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Full Length Research Paper

Immunomodulatory role of *Rhaphidophora korthalsii* methanol extract on human peripheral blood mononuclear cell proliferation, cytokine secretion and cytolytic activity

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Rhaphidophora korthalsii **methanol extract induce the immunomodulatory effect on human peripheral blood mononuclear cell (PBMC). The present study was performed to investigate the immunomodulatory potential of the extract through different tests: MTT assay, BrdU proliferation assay, Trypan blue direct cell counting, cytokine (IL-2, IL-12 and IFN-γ) ELISA assay and Annexin V cytotoxicity assay. Concanavalin A (Con A) and lipopolysaccharide (LPS) were used as positive control. The observed results from all MTT assay, BrdU proliferation assay and Trypan blue direct cell counting showed that the plant extract stimulated the PBMC at 48 and 72 h where 25 µg/ml was the optimum concentration. On the other hand, the results of the cytokines study showed that 25 µg/ml induced the secretion of IL-2 (433 ± 25 pg/ml) and IFN- γ (7336 ± 131.79 pg/ml) but not IL-12 at 72 h only. The activated PBMC also directed the effector cell (K562) in mix culture to undergo apoptosis (12.96 ± 0.57% of early apoptosis and 10.60 ± 0.21% of late apoptosis) at the ratio of 2/1 of PBMC/K562. The present results indicate that** ***R. korthalsii*** **extract showed evidences of immunomodulating activity which may be potential to use as an immunotherapy agent.**

Key words: *Rhaphidophora korthalsii*, immunomodulator, proliferation, cytokine, natural killer.

INTRODUCTION

Over the past two decades, attempts have been made to search for plant derived health-promoting commercial product. Thus, the screening of the bioactivity of the traditional herb has become one of the activities in biomedical field study. For example, Chiu et al. (2006) reported the ethyl acetate extract of Chinese medicinal herb *Patrinia scabiosaefolia* retarded the growth of MCF-7. Besides the cytotoxic study, immunomodulatory role of the plant extract is also one of the widely focus biological activity of the plant. This is due to the various function of the plant mitogen in diverse biological functions such as cell adhesion, cell migration, cellular growth and differential induction of apoptosis.

Rhaphidophora korthalsii (Araceae) (revised to *Epipremnum pinnatum*) is commonly known as "Dragon

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tail" in Malaysia and Singapore. The decoction of the plant leaves has been widely used as a traditional herb to treat cancer and skin disease. Wong and Tan (1996) showed that the ether extract of the plant showed cytotoxic effect towards P388, Molt 4, KB and SW 620 cell lines while the hot water extract was found to induce the weak proliferation on the T cell of the mice splenocytes. The cytotoxicity of the plant may be due to the presence of 5,6-dihydroxyindole (DHI) which showed the cytotoxic effect towards p388 (Toyota and Ihara, 1998). DHI also proved to have the cytotoxic effect on nonmelanocytic cancer cell but not melanocytic cell (Urabe et al., 1994). The hexane extract of the plant was found to exert growth arrests in T-47D cells via a Type II non-apoptotic programmed cell death (Tan et al., 2005). Lau et al. (2004) found that the decoction from the various part of *R. korthalsii* showed strong antioxidant effect and increased the total macrophage cell number. As a result, they concluded that this plant extract may be useful in boosting up the immune system to fight for various diseases including cancer.

Induction of immune cell proliferation is always related to the immunomodulatory property. Few methods are being widely used evaluated the immunomodulating activity based on the assessment of the proliferation (such as 5-bromo-2-deoxyuridine (BrdU) proliferation assay), metabolic activity (such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay) and cell number quantitation (direct cell counting using a hemocytometer). Besides, the activation of immune cell also can be studied by measuring the cytokine production. (Durrieu et al., 2005) IL-12 is a heterodimeric glycoprotein which plays an important role to induce Th1 but suppress Th2 responses which consequently lead to the Interferon-gamma (IFN-γ) production (Wu et al., 2006). On the other hand, T-helper (Th) 1 (Interleukin 2 (IL-2) and IFN-γ) cytokine modulation can affect the Natural killer (NK) cell cytotoxicity (Santarelli et al., 2006). NK cells play an important role in defense against bacterial infection, virus-infected cells and malignant cell. High frequency of infections and cancer always related to the reduction of NK cells number or cytolytic activity. The functional assay involves lysis of NK cell towards the K562 (NK cell-sensitive erythroleukemia cell line) target cell line (Gharehbaghian et al., 2002).

Previous immunomodulatory study for this plant extract was only carried out using mice splenocytes (Wong and Tan, 1996). The immunomodulation effect of the extract on human PBMC cell cycle progression and cytolytic activity against human hepatoblastoma HepG2 cell also have been reported (Yeap et al., 2007). However, the immunomodulating effect of this plant extract toward human immune cell proliferation, cytokine expression and cytotoxicity against K562 has not been studied. In this study, we examined the influence of R. korthalsii methanol extract on human peripheral blood mononuclear cells (PBMC) *in vitro*, Th1 (IL-2 and IFN-γ) and IL-12 cytokines in an in-vitro model. The NK cell activity of the activated PBMC was tested on K562 in 1:1 ratio by Annexin V.

MATERIALS AND METHODS

Plant material

Leaves of *R. korthalsii* was collected in Georgetown, Penang in June 2006, and were identified by science officer Lim Chung Lu (Kepong, Selangor) from Forestry Division, Forest Research Institute Malaysia (FRIM). The voucher number of *R. korthalsii* is FRIM 33687. The leaves of the plant was air-dried in shade and finely powdered. The leaf extract was prepared by keeping 62.5 g of leaf powders soaked in 250 ml methanol (J.T. Baker, USA) for 72 h and repeated for three times. The extracts were filtered with Whatman filter paper no 1 and evaporated to dry under reduced pressure by using Aspirator A-3S (EYELA, Japan) at < 40°C. The process was repeated three times (yield 27.3%, w/w). The dried residue was suspended in DMSO (Fisher Scientific, UK) as a plant extract stock. Briefly, the concentrated plant extracts were dissolved in dimethylsulphoxide (DMSO) (Sigma, USA) to get a stock solution of 10 mg/ml. The substock solution of 0.2 mg/ml was prepared by diluting 20 µL of the stock solution into 980 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°C.

Cell lines

Suspension target cell line, K562 (NK cell-sensitive erythroleukemia cell line) were obtained and thawed from Animal Tissue Culture Laboratory, Department of Cell and Molecular Biology, UPM and were maintained in RPMI 1640 respectively with 10% FBS (PAA, Austria) at 37°C, 5% CO_2 and 90% humidity throughout the study. The cell viability was assessed by the trypan blue exclusion method.

Isolation of human PBMC

Blood (20 to 25 ml) was taken from four different healthy donors throughout this research by using the 25 ml syringe. The blood sample was diluted with the same volume of PBS-BSA-EDTA. Separation of blood cells was performed using density centrifugation. Briefly, the diluted blood sample was carefully layered on Ficoll-Paque Plus (Amersham Biosciences, USA). The mixture was centrifuged under at 400x g for 40 min at 18 to 20°C. The undisturbed lymphocyte layer was carefully transferred out. The lymphocyte was washed and pelleted down with three volume of PBS-BSA-EDTA for twice and resuspended RPMI-1640 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 with equal volume of trypan blue.

MTT Cell viability assay

The effect of the extract on cell viability of PBMC was first determined by using a colorimetric technique which is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 100 µL of RPMI-1640 media with 10% of FBS was added into the all the well except row A in the 96 well plate (TPP, Switzerland). Then, 100 µL of diluted extract at 200 µg/ml was added into row A and row B. A series of two fold dilution of extract was carried out down from row B until row G. Row H was left

untouched and the excess solution (100 µl) from row G was discarded. Hundred µl of lymphocyte (from human peripheral blood) with cell concentration at 5×10^5 cells/ml was added into all wells in the 96 well plate to make up the final volume of 200 µl and thus diluted the extract into the concentration range of 100 to 1.56 µg/ml. All the plates were incubated in 37°C, 5% CO_2 and 90% humidity incubator for selected period (24, 48 or 72 h). After the corresponding period (either 24 , 48, or 72 h), 20 µl of MTT (Sigma, USA) at 5 mg/ml was added into each well in the 96 well plate and incubated for four hours in 37°C, 5% CO_2 and 90% humidity incubator. 170 µL of medium with MTT was removed from every well and 100 µL DMSO (Fisher Scientific, UK) was added to each well to extract and solubilize the formazan crystal by incubating for 20 min in 37°C, 5% CO_2 incubator. Finally, the plate was read at 570 nm wavelength by using µ Quant ELISA Reader (Bio-tek Instruments, USA). The results of the plant extract were compared with the result of ConA (1 µg/ml) and LPS (1 µg/ml) and without mitogens or extract by using same method. Each extract and control was assayed in triplicate in three independent experiments. The percentage of proliferation was calculated by the following formula:

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

BrdU proliferation assay

The proliferation effect of the extract was studied by using Brdu Cell Proliferation Kit (Chemicon, USA). Briefly, Cell concentration of PBMC was adjusted to 4×10^5 cells/ml. Then, 100 µL of media with plant extract at various concentrations (50, 25, and 1 µg/ml) was added into a 96 well plate (TPP, Switzerland). Three different types of control cultures, without extract, with only media and with positive control with ConA (1 µg/ml) and LPS (1 µg/ml), were prepared simultaneously. 100 µl of PBMC was added into all wells and incubated in 37°C, 5% CO_2 and 90% humidity incubator for selected period (24, 48, 72 h). Two hours before the end of corresponding period, 20 µL of BrdU reagent was added into all well except 3 well as unstained control. The culture was incubated for another 16 h and was pelleted down. The culture was then fixed, washed and added with BrdU detection antibody. Then, the culture was washed again and added with Goat anti-mouse Ig G, Peroxidase labeled conjugate. The conjugate will be targeted with 3,3',5,5''-tetramethylbenzidine (TMB) Peroxidase substrate in dark for staining. Finally, 2.5N sulfuric acid stop solution was added and the plate was read at 570 nm wavelength by using µ Quant ELISA Reader (Bio-tek Instruments, USA) at Animal Tissue Culture Laboratory, FBBS, UPM. Each extract and control was assayed in three times

Trypan blue exclusion method

Cell concentration of PBMC was adjusted to 4×10^5 cells/ml and 1 ml of the cell was added into 6 well culture plate (TPP, Switzerland). One ml of FBS (PAA, Austria) with plant extract at various concentrations (50, 25 and 1 µg/ml) was than placed into the plate and incubated for 48 and 72 h in 37°C, 5% CO_2 and 90% humidity. Control cultures without extract and positive control with ConA (1 µg/ml) and LPS (1 µg/ml) were prepared simultaneously. After the incubation period, the cultures were harvested and wash twice with PBS (JRH Biosciences, USA). The cell pellet was then resuspended with 0.5 ml PBS. Then, 10 µL of cell was mixed with equal volume of 0.4% trypan blue (Sigma, USA) and was count by using Neubauer haemocytometer (Weber, England) by clear field microscopy (Nikon, Japan). Only viable cells were counted. Each extract and control was assayed five times in triplicate.

Cytokine (IL-2, IL-12 and IFN-γ) determinations

The expressions of IL-2, IL-12 and IFN-γ were performed by using Cytokine Instant Enzyme Link Immunosorbent Assay (ELISA) kit (Bender MedSystems, Austria). Briefly, the extracted human PBMC was treated with same volume of plant extract at 25 µg/ml in 6 well culture plate (TPP, Switzerland). Control cultures without extract and positive control with ConA (1 µg/ml) and LPS (1 µg/ml) were prepared simultaneously. The culture was than incubated for respective time (24, 48, and 72 h). After the corresponding period, the samples were washed and pelleted. The supernatant was added into the strip on the plate of the kit and incubated for three hours with 200 rpm shaking. Then, the sample was washed and added with 3,3',5,5''-tetramethylbenzidine (TMB) Peroxidase substrate in dark immediately. Finally, 1 M Phosphoric acid stop solution was added and the plate was read at 450 and 620 nm wavelength by using µ Quant ELISA Reader (Bio-tek Instruments, USA) at Animal Tissue Culture Laboratory, FBBS, UPM. The result was compare to the control strip in the kit. Each extract and control was assayed in three times. Data were expressed as pg/ml.

PBMC cytotoxicity assay

PBMC (Target-T) was exposed to the plant extract at 25 µg/ml for 72 h in a 6 well plate. Then, the activated PBMC was cultured with equal volume of K562 (Effector-E) at ratio of T/E at 2/1 and incubated in 37°C, 5% CO_2 and 90% humidity for 24 h. Control culture with only PBMC and K562 was prepared simultaneously. At the end of the co-culture incubation, cells were harvested, washed and stained with Annexin V FITC (BD Biosciences, USA) with propidium iodide (PI). Briefly, the culture was washed with PBS and resuspended in 500 µL total volume that contain 100 µL of cells (5×10^5 cells/ml), 10 µL PI (Sigma, USA), 5 µL Annexin V-FITC (BD Bioscience, USA) and 400 µL binding buffer (BD Bioscience, USA). After 15 min incubation in dark, the cells were analyzed with COULTER EPICS ALTRA flow cytometer (Beckman Coulter, USA) within half hour. Each extract and control was assayed in triplicate in three independent experiments.

Statistical analysis

Results were expressed as Mean ± Standard Error (S.E.M.). Different between means were evaluated using ANOVA test (one way) followed by Duncan test and $p \leq 0.05$ was taken as statistically significant.

RESULTS

After an incubation period of 24 h, only treatment at 100 µg/ml of extract bring significant increase of cell number compare to the control with 111.76 ± 4.88% mean viability (± S.E.M.) in the culture (Table 1). The result from BrdU assay (Figure 1) also showed that all the treatment did not cause the culture to undergo active proliferation. An increase of cell number was observed in both MTT cell viability assay and trypan blue direct cell counting (Figure 2) for both 48 and 72 h. MTT assay showed the highest cell viability at 25 µg/ml when compared to the untreated PBMC at 48 h (144.36 ±

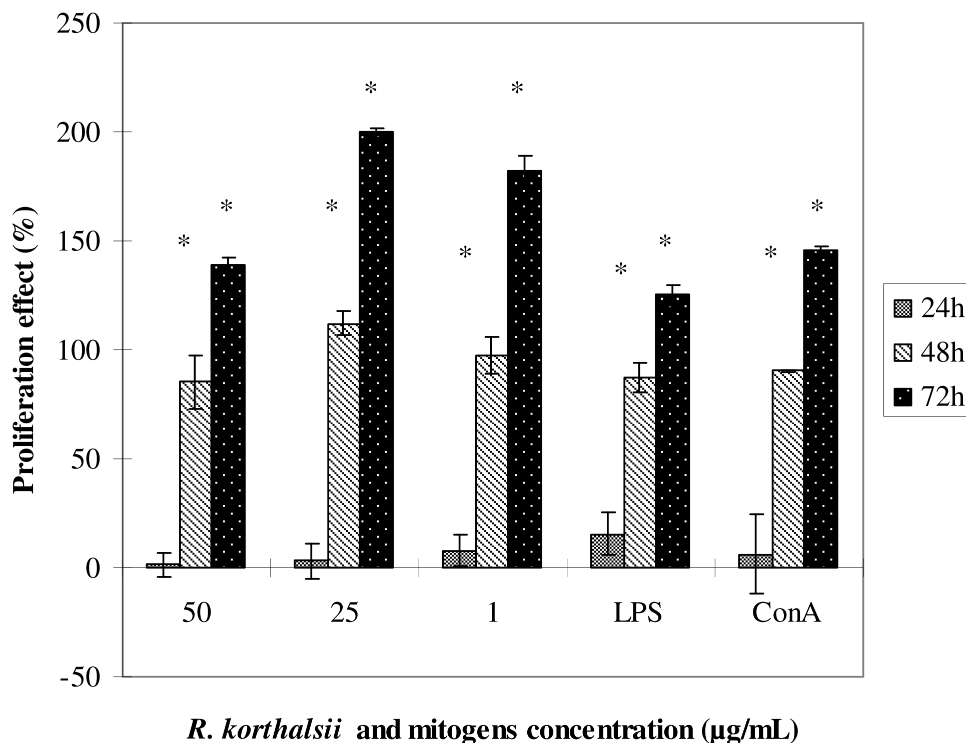

Figure 1. Proliferation Effect of R. korthalsii methanol extract or mitogens on Human PBMC in BrdU proliferation assay compared to the untreated culture at 24 h,48 h and 72 h. Each value represents the means ± S.E.M. triplicate in three independent experiments. The differences between the control group and treated group were determined by one-way ANOVA (*P⩽0.05).

8.95%) and 72 h (151.93 ± 10.88%) (Table 1). The trypan blue dye exclusion cell counting agrees with the MTT assay result where 120.89 ± 1.611 × 10^4 cell/ml was found in the cell population induced with 25 µg/ml at 48 h and 154.67 ± 1.71 × 10^4 cell/ml at 72 h (Figure 2). However, MTT assay and cell count will count both resting and active proliferating cells. Thus, to confirm the time and dosage dependent proliferation effect of the plant extract towards the PBMC, BrdU proliferation test was performed in the three different concentration (50, 25, 1 µg/ml). Figure 1 showed that stimulation of PBMC with 25 µg/ml of *R. korthalsii* methanol extract caused the highest cell proliferation effect on 48 h (112.04 ± 9.78%) and 72 h (199.59 ± 3.15%) compared to other concentrations (Figure 1).

R. korthalsii methanol extract displaying a detectable strong mitogenic response may activate the PBMC by cytokine secretion. Up regulation of cytokine will affect the immune response. In this study, only the secreted cytokine was study by using the ELISA and nonactivated PBMC were used as control. The result of cytokine secretion showed that the plant extract activated PBMC to release high amount of IL-2 (433 ± 25 pg/ml) and IFN-γ (7336 ± 131.79 pg/ml) even compared to ConA and LPS (Figure 3). However, this only happened at 72 h and the secretion of all cytokine at 24 and 48 h was not significant compared to the untreated PBMC. There was no significant IL-12 induction either in extract treated or control group except for LPS at 72 h (138.39 ± 7.14 pg/ml).

In the Annexin V apoptosis study, we observed that the extract itself at 25 µg/ml did not cause any apoptosis to the K562 (93.54 ± 0.67% of cell viability). Besides, the untreated PBMC was also not sensitive towards the K562 (93.39 ± 0.25% of cell viability) (Table 2). On the other hand, the cytolytic activity of PBMC was increase when it was activated with 25 µg/ml of extract where it can induce 4 and 3 fold of early and late apoptosis, respectively, compared to the mix culture of unactivated PBMC and K562 (12.96 ± 0.57% of early apoptosis and 10.60 ± 0.21% of late apoptosis) at the ratio of 2/1 of T/E. However, the activated immune cell only induced low level of necrosis against the effector cell (0.29 ± 0.03%).

DISCUSSION

R. korthalsii methanol extract can stimulate the proliferation (Figure 1) and maintain the cell viability (Figure 2) of PBMC with optimum concentration at 25 µg/ml. The activation of PBMC proliferation is always related to the immunomodulating potential of the extract

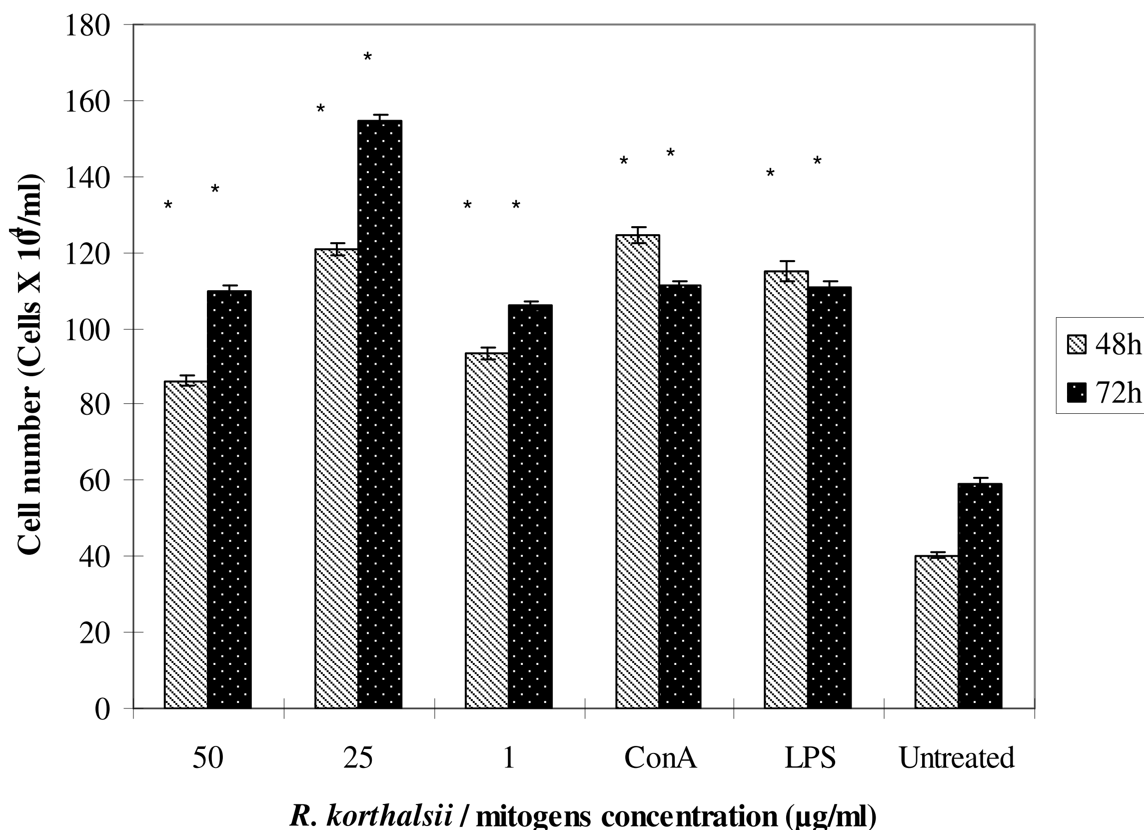

Figure 2. Proliferation effect of R. korthalsii methanol extracts on human PBMC in vitro for 48 h and 72 h. Each value represents the means ± S.E.M. for five independent assays in triplicate each. The differences between the control group and treated group were determined by one-way ANOVA (*P≤0.05).

Table 1. Results of PBMC MTT viability assay for comparison between treated groups and control group. Each value represents the means ± S.E.M. three experiments with triplicate cultures. The differences between the control group and treated group were determined by one-way ANOVA (*P≤0.05),

Concentration of *R. korthalsii* (µg/ml)	Cell viability after the incubation period (%)		
	24 h	**48 h**	**72 h**
100.00	111.76 ± 4.88	116.94 ± 8.47*	142.83 ± 12.17 *
50.00	106.43 ± 5.21	140.98 ± 8.15*	143.07 ± 10.53*
25.00	105.78 ± 5.38	144.36 ± 8.95*	151.93 ± 10.88*
12.50	105.78 ± 4.28	117.32 ± 7.56*	121.42 ± 6.76*
6.25	105.45 ± 2.35	117.59 ± 8.15*	123.31 ± 7.64*
3.13	106.36 ±2.94	120.35 ± 8.56*	129.16 ± 10.54*
1.56	108.54 ± 4.70	120.21 ± 7.70*	125.89 ± 7.91*
LPS 1 ug/ml	113.49 ± 7.55*	144.46 ± 7.77*	118.72 ± 11.28*
Con A 1 ug/ml	122.79 ± 6.94*	151.71 ± 6.96*	121.96 ± 6.28*

(Durrieu et al., 2005). BrdU, MTT and Trypan blue exclusion cell counting were used to measure different parameter of extract induced cell proliferation. BrdU assay was used to study the DNA synthesis of the culture (Durrieu et al., 2005), MTT assay on the other hand was to determine the cell metabolic activity (Mosmann, 1983) while trypan blue dye exclusion was to estimate the total final cell number in the culture (Lezema, 2006). All of these assays agree that this plant extract stimulate the cell proliferation in time (48 and 72 h) and dosage (25 µg/ml) pattern. One µl of *R. korthalsii* has shown higher total cell number and proliferation in both BrdU and Trypan Blue cell counting assay when compared to 50 µl (Figures 2 and 3).

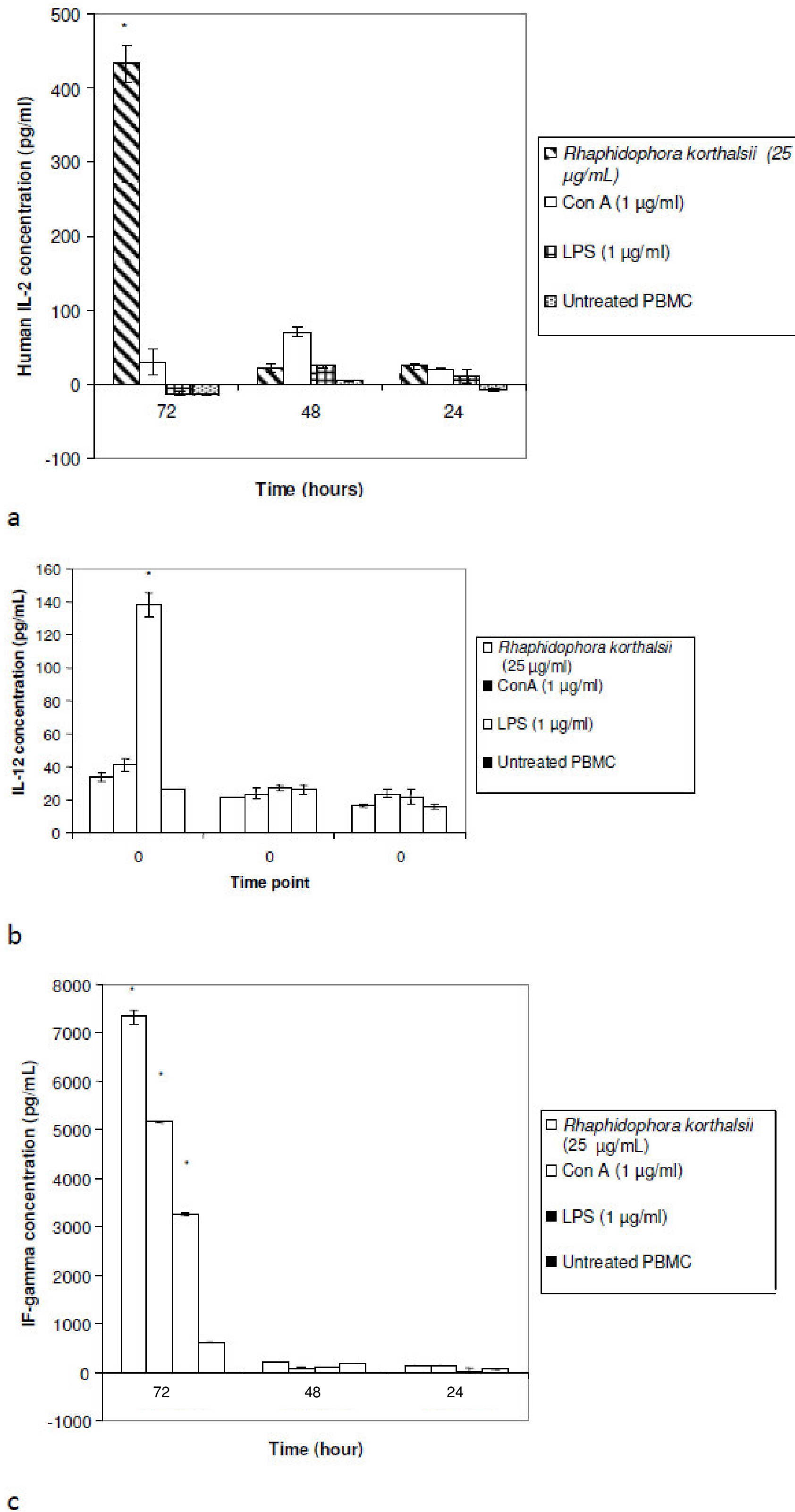

Figure 3. ELISA cytokine assay of *R. korthalsii* methanol extract or mitogens stimulated Human PBMC (a) IL-2 (b) IL-12 (c) IFN-γ at 24, 48 and 72 h. Each value represents the means ± S.E.M. for three independent assays in duplicate each. The differences between the control group and treated group were determined by one-way ANOVA (*P≤0.05).

Table 2. FACS analysis of Annexin V and PI binding of a different culture system for mixed population of PBMC and K562 cells in a ratio 2:1. The values were the means ± SE of three experiments. The differences between the control group and treated group were determined by one-way ANOVA (*P ≤ 0.05).

	Cell population (%)			
	Early apoptosis	**Late apoptosis**	**Necrosis**	**Viable**
PBMC untreated	3.09 ± 0.12	3.30 ±0.26	0.20 ± 0.15	93.40 ± 0.24
PBMC treated with 25 µg/ml *R. korthalsii*	0.99 ± 0.55	1.46 ±0.78	0.01 ± 0.01	97.53 ± 1.33
K562 untreated	1.71 ± 0.21	3.34 ±0.56	0.07 ± 0.04	93.54 ± 0.67
K562 treated with 25 µg/m *R. korthalsii*	2.11 ± 0.59	2.65 ±0.50	0.05 ± 0.03	94.83 ± 0.80
PBMC untreated and K562	3.36 ± 0.19	3.21 ±0.22	0.04 ± 0.02	93.39 ± 0.25
PBMC treated with 25 µg/m *R. korthalsii* and K562	12.96 ± 0.57*	10.60 ±0.21*	0.29 ± 0.03*	76.16 ± 0.50*

However, the MTT viability index of PBMC treated with 1 µl was lower than 50 µl (Table 1). This may be due to lower amount of mitochondrial dehydrogenases was present in the cell that was treated by 1 µl of extract. Besides, BrdU was found to be a more sensitive and most suitable nonradioactive alternative method to detect cell proliferation when compared to MTT which was suggested to be suitable just as a screening tool to detect lymphocyte proliferation (Wagner et al., 1999). In order to obtain precise results, three methods (MTT, BrdU and Trypan Blue cell count) were used to estimate the proliferation effect of the extract. Although, concentration as low as 1.56 µg/ml also induced cell proliferation (Figure 1), 25 µg/ml give the best proliferation effect in MTT, BrdU and trypan blue cell count assays and were selected and recommended in this experiment. This is because this concentration can provide the benefit of both cytotoxic and immunomodulation since previous study has shown that *R. korthalsii* extract induced cytotoxic effect against various cancerous cell line with IC_{50} range between 13 to 50 µg/ml (Wong and Tan, 1996). Previous research has reported that *R. korthalsii* methanol extract enhanced cytotoxicity effect of human PBMC against human hepatoblastoma HepG2 (Yeap et al., 2007). In this study, we have found that this may be contributed by the modulation of cytokine secretion (IL-2 and IFN-γ). IL-2 is a lymphokine product by activated T cell which can stimulate the proliferation of helper and cytotoxic T cell and can induce the differentiation of a precursor cytolytic T or NK cell to become Lymphokine activated killer (LAK) cell *in vitro* (Thompson et al., 1987). The application of IL-2 is at the clinical phase on the patients with renal-cell carcinoma or melanoma. Lower doses of IL-2 are less toxic and could increase the number of NK cell (Dranoff, 2004). Since, the plant extract can induce the secretion of IL-2 (Figure 3), it is possible that the plant extract may be able to replace the recombinant IL-2 which is widely used for the IL-2 treatment.

Deficiency of IFN- γ may promote the tumor formation. IFN- γ plays an important role to inhibit chemical carcinogenesis and to inhibit lymphomas (Dranoff, 2004). IFN-γ modulation can affect the NK cell cytotoxicity (Santarelli et al., 2006). Our IFN-γ secretion result and mix culture Annexin V study showed that with the increase of IFN-γ secretion (Figure 2) after treated with the plant extract, the cytolytic activity of the immune cell towards the effector cancer cell (K562) was increase as well (Table 2).

IL-12 is a heterodimeric cytokine which play an important role in growth stimulation of activated T and NK cells and induction of the secretion of IFN-γ from T and NK cells (Verbik et al., 1996). Activated macrophage by LPS can increase the production of IL-12 which can subsequently induce the expression of IFN-γ by either IL-12 activated Th1 or NK cell (Adam and Hamilton, 1984). Our IFN-γ secretion study result demonstrated that with the presence of LPS, there was an increase of IL-12 compared to the untreated PBMC and a marked increase of IFN-γ secretion. However, the plant extract did not induce any IL-12 secretion but can induce a significant secretion of IFN-γ even compared to the LPS and ConA. This may be due to the direct activation of the plant extract towards either Th1 or NK or indirect effect of IL-2 secreted by activated T cell (Thompson et al., 1987) to stimulate the IFN-γ secretion.

In our study, we showed that the plant extract could stimulate the immune cell cytolytic activity. In this study, the cytotoxicity of extract against NK cell sensitive K562 cell line. Wahlberg et al. (2001) showed that the NK cells were the only effector cells which actively involve in killing of tumor cell target although, there are some other potentially cytotoxic anticancer immune effector cells such as Tumor-specific cytotoxic T lymphocytes and cytotoxic NK-T cells. Thus, the apoptosis of the mix culture tested by using Annexin V may be due to the activation of NK cell activity by the plant extract. NK cell is the distinct subset of large granular lymphocytes which kill the tumor or virus infected target cell by using two mechanisms in the innate immunity (Li et al., 2005). The first mechanism is by secreting granules which contain perforin and granzymes that induce necrosis and apoptosis, respectively. The second mechanism is by Fas ligand (Fas L)/Fas pathway (Li et al., 2004). Our

Annexin V mix culture result showed that the immune cells induce apoptosis rather than necrosis. Thus, it was expected that the induction of the plant extract either directly or indirectly could increase the level of granzyme to kill the K562 through apoptosis pathway. Since, K562 is sensitive towards NK cell, it is expected that the extract has enhanced the cytolytic activity of the NK cell present in PBMC. Although, we do not know the exact mechanism of *R. korthalsii* methanol extract induced NK cell cytotoxicity, it is expected that the activation may be due to the direct binding effect of the extract to NK cell or indirect effect of IL-2 secreted by accessory cells. PBMC cytolytic activity was examined by using flow cytometry Annexin V quantification adopted from Durrieu et al. (2005).

PBMC treated with the extract was used as the background to estimate the possible Annexin V signal contributed by extract treated PBMC. However, this method was not able to differentiate the Annexin V and PI staining was contributed by either the immune cell or cancerous cell. To confirm the effect of R. korthalsii against human NK cell, further test using purified NK cell should be carried out. The active compounds that contributed to the immunomodulatory effect of R. korthalsii are still yet to be identified. Up to now, only 5,6-dihydroxyindole (DHI) which carried cytotoxic effect have been identified (Toyota and Ihara, 1998) and the immunomodulatory effect of this compound is still unknown. As a result, effort to isolate the active compounds that contribute to the immunomodulation effect is needed to develop a potential immunomodulatory agent for tumor immunotherapy. In summary, our study demonstrates that R. korthalsii methanol extract was able to induce the proliferation of immune cell, induced the Th1 cytokine (IL-2 and IFN-γ) and thus increased the cytolytic activity of PBMC.

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