disrupt the TPA-induced biochemical pathways for EBV activation (Murakami *et al.*, 2002). Compounds which possess this group are known to exhibit variety of biological activity (fig. 2). This include inhibiting the growth of tumor cells (Metthes *et al.*, 1980), inducing their differentiation and

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also as a provider of cytoprotective activity (Murakami *et al.*, 2003b).

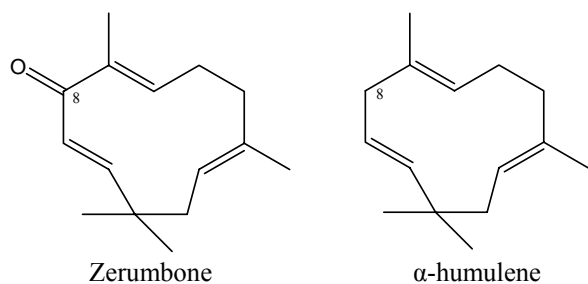


Fig. 1: The chemical structures of zerumbone and α-humulene. Their structural difference lies in the carbonyl group present in zerumbone, but not in α-humulene.

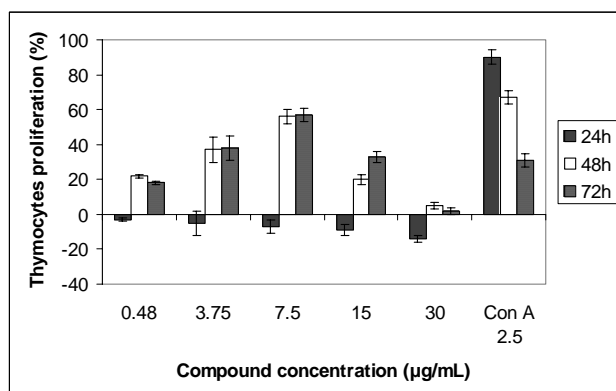


Fig. 2: Effects zerumbone on the proliferative response of mice thymocytes at 24, 48 and 72 h treatment. Data represent means ± S.E.M of triplicate determinations from three independent experiments.

Although the anticancer and the anti-inflammation studies of zerumbone had been carried out intensively, the immunomodulatory effect of zerumbone towards immune cells has not been fully studied yet. Thus, this project was carried out to study the immunomodulatory effect of zerumbone towards the proliferation, cell cycle progression and cytokine (Interleukin 2 and Interleukin 12) induction on the immune cells *in vitro*.

MATERIAL AND METHODS

Compound

The zerumbone (6.65 g, 0.33 %) which isolated from *Zingiber zerumbet* (2 kg) was obtained from Prof. Dr. Hasnah Siraj, Universiti Kebangsaan Malaysia. Zerumbone was identified by Infrared Spectrum using FTIR and Proton and Carbon-13 Resonance Magnetic Nucleus Spectrum (¹H and ¹³C RMN) using Bruker spectrophotometer (Ahmad, 2006).

Animal

ICR mice, 5-8 weeks old, were used in all experiments. The animals were purchased from Animal House, Universiti Putra Malaysia. The animals were housed under standard conditions at 25±2°C and fed with standard pellets and tap water. The mice were avoided from stress or specific control. This work has been approved by Animal Care and Use Committee, Universiti Putra Malaysia (UPM), (Ref: UPM/FPV/PS/3.2.1.551/AUP-R2).

Preparation of mice thymus cell suspensions

The mice were killed by cervical dislocation. The thymus which is located above the heart was removed and quickly washed with Hank's Balanced Salts Solution (HBSS) on the Petri dish. The thymus was minced and pressed through a sterile wire mesh screen with a rubber syringe plunger. The cell suspension was washed with PBS supplemented with 0.1% BSA and 0.06% sodium citrate (PBS-BSA-SC) and spun down at 200 x g for ten minutes. The step was repeated until the pellet was clean (does not contain any debris or contaminant). The supernatant was discarded and 4 mL of Dulbecco's Modified Eagle Medium (DMEM) with 10% heat inactivated serum was added. The pellet was resuspended and cell counting was performed to determine the lymphocyte cell number in the suspension. All of the steps above were carried out under sterile condition in biological safety cabinet to prevent any contamination.

Preparation of Mice Spleen Cell Suspensions

The procedure for mice spleen cell suspensions is quite similar compared to the preparation of mice thymus cell suspensions. Two mice were dissected for each experiment and the spleen which was located right behind the liver was removed and quickly washed with HBSS. The dissection for spleen and thymus can be done simultaneously but should be washed with different HBSS. The spleen was pressed with a rubber syringe plunger and pushed through a sterile wire mesh screen with PBS-BSA-EDTA added when the pressing process was carried out. The cell suspension was centrifuged at 1000 rpm (200 x g) for 10 min. After that, the cells were spun down with 5 mL ammonium chloride to lyse the red blood cells. This process was repeated until the pellet is clear from red blood cells. Finally, the supernatant was removed and 2 mL DMEM was added. The pellet was resuspended and cell counting was performed to determine the lymphocyte cell number in the suspension culture. All of the steps above were carried out under sterile condition in biological safety cabinet to prevent any contamination.

Isolation of peripheral blood mononuclear cells (PBMC)

Venous blood was collected aseptically from healthy donors in preservative free heparin tubes. The blood was diluted with phosphate buffered saline (PBS), pH 7.4 and

layered onto Ficoll plus (Amersham). After centrifugation at 400 x g for 50 min, the lymphocytes were collected at the interface and washed three times with PBS. The cells were resuspended in DMEM with 10% foetal bovine serum and antibiotics.

Lymphocytes proliferation assay

The proliferation assay of lymphocytes (thymocytes, splenocytes and PBMC) was tested according to 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, 100 μ L of DMEM medium were added into each well of flat-bottomed 96 well plate except row A. 100 μ L of diluted zerumbone was added into row A and row B. The solution was mixed by pipetting and starting from row B, 100 μ L was taken from the well and added to the next well in row C. A series of two-fold dilution of the drug down from row B to row G was carried out. The excess 100 μ L was discarded. Row H was left untouched. 100 μ L of lymphocyte cell suspensions (peripheral blood mononuclear cells and thymic lymphocyte) were added into all the wells and incubated at 37°C, 5% CO₂, 90% humidity for 24, 48 and 72 hours. A stock solution of 5 mg/mL MTT in PBS was prepared and 20 μ L of MTT was added into each well. The culture medium was discarded by aspiration and added with 100 μ L of DMSO (Sigma, USA) to lyse cells. Finally, the plate was read on an automated spectrophotometric EL 340 μ Quant ELISA Reader (Bio-tek Instruments, USA) using test and reference wavelength of 570 nm. 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 zerumbone on human PBMC cell cycle progression. PBMC was chosen in order to evaluate the capability of zerumbone to stimulate the proliferation of lymphocytes *in vitro*. In this study, PWM was used as positive control. The active concentration chosen for zerumbone and PWM in order to stimulate the proliferation of PBMC was 30 μ g/mL, and 50 μ g/mL, respectively. In this study, 1 mL of PBMC with a density of 1 x 10⁶ cells/mL, respectively were treated with 1 mL of zerumbone and PWM according to their active concentrations as mentioned above. The treatments were carried out in 6 well plates (Nunc) with 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 4°C 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 consisting of 0.1% triton X-100, 10 mM EDTA, 50 μ g/mL RNase and 3 μ g/mL propidium iodide (PI). This process was done in the dark because PI is sensitive to

light. The cell was 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

Production of human interleukin-2 and human interleukin-12 was measured by enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (Bendemedsystems, Vienna, Austria). All the experiments were carried out in 96 well flat-bottomed microtitre plates. Briefly, 150 μ L of distilled water was added to all standard wells and blank wells. Then, 100 μ L of distilled water was added into the sample wells, followed by adding 50 μ L of culture supernatants (PBMC treated with zerumbone) and the positive control (PWM) into each of the designated wells. The culture supernatants of the control and test compounds were used without dilution. The plate was covered and incubated at room temperature (18°C to 25°C) for 3 hours on a microplate shaker. After that, the plate was washed three times with approximately 400 μ L wash buffer per well with thorough aspiration of microwell contents between washes. The plate was then tapped onto absorbent pad to remove excess wash buffer. After washing, 100 μ L of TMB substrate solution was added and incubated at room temperature on a microplate shaker at 100 rpm. After 10 minutes, 100 μ L of stop solution was pipetted into each well and the result was read immediately using a Benchmark microtitre plate ELISA reader (Bio-Tek instruments Inc) at 450 nm with reference wavelength at 620 nm.

STATISTICAL ANALYSIS

All experiments were performed in triplicates and the results were expressed as mean \pm S.E. Student's *t*-test was used to analyze statistical significant of the differences between the control and the treated values.

RESULTS

Mitogenic activity of zerumbone on Mice thymocytes

In this study, mice thymocytes were used to evaluate the effect of zerumbone on the proliferation of T cells *in vitro*. As shown in Fig. 2, zerumbone significantly ($p < 0.0001$) stimulated the proliferation of mice thymocytes in a time and dose-dependent fashion. Obviously, it showed the reduction of thymus cell population at 24 h treatment. The proliferation of mice thymocytes was only stimulated after 48 and 72 h treatment. Zerumbone exhibited better proliferation of mice thymocytes at concentration 7.5 μ g/mL. The proliferation rate of mice thymocytes treated with zerumbone was less than that of Con A at all

concentrations tested after 48 h treatment. Nevertheless, zerumbone stimulated better proliferation of mice thymocytes after 72 h treatment at concentration 7.5 $\mu\text{g/mL}$ with value of 56.26%. This data suggested that zerumbone stimulated better proliferation of mice thymocytes after longer incubation hour and most active at concentration 7.5 $\mu\text{g/mL}$.

Mitogenic activity of zerumbone on Mice splenocytes

In this study, the proliferation effect of zerumbone was evaluated towards mice splenocyte at different incubation hour. Mice splenocytes which was isolated from spleen cells were reported to have a nearly equal percentage of B and T cells with value of 60% of B cells and 40% is T cells (Cerqueira *et al.*, 2004). As shown in fig. 3, zerumbone stimulated mice splenocytes in a time and dose-dependent fashion. Zerumbone did not stimulate the proliferation of mice splenocytes at 24 h treatment at all concentrations tested. Nevertheless, it showed to stimulate mice splenocytes after 48 and 72 h treatments. Zerumbone has significantly induced highest proliferation of mice splenocytes up to 41.99% after 48 h treatment at 7.5 $\mu\text{g/mL}$. However, it inhibited mice splenocytes at concentration more than 15 $\mu\text{g/mL}$ after 72 h treatment and exhibited better proliferation at concentration 7.5 $\mu\text{g/mL}$. Zerumbone exhibited active proliferation at concentration 7.5 $\mu\text{g/mL}$.

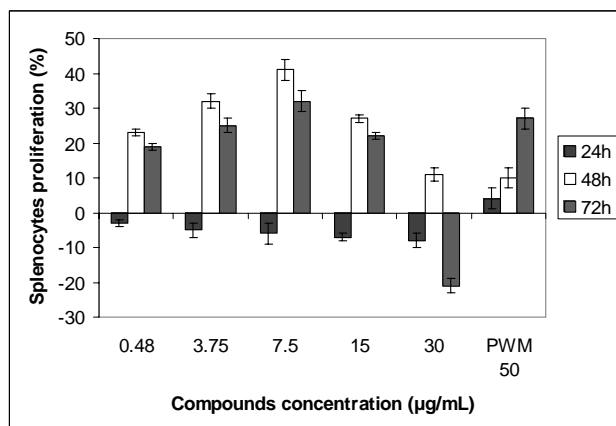


Fig. 3: Effects of zerumbone on the proliferative response of mice splenocytes at 24, 48 and 72 h treatment. Data represent means \pm S.E.M of triplicate determinations from three independent experiments.

Mitogenic activity of zerumbone on Human Peripheral Blood Mononuclear Cells (PBMC)

PBMC were isolated and treated to determine the capability of zerumbone in inducing the proliferation of human lymphocytes. The study was carried out to evaluate the impact of zerumbone towards human health. Zerumbone showed to stimulate the proliferation of PBMC in a time and dose-dependent fashion (fig. 4). Zerumbone did not stimulate the proliferation of PBMC at 24 and 48 h treatments. In fact, a reduction of cells

number was observed at both treatment periods. It started to stimulate the proliferation of PBMC after 72 h treatment, thus suggesting that this compound was active after a longer incubation hour. Similar to the mice splenocytes result, zerumbone was active in stimulating the proliferation of PBMC at concentration 7.5 $\mu\text{g/mL}$. In fact, the proliferation response of PBMC treated with zerumbone was less if compared to positive control, PWM. In conclusion, zerumbone may be toxic to PBMC at 24 and 48 h treatments at all concentrations tested. Zerumbone, in addition, also inhibited PBMC proliferation at concentration higher than 7.5 $\mu\text{g/mL}$ after 72 h treatment.

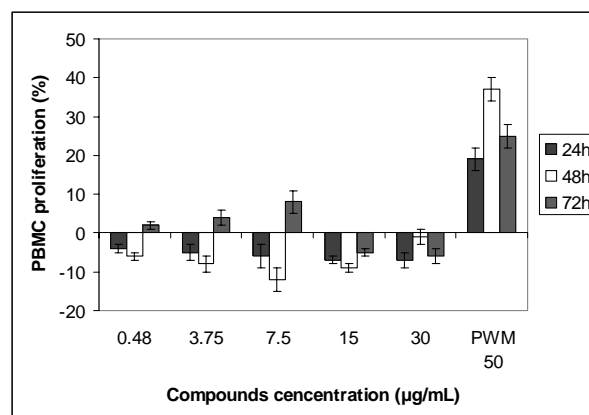


Fig. 4: Effect of zerumbone on the proliferative response of human peripheral blood mononuclear cells (PBMC) at 24, 48 and 72 h treatment. Data represent means \pm S.E.M of triplicate determinations from three independent experiments, * $p < 0.0001$, ** $p < 0.001$ (Student t-test) compared with untreated control.

Flow cytometry analysis of cell cycle distributions on PBMC based on Proliferation Effect of zerumbone and PWM at 24, 48 and 72 h of incubation time

This study was carried out to evaluate the effect of zerumbone and pokeweed mitogen to stimulate the proliferation of PBMC at different incubation hours. Cell cycle analysis also allow the evaluation of cells subpopulations in a mix cells sample such as healthy cells and apoptosis cells without needing extra separation step (Loken, 1980). The significant phase in evaluating the proliferation of cell is at G2/ Mitosis phases. At this phase, cells are divided into two daughter cells, which indicating the proliferation of cells has occurred.

From table 1, the percentage of cells treated with zerumbone entered into G2/Mitosis has increased gradually from 24, 48 and 72 h treatment with value of 15.87%, 35.59% and 56.85%, respectively (fig. 4). Therefore, the highest proliferation of cells treated with zerumbone occurred at 72 h (56.85%) which was 3.6-fold higher compared to untreated cells. Zerumbone, in addition at early incubation contributed to a high

apoptosis rate with value of 13.88%, and significantly reduced after 48 and 72 h treatment with value of 2.5% and 2.0%, respectively.

As demonstrated in fig. 4, the proliferation of PBMC treated with pokeweed mitogen at 24 hour incubation was significantly higher compared to untreated cells with value of 9.26% and 4.63%, respectively. Yet, the percentage of cells treated with pokeweed mitogen entered into G2/M had increased gradually from 24, 48 and 72 h treatment with value 9.26%, 30.48% and 41.91%, respectively. At early incubation hour, the low percentage of cells entered into G2/M phase corresponded to an increase of cells entered into Sub G1 phase, thus indicating that PWM may cause higher apoptosis at early incubation time particularly at 24 and 48 h treatment with value of 18.84% and 13.25%, respectively. However, PWM exhibited better proliferation of PBMC with 2.6-fold higher compared to control after 72 h treatment.

The production of human Interleukin-2 and human Interleukin-12

As shown in fig. 5, zerumbone significantly exhibited to induce the production of human IL-2 in a time-dependent manner. Interestingly, zerumbone has promoted higher induction of human IL-2 after 24 h of incubation with value of 531.66 pg/mL, compared to negative control with value of 20 pg/mL. These results indicated that zerumbone induced the production of human IL-2 after 24 h treatment with 26.5-fold higher compared to control. This result was 1.5 times higher compared to positive control, PWM after 24 h treatment. Nevertheless, the production of human IL-2 by zerumbone decreased drastically after 48 and 72 h treatment with 2.5-fold and 1.3-fold, respectively. PWM, however showed better induction of human IL-2 after 48 h treatment with 25.33-fold higher compared to control. In addition, it also demonstrated a steady induction of human IL-2 throughout the treatment period.

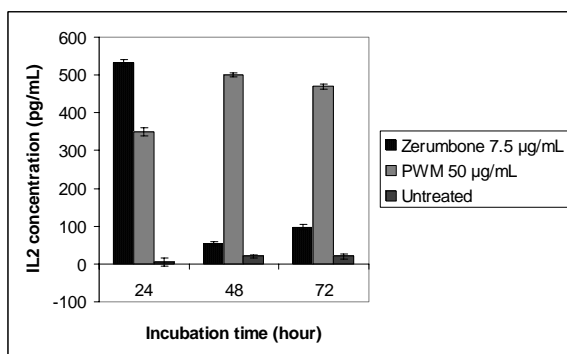


Fig. 5: The production of human IL-2 in culture supernatants upon stimulation of PBMC by zerumbone and PWM. PBMC were isolated and incubated at 24, 48 and 72 h with active concentrations (zerumbone at 7.5 µg/mL; PWM at 50 µg/mL) and IL-2 induction was specifically determined by ELISA.

As shown in fig. 6, the production of human IL-12 by zerumbone was quite high if compared to negative control human IL-12 with value of 12 pg/mL. Zerumbone exhibited to induce higher production of human IL-12 after 72 h treatment with a value of 53.66 pg/mL or 4.4-fold higher compared to negative control. LPS which was used as positive control showed to induce higher production of human IL-12 at 24 h treatment with value of 140.12 pg/mL indicating 11.67-fold higher compared to negative control. However, the production of human IL-12 induced by LPS decreased sharply after 48 and 72 h with a value of 26.93 pg/mL or 2.24-fold and 22.06 pg/mL or 1.8-fold higher, respectively compared to negative control. In conclusion, the capability of zerumbone to induce the production of human IL-12 is considered low if compared to positive control throughout the treatment periods.

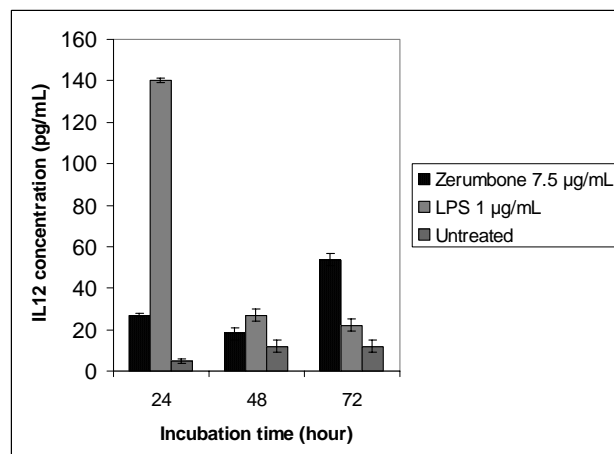


Fig. 6: The production of human interleukin-12 in culture supernatants upon stimulation of PBMC by zerumbone and LPS. PBMC were isolated and incubated at 24, 48 and 72 h with active concentrations (zerumbone at 7.5 µg/mL; LPS 1 µg/mL) and IL-12 induction was specifically determined by ELISA.

DISCUSSION

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 as 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).

In vitro immunomodulatory study exhibited that zerumbone was able to stimulate the proliferation of mice thymocytes, mice splenocytes and PBMC in a time and dose dependent manner. It was demonstrated that

Table 1: Flow cytometry analysis of cell cycle distribution on PBMCs based on proliferation effect of zerumbone and PWM. The values were the means \pm S.E. of three experiments

Compounds	Percentage of cell cycle distribution (%)	Incubation periods (h)		
		24	48	72
Untreated cell	Sub-G1 (apoptosis)	7.71 \pm 0.69	5.59 \pm 0.37	1.80 \pm 0.03
	G0/G1	5.82 \pm 0.52	36.09 \pm 2.43	58.71 \pm 1.12
	Synthesis	67.25 \pm 6.02	24.02 \pm 1.59	27.58 \pm 0.53
	G2/Mitosis	4.94 \pm 0.44	36.59 \pm 2.46	15.79 \pm 0.30
Zerumbone	Sub-G1 (apoptosis)	11.60 \pm 3.22	2.40 \pm 0.13	1.93 \pm 0.09
	G0/G1	41.6 \pm 11.55	42.13 \pm 2.34	24.07 \pm 1.15
	Synthesis	14.12 \pm 3.92	12.53 \pm 0.70	28.94 \pm 0.67
	G2/Mitosis	13.26 \pm 3.68	32.31 \pm 1.8	56.85 \pm 1.15
PWM	Sub-G1 (apoptosis)	17.10 \pm 2.46	11.64 \pm 2.27	0.65 \pm 1.02
	G0/G1	7.70 \pm 1.11	37.53 \pm 7.33	39.49 \pm 1.11
	Synthesis	45.11 \pm 6.49	12.04 \pm 2.35	24.02 \pm 0.67
	G2/Mitosis	8.40 \pm 1.20	26.78 \pm 5.23	41.09 \pm 1.15

zerumbone, at lower concentration ≤ 7.5 $\mu\text{g/mL}$ stimulated *in vitro* proliferation of mice and human lymphocytes. In contrast, zerumbone at concentrations higher than 30 $\mu\text{g/mL}$ suppressed mice and human lymphocytes. Interestingly, zerumbone showed higher stimulation of mice thymocytes, mice splenocytes and PBMC at concentrations 7.5 $\mu\text{g/mL}$ with the proliferation value of 56.26%, 33.77% and 8.57%, respectively after 72 h of incubations. The inhibition effect of zerumbone at concentrations higher than 15 $\mu\text{g/mL}$ was due to the compound exerting either stimulatory or inhibitory activity to the immune system. In addition, this study provided evidence that the same compound can exert both effects, depending on applied concentration. However, this phenomenon of stimulatory or inhibitory activity might be in accordance with previous publication that certain plant extracts or natural pure compounds possess cytotoxic and cytostatic activities. According to Wagner (1999), compounds which demonstrated higher cytotoxic effects towards cancer cells may exert immunomodulatory effect at lower concentration. At the moment, it could be speculated that the observed stimulatory and inhibitory effects of zerumbone towards mice and human lymphocytes is related to hormesis, a phenomenon observed with dose-response curve. Hormesis was defined as the response of a biological entity to an effector, with stimulatory results at low doses and inhibitory or harmful at high doses, thus explaining a molecule showing hormesis has the opposite effect in small doses than in large doses (Murado and Vazquez, 2007).

Cell cycle analysis demonstrated that zerumbone after 24 h treatment induced apoptosis with approximately 13.88% cells entered Sub G1 in the cell cycle distribution, thus reducing the percentage of the cells entering G2/M phase. After 48 h treatment, there was an increase of cells entered into G2/M phase with value of approximately

33.59%. However, this percentage of cells was quite low if compared to the control cells after 48 h of incubation which showed the proliferation value of 34.85%. In addition, the percentage of cells entered G2/M phase was higher after 72 h treatment with value of 56.85%. Only two percent of cells undergone apoptosis at this stage. Results obtained from cell cycle analysis clearly supported the result from lymphocyte proliferation assay on PBMC which showed zerumbone at 24 and 48 h treatment did not stimulate the proliferation of lymphocytes at all concentrations tested. This situation was due to some cells would have undergone apoptosis stages. Obviously, zerumbone was more active to stimulate lymphocytes proliferation after longer incubation h. The pattern of stimulatory effect of zerumbone towards PBMC was similar with the commercial mitogen, PWM.

Current result reflected that PWM stimulation in this experimental conditions reduced cell viability in the culture after 24 and 48 h treatments. The initial loss of cells was due to cytotoxicity effect of PWM towards PBMC which caused higher apoptosis rate in the cell cycle analysis (table 1). Nevertheless, there was an increase of the proliferation rate of PWM towards PBMC with value of 41.91% after 72 h treatment, thus suggesting that PWM was more active after longer incubation h.

Cytokines have been known to play an important role in regulating the proliferation and differentiation of lymphocytes, therefore the modulatory effect of zerumbone on the production of human IL-2 and human IL-12 upon stimulation of lymphocytes was of interest. It was surprising that zerumbone had significantly induced higher production of human IL-2 with the value of 531.66 pg/mL , followed by a sharp reduction after 48 and 72 h treatment with value of 56 pg/mL and 91.33 pg/mL ,

respectively (fig. 5). Interestingly, the human IL-2 concentration induced by zerumbone was much higher than that of PWM after 24 h treatment (fig. 5), thus suggesting that zerumbone might, partly or indirectly be responsible for promoting the proliferation of T-lymphocytes in PBMC. However, according to Akmal *et al.* (2005), the high production of IL-2 in the individual body with cancer may cause liver toxicity, skin toxicity, thrombocytopenia, respiratory problem as well as suppression of the immune system. Undoubtedly, the higher production of IL-2 may be toxic to the cells since the apoptosis rate is quite high in PBMC treated with zerumbone after 24 h treatment as shown in cell cycle analysis study.

In contrast, zerumbone was found to induce the production of human IL-12 in a time-dependent fashion, which it showed fluctuation from 24, 48 and 72 h with value of (25±3.6 pg/mL), (17.33±10.21 pg/mL) and (53.66±7.76 pg/mL), respectively. Nevertheless, the production of human IL-12 by Zerumbone was considered low throughout the treatment periods if compared to positive control human IL-12 with value of 200.35 pg/mL. The capability of zerumbone to induce low production of human IL-12 did support the previous findings which reported that zerumbone possessed strikingly anti-inflammatory agent (Murakami *et al.*, 2003(a); Tanaka *et al.*, 2001). This finding is relevant since IL-12 was known as pro-inflammatory cytokine. In addition, zerumbone was reported to possess anti-inflammatory effect through the inhibition of pro-inflammatory protein expression (iNOS/COX-2) and TNF- α released in RAW 264.7 cells (Murakami *et al.*, 2002). These suppressive events were accompanied by reduction in the production of NO and PGE₂, while the expression of COX-1 was unchanged.

As demonstrated by previous studies, NF- κ B is an important transcriptional factor for the expression of various inflammatory mediators. It could be speculated that the low production of human IL-12 upon stimulation of PBMC by zerumbone is correlated to the inhibition nuclear translocation of NF- κ B. Moreover, Murakami *et al.* (2003b) had demonstrated that zerumbone significantly inhibited the expression of NF- κ B. Meanwhile, it could be speculated that the induction of low concentration of human IL-12 throughout the treatment period was due to its nucleophilic properties that enable zerumbone to react with biological nucleophiles, such as I κ B kinase thereby modifying the cystein residue through attenuating I κ B phosphorylation to abolish the NF- κ B activity (Murakami *et al.*, 2002). NF- κ B could be activated by a number of physiological and nonphysiological stimuli, including cytokines, mitogens, viruses, mechanical, oxidative stress and a variety of chemicals agents. Thus, these findings suggested that zerumbone can act as promising novel

therapeutic agent in maintaining the immune system and in controlling inflammation.

Although zerumbone showed encouraging results as an immunomodulator, further studies are needed to elucidate the exact mechanism of action involved in zerumbone. At the same time, it is vital to analyze multiple assays to allow a comprehensive evaluation of the immunomodulatory efficacy of this compound. In addition, pharmacokinetic studies are also fundamental to further evaluate the effective doses and toxicity effects of these four compounds. Once the mechanism of its action and comprehensive bioassays are elucidated, zerumbone could be used as a lead molecule for a new generation of drugs in cancer treatment particularly in boosting up the immune system.

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