Macrothink Institute™

Journal of Applied Biotechnology
ISSN 2327-0640
2015, Vol. 3, No. 2

The Immunomodulation of Mangiferin on the Proliferation and Interleukin-2 Receptors Expression of *in Vitro* Culture of T lymphocytes Derived from Mammary Tumor Bearing Mice

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Received: October 18, 2014 Accepted: February 28, 2015 Published: March 18, 2015

Doi: 10.5296/jab.v3n2.6423 URL: http://dx.doi.org/10.5296/jab.v3n2.6423

Abstract

The development of a tumor has a strong relationship with cellular immunity mediated by T lymphocytes. Some research showed that mangiferin can increase host immunity but its scientific base was still unclear. The purpose of this study was to explain the role of mangiferin as an immunostimulator on *in vitro* T lymphocytes culture of mammary tumor bearing mice. T

lymphocytes were isolated from the spleen of mice after two weeks of mammary tumor transplantation. The isolation was performed by using sterile nylon net and passed through nylon wool. Isolated T lymphocytes were cultured *in vitro* on RPMI 1640 medium. The culture of T lymphocytes was divided into three groups containing 5, 25 and 50 mg/L of mangiferin, and a control group (RPMI medium). The proportion of T lymphocytes and IL-2Rα (CD25) expressed T cells were evaluated using immunoflouorescens techniques after one and three days of lymphocytes cultures. Our study showed that 5, 25 and 50 mg/L mangiferin was effective to enhance IL-2Rα (CD25) on T lymphocytes culture and T lymphocytes prolifferation.

Keywords: T lymphocytes, Interleukin-2 receptors, Mouse mammary tumor, Mangiferin

1. Introduction

Breast cancer is a malignant disease that affects women, including women in Indonesia. Of all malignant disease that attacks the Indonesian women, breast cancer ranks second (28.03%), slightly below the cervical cancer (28.16%). Treatment and management of breast cancer patients is quite complicated and expensive. In an effort to find ways of treatment with the potential use of natural ingredients Indonesia, needs to be done comprehensive research into the molecular level by studying the interactions between cells involved in the healing process (Prestwich et al., 2008; deVisser et al., 2006).

The treatment was complicated for people with breast cancer, also associated with the immune system because as general malignant disease associated with poor immunologic surveillance of patients. A discussion of immunologic surveillance can not be separated from the discussion of the role of T lymphocytes (Vesely et al., 2011; Grivennikov et al., 2010).

The Immune response was the result of interaction between antigen with immunocompetent cells, including their mediators. Lymphocytes are the basic units due to the formation of the immune response is able to differentiate into other series, as well as play a role in recognizing at once react with antigens (Macchetti et al., 2006).

T lymphocytes can act as effectors in the immune response, but can also act as regulators of the immune response because of its ability to influence the activity of other immunocompetent cells through the release of lymphokines. T-helper lymphocytes (Th) and T-suppressor (Ts) affects the production of immunoglobulin by B lymphocytes.

After antigen contact with B lymphocytes proliferate later, partially differentiate into plasma cells that synthesize and secrete immunoglobulin, and some B lymphocytes into memory Curigliano et al., 2011).

In the *in vivo* processing of tumor antigens would involve both humoral and cellular immune responses. Effector components of the immune system which has the ability to react with tumor cells are: T lymphocytes, antibody-dependent cellular cytotoxicity (ADCC), natural killer (NK) cells and macrophages. To perform these functions properly, they need to go through the process of T lymphocyte activation previously. T lymphocyte activation occurs due to the introduction of antigens that have been processed by macrophages as antigen presenting cells (APC). In addition to the role of antigen in activating T lymphocytes, an

Journal of Applied Biotechnology
ISSN 2327-0640
2015, Vol. 3, No. 2

immunostimulator also can play a role in this process (Sheu et al., 2008; Gooden et al., 2011).

The immunostimulator contained in natural materials, was researched and developed as an alternative cancer treatment. One alternative natural materials that can be explored was the mango leaves. Mango fruit contains mangiferin and other polyphenols. Both mangiferin and polyphenols contained in mango fruit promote to study its use for alternative cancer therapies. The potential report of mangiferin as a potent antioxidant in breaking free radicals caused by the administration of carcinogenic compounds (Chattopadhyay et al., 1987).

This study showed that administration of mangiferin extracted from mango fruit using water and ethanol solvents on the *in vitro* culture can increase the expression of interleukin-2 receptors on T lymphocytes and T lymphocytes prolifferation

2. Methods

2.1 The Extraction of Mangiferin

A total of 150 grams of rice dumplings fresh mango fruit peel was dried and pulverized extracted by maceration using ethanol or water 1500 ml (1: 10), performed maceration for 24 hours at room temperature. The filtrate was filtered and then concentrated by rotary vacuum evaporator at a temperature of 50oC. Viscous filtrate were then dried by the freeze drier to remove residual solvent remaining. Mangiferin isolation method refers to a method performed Garcia et al. (2002). A total of 10 g of dry powder of mango fruit peel (*Mangifera foetida* Lour) was placed in Soxhlet with petroleum ether (60-800C) for 12 hours. The plant material without fat then extracted with ethanol by means of reflux for 16 hours, then removed again defatted with petroleum ether and crystallized with ethyl acetate (Morgan & Darling, 1993).

2.2 The Experimental Animals

The experimental animals used were mice strain C3H, 4 months old, weighing 20-25 g.

2.3 Treatment on Mangiferin

Testing the test solutions were divided into four groups with three repetitions.

Group I (control group): suspension lymphocyte 1×10^6 cells / ml of 200 mL in RPMI medium. Group II: lymphocyte cell suspension 1×10^6 cells / mL of 180 mL in RPMI medium and 20 µl of mangiferin 5 mg / L. Group III: lymphocyte cell suspension $1x10^6$ cells / mL of 180 mL in RPMI medium and 20 µl mangiferin 25 mg / L. Group IV: lymphocyte cell suspension 1×106 cells / mL of 180 mL in RPMI medium and 20 µl mangiferin 50 mg / L. Each group was filled into the microwell and incubated in 5% carbon dioxide incubator at a temperature of 37 °C. Observations were made for 2 times (day-1 and day-3) after treatment acetate (Morgan & Darling, 1993).

2.4 T lymphocytes Cultures

T lymphocytes as effector cells isolated from the spleen of normal and mamary tumor bearing mice, 2 weeks posttumor transplantation. The mice spleen placed at 60 mm sterile dish containing 5 ml of serum-free medium. To obtain cell suspensions of lymphocytes, cells were

passed on sterile nylon net. Ammonium chloride buffer was used for lysis of erythrocytes.

T lymphocytes enrichment was done by passing the cell suspension on nylon wool columns. First, nylon wool (Biotest) soaked in 2M HCl, washed with flowing aquadest 5-6 times and dried. Nylon wool fibers filled into polyethylene plastic syringe (Terumo). Each 0.6 g of nylon wool, loaded on a 10 ml syringe as a column of spleen cell suspension or 0.3 g of nylon wool, loaded on a 5 ml syringe column of macrophage suspension.

The column was preincubated with RPMI 1640 medium containing 5% fetal bovine serum (FBS) two times the volume of the syringe. Resuspended cell suspension into 5.10^6 cells/ml. The cell suspension was dripped through the column and incubated 60 min at 37 °C. Lymphocytes elution that do not bind to the column was done by adding the medium to the column as much as one-time syringe volume. T lymphocytes eluent were washed 2 times by centrifugation 200 g for 5 minuttes.

The cell suspensions were cultured in RPMI medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 50 mM 2-mercaptoethanol. Mangiferin extract with different doses selected were 100 ul added to each culture well of T lymphocytes (Garcia et al., 2002).

2.5 The Expression of IL-2 Receptor Alpha Evaluation

The $CD4^+$ T-lymphocyte activity was measured by the expression of IL-2 receptor alpha (IL-Rα) existing in 24, 48 and 72 hours *in vitro* culture of spleen T lymphocytes of mammary tumor bearing mice by using immunofluorescence techniques.

2.6 Analysis of Data

To determine differences between the groups was performed one-way ANOVA followed by Tukey test.

3. Result and Discussion

3.1 T Limphocyte Prolifferation

In vitro culture or T lymphocytes derived from mammary tumor bearing mice was cultured on microwell plate (Figure 1A) and prolifferation was count on haemocytometer (Figure 1B). Figure 2 showed an increased number of T lymphocytes per ml with mangiferin administration of a dose of 5, 25 and 50 mg/L. According to analysis of variance indicated that there was a significant difference (p = 0.02) between treatments groups ang control group. The tests were followed by Tukey HSD test, showed that the significant difference between the control group with other groups. That means the provision of mangiferin impact on increasing the number of T lymphocytes in cell culture either on the first day and on the third day.

Journal of Applied Biotechnology
ISSN 2327-0640
2015, Vol. 3, No. 2

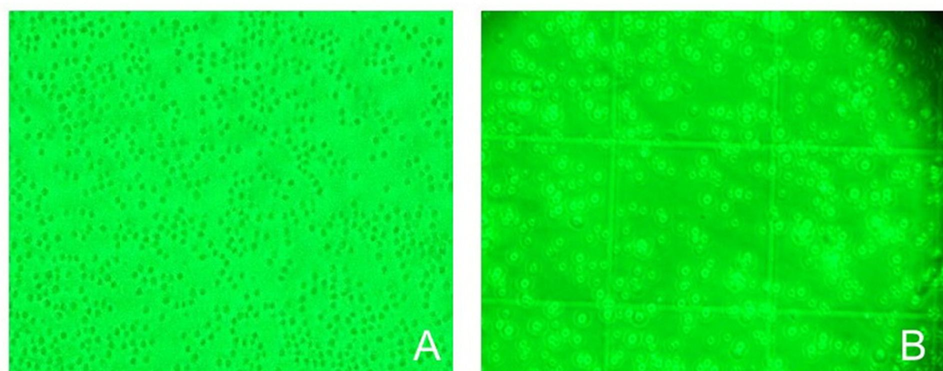

Figure 1. T lymphocytes derived from cultures on days one and three on the glass object (A, 40×) and hemocytometer (B, 100×) was observed using phase contrast microscopy

The test is also shown that administration of mangiferin with a dose of 5, 25 and 50 mg/L did not result in increased proliferation, although from the graph shown in Figure 2 showed an increase with increasing dose, especially on the third day prolifferation. The opposite occurs on the first day which showed a slight decrease as showed in Figure 2, although statistically not showed significant differences.

Thus, administration of mangiferin in cultured lymphocytes isolated from the spleen of mammary tumor bearing mice either in the first or third day resulted in increased proliferation, but it does not showed dependency on the dose (dose-dependent). This is more common in studies using natural extracts, where the active compound is not known. Further studies were extrapolated at doses between 5 mg/L to 25 mg/L is useful to determine the optimum dose that can increase the number of T lymphocytes.

The results of this study, in line with previous studies, which linked the provision of mangiferin with increased immune response, using either experimental animals or *in vitro* using cell lines that most of the results of these studies resulted in an increase in the specific immune response mediated by T lymphocytes and lymphocytes in general, especially, as well as nonspecific immune system include macrophages, polymorphonuclear cells, and dendritic cells. This study was also in line with research that showed the relationship between the administration of mangiferin with the ability of cells that act as antigen-presenting cells (APC) in presenting antigens to T helper lymphocytes (Hernandes et al., 2007; Sanchez et al., 2000; Garido et al., 2004a; Garido et al., 2004b; Garcia et al., 2003).

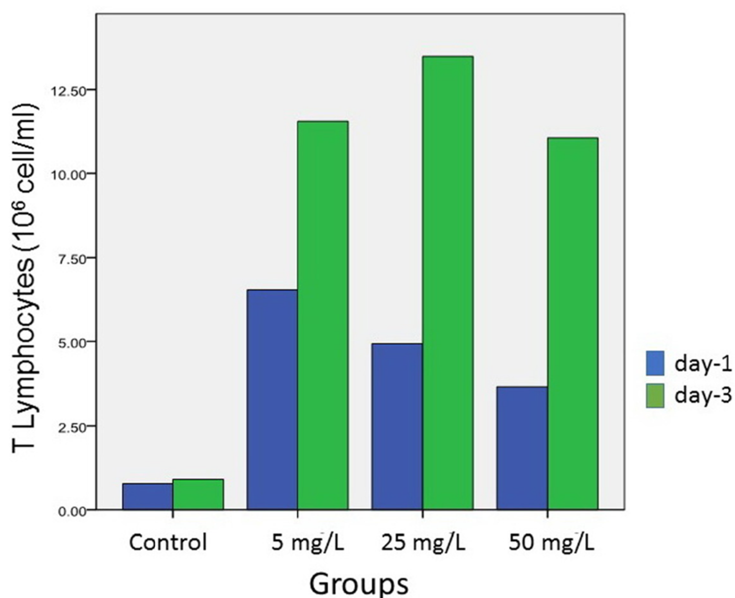

Figure 2. The number of T lymphocytes (10^6 cells/ml) of four groups after 1 and 3 days administration of mangiferin

3.2 The expression of Interleukin-2 Receptor on T lymphocytes

By indirect immunofluorescence staining using mouse anti-interleukin-2 receptor, as shown in Figure 3, showed the interleukin-2 receptor expressed on the cell membrane of T lymphocytes.

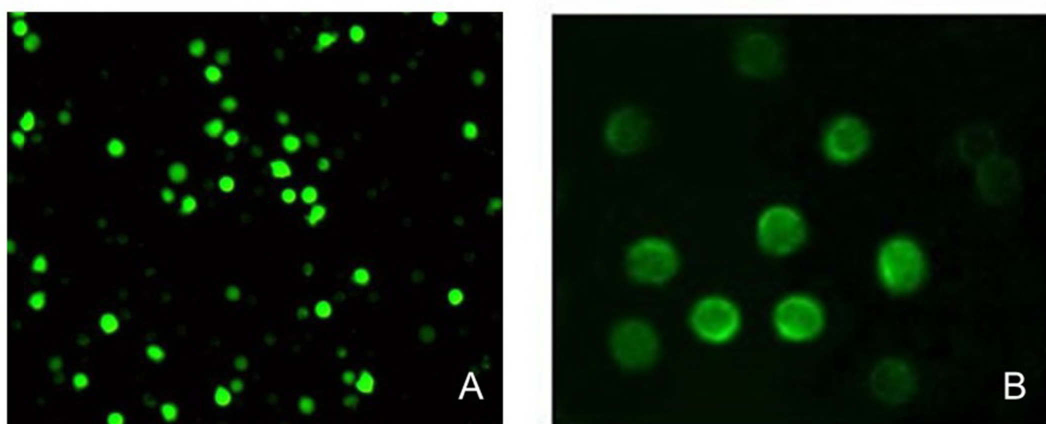

Figure 3. Direct immunoflourescens staining with rabbit anti-mouse interleukin-2 receptors antibody on T lymphocytes derived from the spleen of mammary tumor bearing mice, of dosage group of 25 mg/L (A, strong expression (3+), magnification 100×) and 50 mg/L (B, moderate expression (2+), magnification 400×)

With Kriskal-Wallis test indicated there were significant differences in the interleukin-2 receptor expression between the control group with both dose groups of mangiferin 5, 25 or 50 mg / L in T lymphocytes cultured either one day or three days. This was also showed by Figure 4, which suggests that in all three treatment groups, indicating that the same bar chart, the moderate to strong expression in cultured three days between weak and strong until one day old cultures. It was thus gives results consistent with the increased proliferation of T lymphocytes.

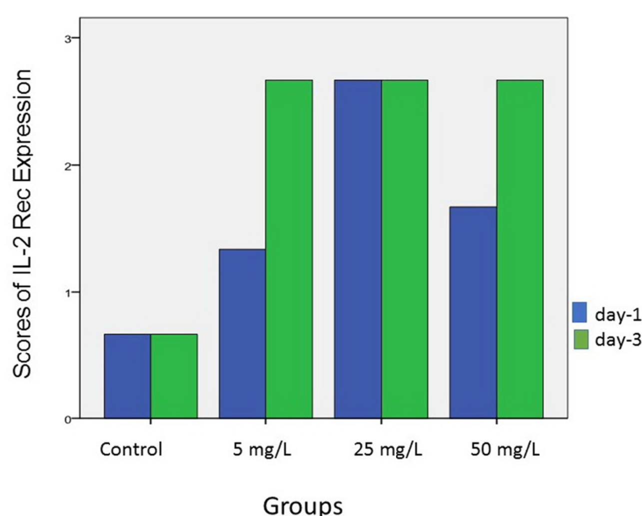

Figure 4. Expression of interleukin-2 receptor (IL-2 rec) on T lymphocytes after administration of mangiferin 1 and 3 days using immunofluorescence (0: negative, 1: weak, 2: moderate and 3: strong)

To see the relationship between the prolifferation of T lymphocytes with interleukin-2 receptor expression was analyzed using correlation Kendall test. According to the test, there was a correlation between the prolifferation of T lymphocytes with interleukin-2 receptor expression on T lymphocytes. This fact illustrates that proliferation was enhanced with an increase in interleukin-2 receptor. According to many studies presented that the activities of T lymphocytes as effector function, characterized by the increased expression of the interleukin-2 receptor alpha (Muruganandan et al., 2005a; Muruganandan et al., 2005b; Agarwala et al., 2012; Viswanadh et al., 2010).

4. Conclusion

Administration of mangiferin in cultured lymphocytes isolated from the spleen of mammary tumor bearing mice either in the first or third day resulted in increased proliferation, but it was not dose-dependent. There was a correlation between the prolifferation of lymphocytes with interleukin-2 receptor expression on T lymphocytes.

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