Dose dependent contradicting immunoproliferative effect of curcumin in MTT assay

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ABSTRACT

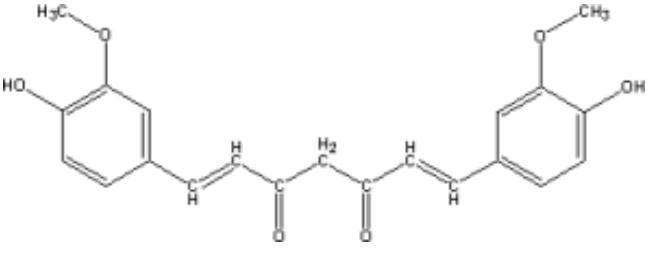
Context: MTT assay is a common biochemical test to screen for immunoproliferative and cytotoxic of a study compounds. However, colour of the study compound may interfere with reading of the MTT assay thus lead to false positive or negative without a proper background blank control. **Aim:** To study the important of background blank control for quantification of immune cell proliferation after treated with colour compound. **Settings and Design:** Curcumin which is the yellow pigment isolated from turmeric was used in this study. It has been previously identified as an immunosuppressor toward T lymphocyte. MTT assay was carry out on the curcumin in the present and absent of mice splenocyte and thymocytes after corresponding treatment period. Colourless concanavalin A and poke weed mitogen were used as positive control in this study. **Methods and Material:** Colourimetric MTT assay was use to quantify the cell proliferation in this study. **Statistical analysis used:** One way ANOVA test followed by Duncan test was used in this study. **Results:** Without a proper background blank control, colour of curcumin contribute to false positive immunoproliferative effect on both splenocyte and thymocyte. However, low concentration of curcumin was able to stimulate splenocyte and thymocyte proliferation at all the studied time points. **Conclusions:** Proper background blank control is important in colorimetric base study to study the proliferation of colour compound.

Key words: Immunomodulatory, Curcumin, MTT assay, proliferation. **Key Messages:** Background blank control is necessary to study the effect of colour compound using colorimetric base study.

INTRODUCTION

A fully functioning immune system is one of the most important criteria for a healthy person. However, the immunological protection against diseases can be influenced and reduced by many anthropogenic factors such as environment condition, malnutrition and seasonal changes[1]. Immunomodulator is the compound that capable of modifying or regulating one or more immune functions. The goal of using immunomodulator can be subdivided into three types which are: suppression, stimulation and restoration of immune system[2]. One of the way to measure immunomodulatory effect of compounds was by measuring the capacity of these compounds to induce or inhibit the proliferation of immune cells such as peripheral blood mononuclear cell (PMBC), splenocytes and thymocytes[3-7].

Curcumin (diferuloylmethane) (figure 1) is a natural occurring yellow pigment biologically active ingredient of Turmeric which is a member of *Curcuma* botanical group. The Turmeric was produced by crushing and powdering of the root and rhizome of the *Curcuma longa L.* Since long time ago, turmeric has been used as a folk medicine for the treatment of variety of illnesses.



$C_{21}H_{20}O_6$ Molecular chemical formula of Curcumin[14]

Figure 1: Chemical structure of curcumin

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Nowadays, the potential of curcumin have been discovered continuously. The possible pharmacological properties of curcumin are anti-HIV, anti-oxidant, anti-inflammatory, anti-tumor, anticoagulant, anti-diabetes, anti-arthritis and antifungal[8,9]. Gao et al. proved that curcumin is a powerful cytotoxic and anti-inflammatory agent[10]. Added to this, they also reported that curcumin inhibits the mitogen induced proliferation of mice spleen lymphocytes. Yadav et al. also reported that curcumin inhibits PHA-induced T-cell proliferation[11]. Although many studies have indicated that curcumin carry the immunosuppressive effect, improper in situ calorimetric base proliferation study such as MTT assay may also recorded the immunoproliferative effect of curcumin at high concentration. This study evaluated the immunoproliferative effect of curcumin using MTT assay with and without the proper elimination of background colour.

SUBJECTS 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. Curcumin, Concanavalin A (ConA) and Poke weed mitogen (PWM) were purchased from Sigma Chemical Co. (St. Louis, MO). Concanavalin A (ConA) and pokeweed mitogen (PWM) (Sigma, USA) were used as a positive control for thymocytes and splenocytes, respectively. Curcumin, ConA and PWM were prepared by dissolving it with DMEM medium (Sigma, USA) and store in -80°C prior usage.

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).

Preparation of splenocytes and thymocytes

The mice were anesthetised with 2% isoflurane (Merck, USA) and sacrificed by cervical dislocation. The thymus and spleen were removed and washed with Hank's Balanced Salts Solution (HBSS) (Sigma, USA) on the Petri dish. 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 minutes. For spleen 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 above were carried out under sterile conditions in biological safety cabinet to prevent any contamination.

Lymphocyte proliferation assay

The proliferation of thymocyte and splenocyte were determined using colorimetric technique 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay. All type of lymphocytes were treated with curcumin (30, 15, 7.5, 3.751. 875, 0.9375, 0.46875 and 0 μg/ml) and PWM 50 μg/ml at 2 $X10^5$ cells/200 μL/96 well and incubated in 37°C, 5% CO_2 incubator for 24 hours, 48 hours and 72 hours. After the corresponding period, 20 μl of MTT (5 mg/ml) was added into each well and incubated for four hours in 37°C, 5% CO_2 incubator. Then, medium with MTT (170 μl) was removed from every well and DMSO (100 μl) extraction buffer was added to each well to extract and solubilize the formazan crystal. The solution was vigorously mixed to dissolve the reacted dye and incubated for 20 minutes in 37°C, 5% CO_2 incubator. Finally, all plate was read on a Powewvex 340 Microtitier-Plate ELISA Reader at UPM Faculty of Biotechnology and Biomolecular Sciences Laboratory of Animal Tissues Culture with 570 nm wavelength. In this experiment, treatment-free culture was used as negative control while ConA (Sigma, USA) at 2.5 μg/mL and PWM (Sigma, USA) at 50 μg/mL were used as positive control for thymocytes and splenocytes, respectively. On the other hand, a set of serial diluted curcumin (30, 15, 7.5, 3.751. 875, 0.9375, 0.46875 and 0 μg/ml) without any cells was used as background control. This background control was added with MTT solution and subjected to DMSO solubilization as the other treated culture. Each treatments and control were assayed in triplicates in three experiments. Results are expressed as mean ± standard error. The percentage of cell proliferation was first calculated using the following formula:

$$\% \text{ of cell viability} = \left[\left(\frac{\text{OD curcumin}}{\text{OD control}}\right) \times 100\right] - 100\%$$

Then, the percentage of cell proliferation was calculated by using OD of cells treated with curcumin minus off the OD of background value contributed by that particular concentration of curcumin in the MTT assay:

$$\%\ \text{of cell viability} = \left[\left(\frac{\text{OD curcumin} - \text{OD curcumin background}}{\text{OD control}}\right)\times 100\right] - 100\%$$

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 AND DISCUSSION

Effects on splenocytes proliferation by MTT assay

The immunoproliferative effect of curcumin from figure 2 showed that high concentrations stimulate cell proliferation after short incubation time (24 hours) while low concentrations are more effective after long incubation time (72 hours). Unlike 24 and 72 hours, 48 hours of incubation only induce significant proliferation at 30 µg/mL. However, curcumin is a yellow colour pigment and this colour may contribute to the increase of absorbance reading and thus interfere with the results and thus recorded the false positive effect in term of immunoproliferative effect. In this study, we have prepare a background blank control that only contain the serial diluted curcumin with the same range of concentration as we tested but without the present of the cancerous cell. Figure 3 shows the immunoproliferative effect of curcumin after we minus off the reading obtained from the tested sample with the background blank control. Unlike figure 2, concentrations range from 1.87 to 30 µg/mL inhibited splenocyte proliferation at all tested time pointes. Only concentration at 0.47 µg/mL was successfully stimulated the proliferation of splenocyte at 72 hours. This result was similar with the previous reportes where high concentration of curcumin was able to inhibit mitogen stimulated proliferation while low concentration was on the other hand stimulate the DNA synthesis of splenocytes[10,12].

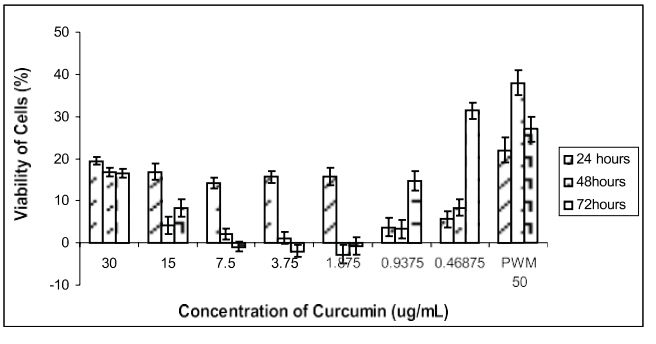

Figure 2: Proliferative effect of curcumin (30, 15, 7.5, 3.75, 1.875, 0.9375, 0.46875, 0 µg/mL) and PWM (50 µg/mL) towards mice splenocytes after 24, 48 or 72 hours incubation time evaluated by MTT assay. Each value represents the means ±S.E.M. triplicate in three independent experiments. The differences between the control group and treated group were determined by one-way ANOVA (*P ≤ 0.05).

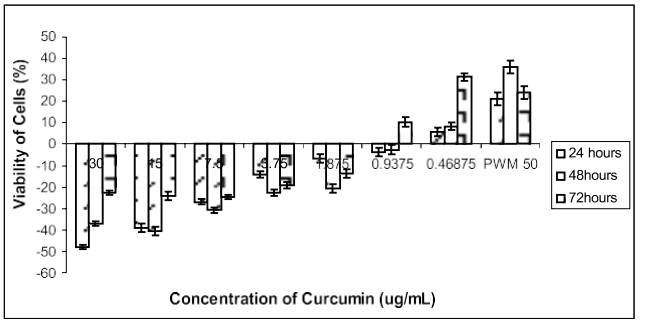

Figure 3: Proliferative effect of curcumin (30, 15, 7.5, 3.75, 1.875, 0.9375, 0.46875, 0 µg/mL) and PWM (50 µg/mL) after substitute with the respective colour background towards mice splenocytes after 24, 48 or 72 hours incubation time evaluated by MTT assay. Each value represents the means ±S.E.M. triplicate in three independent experiments. The differences between the control group and treated group were determined by one-way ANOVA (*P ≤ 0.05).

Effects on Thymocytes proliferation by MTT assay

The immunoproliferation effect of curcumin is shown on figure 4 while figure 5 shows the effect after minus off the background colour contributed by the colour of curcumin. In comparison to effect against splenocytes, curcumin did not show significant proliferative effect on figure 4 and shows inhibition effect against thymocytes figure 5. Thymus is a primary lymophoid tissue that responsible for T cell development[13]. Thus, evaluation of immunoproliferative effect on thymocytes isolated from thymus allows the understanding of the effect of curcumin on T cell. The inhibitory effect of curcumin on thymocytes after minus the background colour was similar with the effect presented by figure 4. However, at concentration lower than 2 µg/mL, curcumin was able to stimulate proliferation of thymocytes after 72 hours of incubation. Level of inhibition by curcumin on both

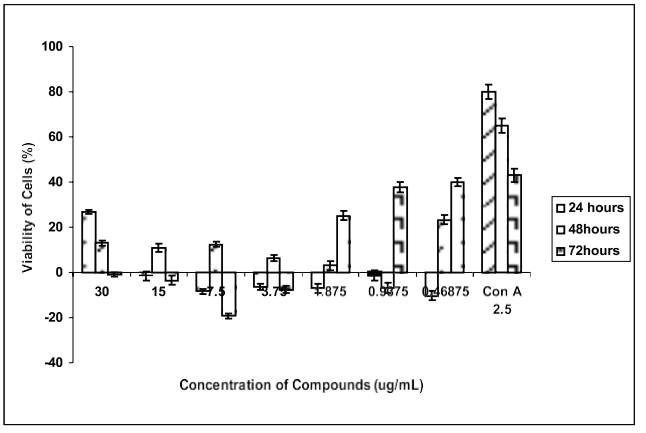

Figure 4: Proliferative effect of curcumin (30, 15, 7.5, 3.75, 1.875, 0.9375, 0.46875, 0 μg/mL) and ConA (2.5 μg/mL) towards mice thymocytes after 24, 48 or 72 hours incubation time evaluated by MTT assay. Each value represents the means ±S.E.M. triplicate in three independent experiments. The differences between the control group and treated group were determined by one-way ANOVA (*P≤0.05).

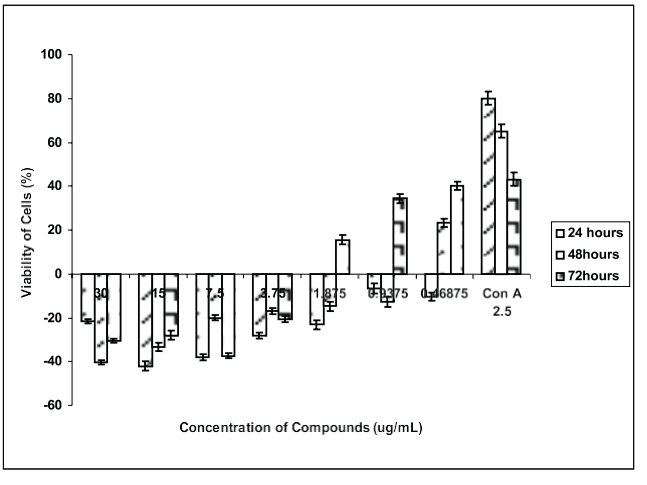

Figure 5: Proliferative effect of curcumin (30, 15, 7.5, 3.75, 1.875, 0.9375, 0.46875, 0 μg/mL) and ConA (2.5 μg/mL) after substitute with the respective colour background towards mice thymocytes after 24, 48 or 72 hours incubation time evaluated by MTT assay. Each value represents the means ±S.E.M. triplicate in three independent experiments. The differences between the control group and treated group were determined by one-way ANOVA (*P≤0.05).

splenocyte and thymocyte was similar (figure 3 and 5) and this suggested that inhibition effect of curcumin on splenocyte may mainly due to the suppression on the T cell population present in splenocyte.

Curcumin is compound which showed the immunosuppressive effect at high concentration while immunoproliferative effect at concentration lower than 1 μg/mL toward lymphocytes especially T lymophcytes. Results from MTT assay showed the false immunoproliferative effect of curcumin at all dose and time tested. Thus, proper background blank control should be assay together when the tested compound with colour to avoid for false positive results.

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