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Full Length Research Paper

Immunomodulatory effect of *Rhaphidophora korthalsii* on mice splenocyte, thymocyte and bone marrow cell proliferation and cytokine expression

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Rhaphidophora korthalsii **methanol extract was previously found as a potential *in vitro* immunomodulating agent. In this study, the *in vitro* immunomodulatory activity of *R. korthalsii* methanol extract on mice splenocyte were quantified through evaluating T/NK cell population, cytokine expression (IL-2 and IFN-γ) and cytotoxicity against Yac-1 cell. *In vitro* MTT proliferation study showed that *R. korthalsii* methanol extract stimulate significant proliferation of splenocyte, thymocyte and bone marrow cell at 25 µg/ml after 72 h incubation as compared to rIL-2. Besides, 25 µg/ml of extract also enhanced T-helper 1 cytokine (IL-2 and IFN-γ) secretion, natural killer cell ($NK1.1^+CD3^-$) population and cytotoxicity against natural killer sensitive Yac-1 leukemic cell line. It was concluded that *R. korthalsii* methanol extract is a potential immunomodulator agent that could activate the natural killer cell.**

Key words: Immunomodulation, *in vitro*, *Rhaphidophora korthalsii*, cytotoxicity.

INTRODUCTION

Development of new highly safe and effective immunostimulators is a relevant scientific and practical problem of modern immunology and medicine. Herbs have become one of the major nutritional supplements and have exhibited health-beneficial effects against cancer and infectious disease. More and more attention were channelled towards dietary phytochemicals that have played a significant role in drug discovery and development, especially for agents with anti-proliferation, cytotoxic and immunomodulatory effects. Among these, there is a growing interest in identifying plant-based immunomodulator ever since their possible use in modern medicine was suggested (Gupta et al., 2006). Thus, a systematic evaluations of the immunomodulatory activities of most of the plant extracts is needed. Major parameters to evaluate the immunostimulatory effect of the natural products normally include the following methods. Investigation on cell proliferation effect of the treated immune cell is the preliminary study for the discovery of new immunomodulators from herbs.

Rhaphidophora (Araceae) is a large genus of a climbing shrub distributed throughout India and South East Asia (Kiritikar and Basu, 2001). *Rhaphidophora korthalsii* or "Dragon tail" is a large root-climber (Tan et

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al., 2005). The decoction of the plant leaves has been widely used in Chinese traditional medicine to treat cancer and skin disease.

Previously, *R. korthalsii* has shown antioxidative and immunomodulatory effects toward macrophage and murine splenocyte proliferation (Lau et al., 2004; Wong and Tan, 1996). Furthermore, Yeap et al. (2007) have reported that *R. korthalsii* methanol extract stimulated proliferation, T helper 1 (Th1) cytokine expression and lymphocyte-mediated cytotoxicity in human PBMC. However, the detail *in vitro* stimulating effect of this extract on splenocyte T and NK cell population, cytokine expression and cytotoxicity are still unclear. The aim of this study is to further evaluate the *in vitro* immunomodulatory effects of *R. korthalsii* methanol extract in mice splenocyte in comparison with *in vitro* immunomodulatory effects in human PBMC.

MATERIALS AND METHODS

Reagents and chemicals

Medium Dulbecco's modified eagle medium (DMEM) (Sigma, USA) and Foetal bovine serum (FBS) (PAA, Austria) were used in all the studies. Recombinant murine Interleukin 2 (rIL-2) (Dako, USA) and pokeweed mitogen (PWM) (Sigma, USA) was used as a positive control. This commercial T and NK cell activator was prepared by dissolving it with DMEM medium (Sigma, USA) and store in -80°C prior usage. Fluoroisothiocyanate (FITC)-labeled CD3 anti-goat monoclonal antibody and phycoerytherin (PE)-labeled CD56 anti-goat monoclonal antibody were purchased from Biolegend, USA.

Preparation of plant material

Leaves of *R. korthalsii* (Araceae) 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 placing the leaf powders in percolator with 250 ml of methanol (J.T. Baker, USA) for 72 h. The extracts were collected and the same process of extraction was repeated three times. The total extract was collected, filtered with Whatman filter paper no. 1 and evaporated to dry under reduced pressure by using Aspirator A-3S (EYELA, Japan) at < 40°C (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.

Animal

Balb/c mice, 8 weeks old, were used in all experiments. The animals were purchased from Animal House, Institute for Medical Research (IMR) (Kuala Lumpur, Malaysia). The animals were housed under standard conditions at 25 ± 2°C and fed with standard pellets and tap water. 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).

Cell lines and cell preparation

Yac-1 cell line was purchased from ATCC, US. Yac-1 cells were maintained in DMEM (Sigma, USA) with 10% FBS (PAA, Austria) at 37°C, 5% CO_2 and 90% humidity. The cell viability was assessed by the trypan blue exclusion method.

Preparation of mice splenocytes, thymocytes and bone marrow cell suspensions

The mice were anesthetised with 2% isoflurane (Merck, USA) and sacrificed by cervical dislocation. The thymus and spleen were removed and quickly washed with Hank's balanced salts solution (HBSS) (Sigma, USA) on the Petri dish. Femur was removed from mice, epiphysis was cut and the bone marrow was triturated using an 18 gauge needle with HBSS and passed through 80 µm sterile wire mesh. On the other hand, thymus and spleen were minced and pressed through 80 µm sterile wire mesh screen with a rubber syringe plunger separately. All types of cell suspension were washed once with PBS supplemented with 0.1% BSA and 2 mg/ml EDTA (PBS-BSA-EDTA) and spun down at 200 *g* for 10 min. For spleen and bone marrow cell suspension, red blood cells were removed by incubating and washing with lysis buffer (8 g NH_4Cl, 1 g Na_2EDTA, 0.1 g KH_2PO_4, pH 7.4). The step was repeated until the pellet was clean. Then, all types of cell suspension were washed with PBS-BSA-EDTA. After that the supernatant was discarded and resuspended in 4 ml of DMEM with 10% heat inactivated FBS. Cell counting was then performed to determine the lymphocyte cell number in the suspension. All of the steps mentioned earlier were carried out under sterile conditions in biological safety cabinet to prevent any contamination.

In vitro splenocytes, thymocytes and bone marrow cell viability assay

The immunoregulatory effect of extract (ranging from 100 µg/ml to 1.56 µg/ml) on different types of mice primary immune cell viability was determined by using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, USA) assay (Mosmann, 1983). Briefly, 100 µl of DMEM media with 10% of FBS was added into all the well except row A in the 96-well plate. 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. The row H was left untouched and the excess solution (100 µl) was discarded and 100 µl of lymphocyte (from mice spleen, thymus or bone marrow cell) with cell concentration at 5×10^5 cells/ml was added into all wells in the 96-well plate and incubated in 37°C, 5% CO_2 and 90% humidity incubator for selected period (24, 48 and 72 h). After the corresponding period, 20 µl of MTT at 5 mg/ml was added into each well in the 96- well plate and incubated for 4 h in 37°C, 5% CO_2 and 90% humidity incubator. One hundred and seventy microlitres of medium with MTT was removed from every well and 100 µl DMSO (Fisher Scientific, UK) was added to each well to extract and solubilise the formazan crystal by incubating for 20 min in 37°C, 5% CO_2 incubator. Finally, the plate was read at 570 nm wavelength using µ-Quant ELISA Reader (Bio-Tek Instruments, USA). In this experiment, extract-free culture was used as negative control while murine recombinant IL-2 (range from 100 to 0.78 U/ml) (BD, USA) was used as positive control. Each extract and control was assayed in triplicates in three experiments. Results are expressed as mean ±

standard error. The percentage of proliferation was calculated using the following formula:

$$\% \text{ of cell viability} = \left(\frac{\text{OD sample}}{\text{OD control}}\right) \times 100$$

In vitro NK cell immunophenotyping for mice splenocytes

The T cell and NK population in the controls and extract treated splenocytes was identified through flow cytometer immunophenotyping. Briefly, cell concentration of splenocytes was adjusted to 2 X 10^5 cells/ml and was treated with same volume (1 ml) of plant extract at 50, 25 or 1 µg/ml (1 ml) in a 6 well culture plate. Untreated control cultures (without extract) and positive control (with 50 U/ml murine rIL-2) were prepared simultaneously. The culture was then incubated for respective period (24, 48 and 72 h). After the corresponding periods, the samples were washed and transferred to a centrifuge tube. For each sample, 1×10^6 cells/ 100 µl PBS were stained with 10/10 µl Fluorescein (FITC) conjugated anti-mouse CD3 (17A2) (Isotype control: IgG2b, κ) (Biolegend, USA) and 12.5 µg/10 µl Phycoerythrin (PE) conjugated anti-mouse NK-1.1 antibodies (PK136) (Isotype control: IgG2a, κ) (Biolegend, USA). After that cells were washed three times (300 *g*, 10 min) and resuspended in PBS-BSA-EDTA at 1 ml of final volume for flow cytometric analysis by using COULTER EPICS ALTRA flow cytometer (Beckman Coulter, USA).

In vitro splenocyte cytokine (IL-2, IL-12 and IFN-γ) determinations

The production of IL-2 and IFN-γ were performed using murine cytokine instant enzyme link immunosorbent assay (ELISA) kit (BioLegend, USA). Briefly, the extracted murine splenocytes (1 X 10^5 cells/ml) was treated with same volume of the plant extract at 50 and 25 µg/ml in 6 well culture plate (BD Biosciences, USA). Control cultures without extract and positive control with murine rIL-2 (50 U/ml) (for the IFN-γ ELISA) and PWM (1 µg/ml) (for IL-2 ELISA) were prepared simultaneously. The culture supernatants used for cytokine assay were collected after 24, 48 and 72 h of incubation of splenocytes with treatment. Capturing antibody specific to murine IL-2 or IFN-γ was coated onto the wells of the microtitre strips provided one day before the assay at 4°C. Then, 100 µl of diluted IL-2 or IFN-γ standard (range from 125 to 2 µg) and supernatant were added into the precoated microtitre plate and incubated for 2 h at room temperature. The plates were then washed and 100 µl of diluted biotinylated detection antibody was added and incubated for 2 h. Revelation step included Avidin-Horse Radish peroxidase and 3,3',5,5' tetramethylbenzidine (TMB) as chromogen. OD were read immediately at 450 nm wavelength by using µ-Quant ELISA reader (Bio-Tek Instruments, USA) at animal tissue culture laboratory, FBBS, UPM. The result was compared with the control prepared simultaneously. Each extract and control was assayed three times. Data are expressed as pg/ml.

In vitro splenocytes cytotoxicity activity determination

Splenocytes treated with 50 or 25 µg/ml of *R. korthalsii* or 50 U/ml of murine rIL-2 at cell concentration of 1 X 10^5 cells/ml was subjected to splenocytes cytotoxicity test. The cytotoxicity of effector splenocytes on NK cells-sensitive target (Yac-1) was evaluated at ratio effector: target of 2:1; 10:1 and 50:1 and incubated in 37°C, 5% CO_2 and 90% humidity for 24 h. Control cultures with non-treated splenocyte co-culture with Yac-1, splenocyte with the cell concentration equivalent to each ratio in the co-culture experimental well (effector spontaneous LDH release) and Yac-1 with cell concentration equivalent to the co-culture (for both target spontaneous LDH release and target maximum LDH release lyse by the lysis solution) were prepared simultaneously. The cytotoxicity of mice splenocyte on Yac-1 cell was screened by measuring the release of lactate dehydrogenase (LDH) from degenerating Yac-1 cell using CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, USA) as described by the supplier. LDH is a stable cytosolic enzyme which is released upon cell lysis. Briefly, 45 min prior to the ending of co-culture incubation period, 10 µl of Triton-X lysis solution (10X) was added into the target cell maximum LDH release control wells. The plate was further incubated for 45 min until the target cells maximum LDH release control were completely lysed. Then, the plate was centrifuged (250 *g*, 4 min) and 50 µl of supernatant from each wells was transferred to a new 96 well plate (BD Biosciences, USA). The substrate mixture was prepared simultaneously by reconstituting 1 vial of substrate mixing with 12 ml of assay buffer. After that 50 µl of reconstituted substrate mixture was added into all the experimental wells and incubated for 30 min in dark. Finally, 50 µl of 2.5 N sulfuric acid stop solution was added into each well and the plate was read at 490 nm wavelength immediately by using µ Quant ELISA Reader (Bio-Tek Instruments, USA). LDH converts substrate mixture into a yellow formazan product. The intensity of color formed (recorded as absorbance at 496 nm) is proportional to the number of lysed cells. Percentage of LDH release was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \frac{OD_{sample} - (OD_{target\ spontaneous} + OD_{effector\ spontaneous})}{OD_{target\ maximum} - OD_{target\ spontaneous}} \times 100\%$$

Statistical analysis

Results are expressed as mean ± standard error (S.E.M.). Differences between means were evaluated using ANOVA test (one way) followed by Duncan test and $p \leq 0.05$ was taken as statistically significant.

RESULTS

In vitro immune cell viability assay

The effect of *R. korthalsii* methanol extract on proliferation of splenocytes, thymocytes and bone marrow cells was examined through MTT cell viability assay. The results presented in panels a, b and c of Figure 1 demonstrates the viability of the immune cells after 24, 48 or 72 h treatments. The percentage of untreated cell viability was calculated as 100%. There was a significant increase in number of viable splenocyte, thymocytes and bone marrow cells after treated with *R. korthalsii* methanol extract for 24 h when compared with the untreated cells. The increase of cell number on all tested immune cells was found to be highest at those treated with 25 µg/ml extract and the mouse rIL-2. The concentration of 100, 50 and 25 µg/ml of the methanol extract increased the total number of viable thymocytes (144 ± 2.1, 141 ± 4.5 and 157 ± 4.1%, respectively) at 72 h

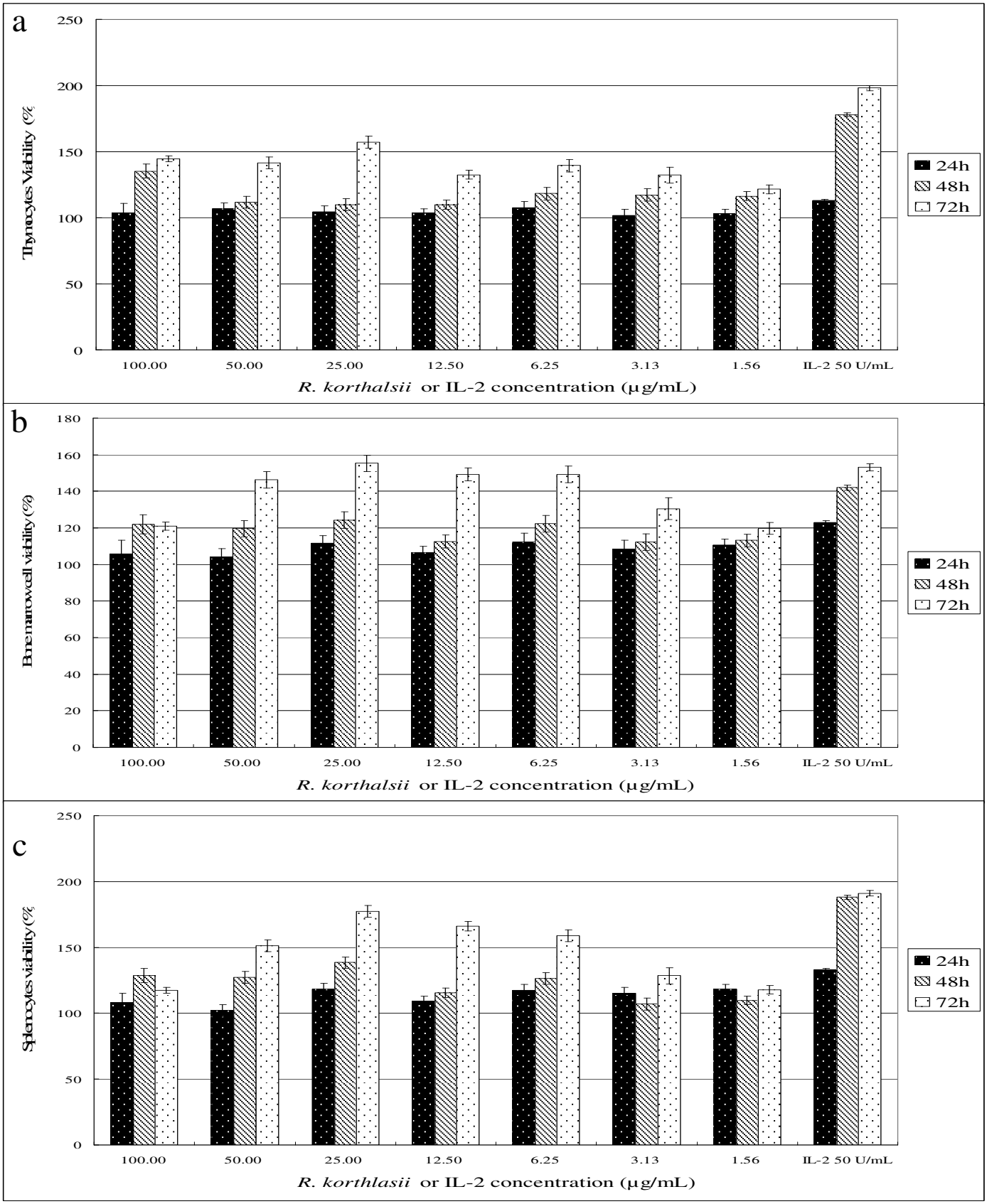

Figure 1. Effect of *R. korthalsii* methanol extract or mouse rIL-2 on mice (a) thymocytes, (b) bone marrow cell and (c) splenocytes viability at 24, 48 and 72 h. Note: The values were the means ± SE of three experiments.

Table 1. NK cell immunophenotyping on mice splenocyte after treated with various concentrations of *R. korthalsii* methanol extract or mice rIL-2 for 24, 48 and 72 h.

Parameter	NK cell population ($NK1.1^+/CD3^-$; %) in splenocyte		
	24 h	48 h	72 h
Control	5.1 ± 1.9	5.4 ± 0.7	4.9 ± 1.8
R. korthalsii (50 µg/ml)	8.2 ± 2.1*	8.8 ± 1.4*	9.1 ± 1.2*
R. korthalsii (25 µg/ml)	8.1 ± 2.5*	9.1 ± 1.5*	12.3 ± 2.3*
R. korthalsii (1 µg/ml)	6.2 ± 2.4	5.9 ± 0.8	5.8 ± 1.7
Mice rIL-2 (50 U/ml)	9.7 ± 1.4*	12.5 ± 1.1*	14.2 ± 1.1*

Each value represents the means ± S.E.M. of three assays in duplicate each. The differences between the control group and treated group were determined by one-way ANOVA (*P ≤ 0.05).

more significantly when compared with the cells which were treated with the lower concentration of the extract. However, mouse rIL-2 showed much higher number of viable thymocytes at both 48 and 72 h (178 ± 1.7 and 198 ± 2.1%, respectively) when compared to all concentration of *R. korthalsii* methanol extract. In splenocyte culture, 25 µg/ml was the best dosage of *R. korthlasii* methanol extract to increase the number of viable cell at 72 h. The increase in the number of viable cell induced at this concentration (177 ± 2.7%) was close to the effect induced by mouse rIL-2 (191 ± 2.6%) after 72 h culture. In bone marrow culture exposed to the extract and mouse rIL-2, both treatments effectively increased the number of viable bone marrow cell when compared with untreated culture. Unlike the effect on thymocytes, treatment with 50, 25, 12.5, 6.25 µg/ml extract and mouse rIL-2 after 72 h showed significant higher percentage of viable bone marrow cell when compared with control.

In vitro NK cell immunophenotyping

From the MTT assay results, both extract and mouse rIL-2 enhanced the proliferation of the immune cells which contributed to the increase of viable cell number in the culture after 72 h when compared with control. Flow cytometer immunophenotyping was carried out to identify the changes of T and NK cell population in mice splenocytes after treated with the extract. In mice, NK cells which play a more important role in lysing tumor target in the innate immunity can be identified by evaluating the expression of NK1.1. Although, a small fraction of T cells also express NK1.1 (Reichlin and Yokoyama, 1998), but the NK cells can be distinguished from these cells by targeting the population in the absence of CD3 expression (Table 1). By using flow cytometric analysis, the phenotype of non-stimulated and stimulated NK cells with NK1.1 was determined after 24, 48 and 72 h culture. The untreated splenocytes did not show any significant changes of NK cell population throughout the study time points. However, *R. kothalsii* methanol extracts increased the NK cell population *in vitro* in a dose and time dependent manner where both 50 and 25 µg/ml but not the low concentration (1 µg/ml) of extract was able to enhance the NK cell population most significantly at 72 h.

In vitro mice splenocyte IL-2 and IFN-γ secretion

Lymphocyte proliferation and the activation of NK cells are cytokine dependent (Gao et al., 2004). The type of cytokine upregulated affects the course of immune response and the whole network of immune regulation. The enhancement of this immune response from the proliferation and NK cell immunophenotyping results suggests that it may also improve the production of cytokines. As shown in Figure 2, the effect for both cytokines (IL-2 and IFN-γ) production after stimulation was time dependent and the major effect was observed 72 h after culture period. Extract treated splenocytes released high amount of IL-2 and IFN-γ at 50 and 25 µg/ml, but not the 1.5 µg/ml after 72 h. Unlike *R. korthalsii* extract, pokeweed mitogen (PWM) which was used as positive control in IL-2 secretion ELISA test showed significant up-regulation of IL-2 production at all the time point. Besides, mouse rIL-2 which was the positive control for IFN-γ ELISA study also showed significant higher production at all the tested time point.

In vitro splenocytes cytotoxicity assay

Decrease in antitumor immune potential of the spleen cells is always associated with progression of tumor growth (Mokyr et al., 1985). Thus, evaluation of the splenocytes may give the idea of the immune potential of the extract to cancer prevention and even contributing to antitumor activity. Figure 3 shows that *R. korthalsii* methanol extract could induce the splenocytes to achieve anti-tumor activity in an extent similar to rIL-2 stimulation. The tumourcidal activity increases from 13 ± 1.7% (non-stimulated) to 69 ± 2.7% and 72 ± 2.4% by rIL-2 and extract stimulation at the co-culture ratio of 2:1, respectively. Increase in the ratio of splenocytes from 2 to

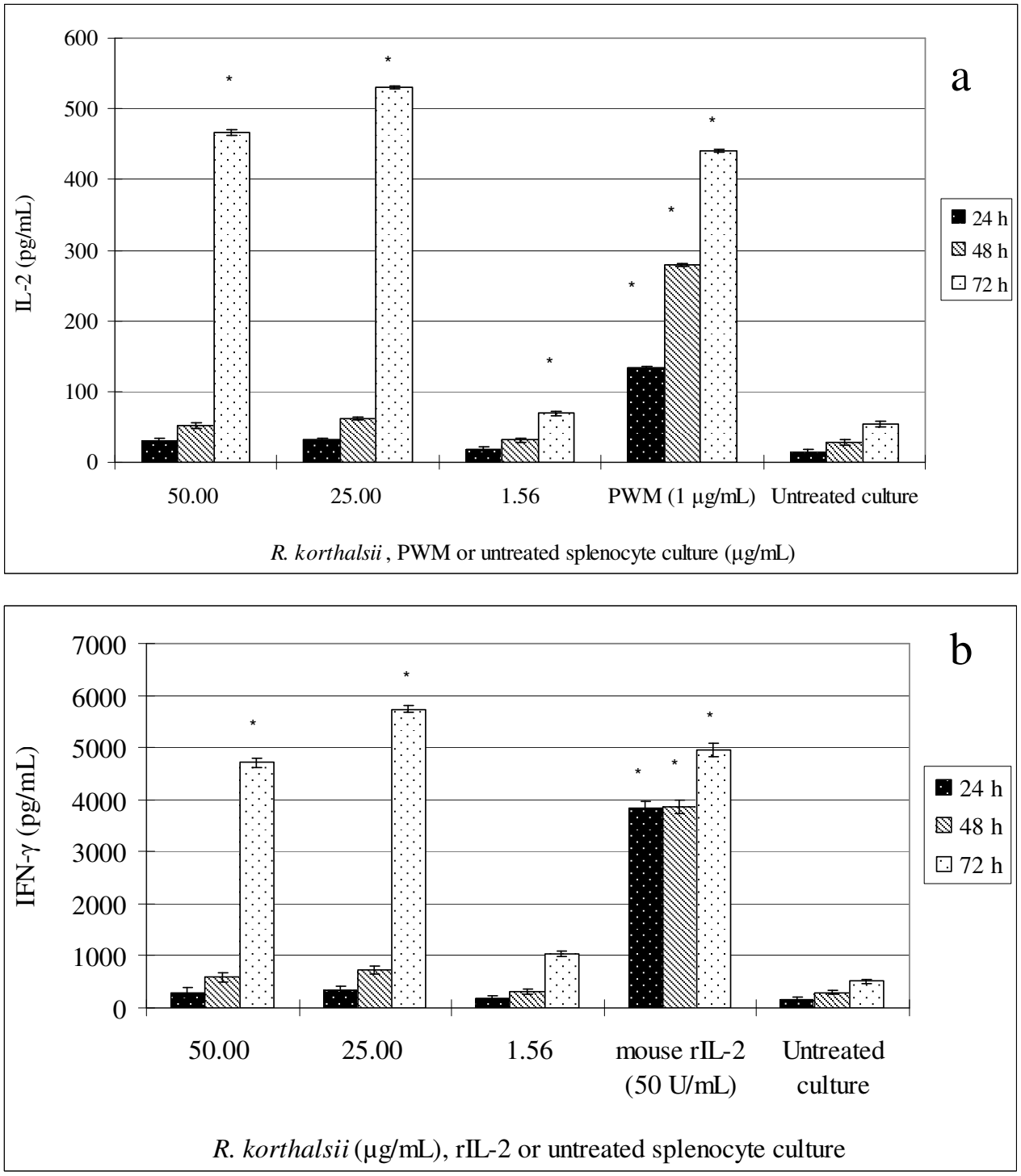

Figure 2. ELISA cytokine assay of *R. korthalsii* methanol extract or mouse rIL-2 stimulated mice splenocytes (a) IL-2 concentration *in vitro* (b) IFN-γ concentration *in vitro* at 24, 48 and 72 h. Note: Each value represents the means ± S.E.M. for three assays in duplicate each. The differences between the control group and treated group were determined by one-way ANOVA (*P ≤ 0.05).

50 enhanced the cytotoxicity of non-stimulated splenocytes from 13 ± 1.7 to 48 ± 2.1%. In term of rIL-2 and extract treated culture, increase in the ratio of splenocytes also enhance the cytotoxicity to 86 ± 3.2 and 90 ± 2.9%. When compared between the untreated and treated group, increase in the cell number of splenocytes

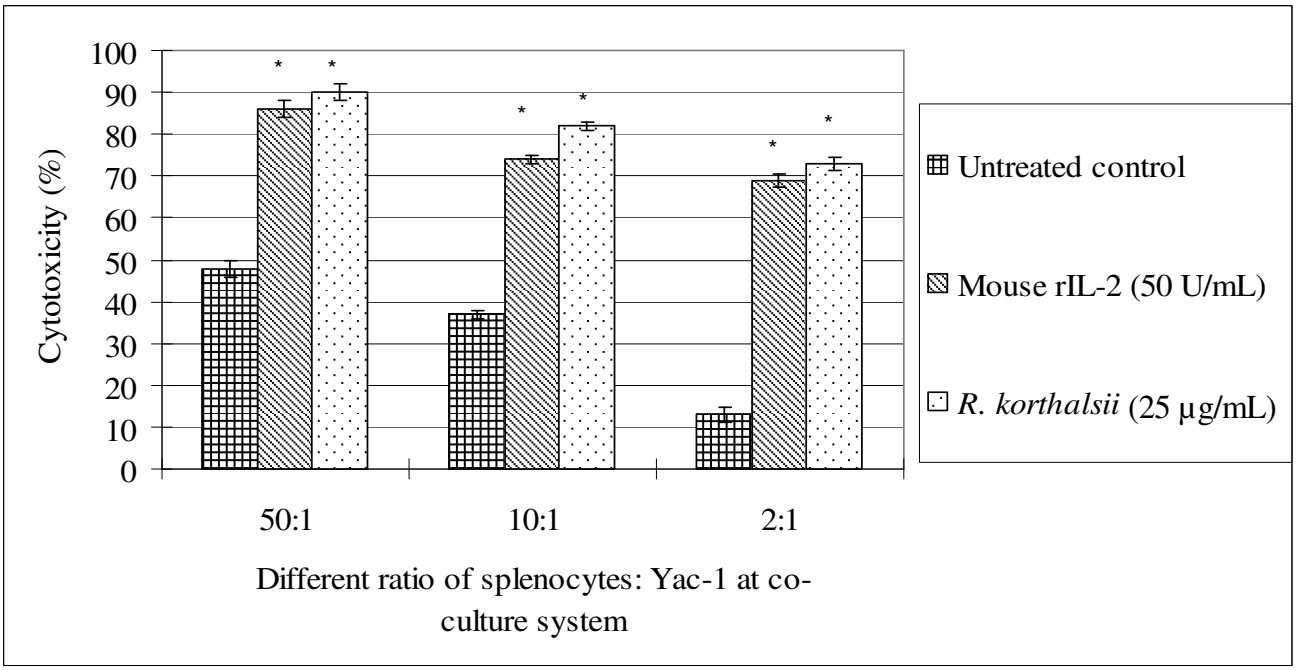

Figure 3. Viability of Yac-1 after treated with different ratio of *R. korthalsii* extract or mouse rhIL-2 activated splenocytes *in vitro*. Note: Each value represents the means ± S.E.M. for three assays in triplicate each. The differences between the control group and treated group were determined by one-way ANOVA (*P ≤ 0.05).

gives higher fold change (3.7 fold) of the cytotoxicity toward Yac-1 cells.

DISCUSSION

Searching for substance which can attribute to the immunostimulative or immunorestorative effects could contribute to the maintenance of immune system which may prevent the progression of tumor establishment (Kadhim et al., 2000). Evaluation on the immune cells especially the bone marrow cells, splenocytes and thymocytes proliferation is always used to screen on the potential immunomodulatory effect of a substance. This is because thymus is the major primary lymphoid organ for T cell development, while bone marrow is for B and NK cells. These immune cells then migrate to the spleen which is the secondary lymphoid tissue and respond to antigens in there. Therefore, the regulation of thymus and spleen cell proliferation which is closely related to maintaining immune homeostasis can be considered as an important marker for immune response control (Seo et al., 2005). In this study, the immunomodulatory effect of *R. korthalsii* methanol extract was screened by assessing the metabolic activity of immune cells through MTT assay. MTT assay which is more rapid, less costly, less time consuming and non-radioactive was previously proven to show the similar result in cell proliferation as the BrdU assay, because accurate relation between the metabolic activity and cell growth was proven (Durrieu et al., 2005).

In Figure 1, it was found that methanol extract of *R. korthalsii* stimulated cell growth in bone marrow, splenocyte and thymocyte culture. When comparing among the three types of immune cell culture, *R. korthalsii* showed the highest proliferation potential in splenocytes. The possible explanations could be splenocytes which were isolated from the secondary lymphoid organ contain 70 to 80% of lymphocytes with high proliferation capacity and cell cycle ranged from 6 top 15 h, while the bone marrow and thymocytes which were isolated from the primary lymphoid organ only contain 30 to 40% of immune cells with high proliferation capability and longer cell cycle lasting time (15 to 32 h) (Velasco-Lezama et al., 2006). The ability of *R. korthalsii* methanol extract to stimulate mice lymphocyte proliferation as indicated in this study is in agreement with the results obtained by Wong and Tan (1996) who reported that the water extract of this plant stimulated the proliferation of splenocytes *in vitro*. Both results suggest that water and methanol extract may isolate the similar molecules which possessed mitogenic activity.

Lymphocyte proliferation is a complex event involving the participation of IL-2 and IL-2 receptor expression. IL-2 is one of the major regulatory cytokine of the immune system which can further enhance the NK cell and LAK cell growth and activity (Sugiura et al., 2002). This may further contribute to the antitumor activity of the immune cell (Wang and Johnston, 1995). The results from the *in vitro* study give the clue that high proliferation of immune cells especially at 72 h after treatment may be induced by

the high production of IL-2 at 72 h which enhanced the NK cell population, IFN-γ production and cytolytic activity toward Yac-1 at the same time point. On the other hand, mouse rIL-2 which was used as the positive control in this study reacted directly to stimulate the immune cell proliferation and cytokine expression at the earliest 24 h. Thus, production of IL-2 by the extract activated immune cell may be one of the key factors for the immunostimulation. However, the extract by itself may also mimic the role of IL-2 to contribute to the co-stimulation. This is because the detected extra-cellular IL-2 after 24 h of treatment was much lower when compared with the rIL-2 used in the experiment, but both of the treatment succeeded in achieving similar level of activation after 24 h of culture. The application of IL-2 was found to be limited by the associated toxicity at high concentration which showed the most effective cytotoxicity effect *in vitro*. Thus, searching for less or nontoxic natural product which can mimic the IL-2 effect or enhance the host immune system to produce IL-2 may be an alternative to improve this type of immunotherapy.

In this study, stimulation of lymphocytes (especially splenocytes) by *R. korthalsii* methanol extract has significant effects on proliferation, NK cell population and cytokine expression which further enhanced the cytolytic activity of the immune cells. Natural killer cell may be the major target that was activated by the extract. Thus, study of immunomodulatory effect of the extract against isolated natural killer cell and identification of active constituents in the extract should be the direction for further study.

REFERENCES

Durrieu C, Degraeve P, Carnet-Pantiez A, Martial A (2005). Assessment of the immunomodulatory activity of cheese extracts by a complete and easy to handle invitro screening methodology. Biotechnol. Lett., 27: 969-975.

Gao X, Kuo J, Jiang H, Deeb D, Liu Y, Divine G, Chapman RA, Dulchavsky SA, Gautam SC (2004). Immunomodulatory activity of curcumin: suppression of lymphocyte proliferation, development of cell-mediated cytotoxicity, and cytokine production *in vitro*. Biochem. Pharmacol., 68: 51-61.

Gupta A, Khajuria A, Singh J, Bedi KL, Satti NK, Dutt P, Suri KA, Suri OP, Qazi GN (2006). Immunomodulatory activity of biopolymeric fraction RLJ-NE-205 from *Picrorhiza kurroa*. Int. Immunopharmacol., 6: 1543-1549.

Kadhim S, Penney C, Lagraoui M, Heibein J, Attardo G, Zacharie B, Connolly T, Gagnon L (2000). Synergistic anti-tumor activity of a novel immunomodulator, BCH-1393, in combination with cyclophosphamide. Int. J. Immunopharmacol., 22: 659-671.

Kiritikar KR, Basu BD (2001). *Indian Medical Plant*. pp. 3578-3622. Dehradum: Oriental Enterprises.

Lau TT, Ong LB, Fang TH (2004). The antioxidant potential and immunomodulating activity of dragon plant. Retrieve 31 May, 2007 from http://www.science.edu.sg/ssc/events.jsp?artid=5705&type=17&root=268&parent=268&cat=330.

Mokyr MB, Colvin M, Dray S (1985). Cyclophosphamide-mediated enhancement of antitumor immune potential of immunosuppressed spleen cells from mice bearing a large MOPC-315 tumor. Int. J. Immunopharmacol., 7: 111-22.

Reichlin M, Yokoyama P (1998). Natural killer cell proliferation induced by anti-NK1.1 and IL-2. Immunol. Cell Biol., 76: 143-152.

Seo Y, Lee HJ, Kim Y, Youn H, Lee BJ (2005). Effects of several salt marsh plants on mouse spleen and thymus cell proliferation using MTT assay. O. S. J., 40: 209-212.

Sugiura H, Nishida H, Mirbod SM (2002). Immunomodulatory action of chronic exercise on macrophage and lymphocyte cytokine production in mice. Acta Physiol. Scand., 174: 247-56.

Tan ML, Muhammad TST, Najimudin N, Sulaiman SF (2005). Growth arrest and non-apoptotic programmed cell death associated with the up-regulation of c-myc mRNA expression in T-47D breast tumor cells following exposure to *Epipremnum pinnatum* (L.) Engl. hexane extract. J. Ethnopharmacol., 96: 375-383.

Velasco-Lezama R, Tapia-Aguilar R, Roman-Ramos R, Vega-Avila E, Perez-Gutierrez MS (2006). Effect of Plantago major on cell proliferation *in vitro*. J. Ethnopharmacol., 103: 36-42.

Wang PL, Johnston TP (1995). Sustained-release interleukin-2 following intramuscular injection in rats. Int. J. Pharm., 113: 73-81.

Wong KT, Tan BKH (1996). *In vitro* cytotoxicity and immunomodulating property of *Rhaphidophora korthalsii*. J. Ethnopharmacol., 52: 53-57.

Yeap SK, Alitheen NB, Ali AM, Omar AR, Raha AR, Suraini AA, Muhajir AM (2007). Effect of *Rhaphidophora korthalsii* methanol extract on human peripheral blood mononuclear cell (PBMC) proliferation and cytolytic activity toward HepG2. J. Ethnopharmacol., 114: 406-411.