Immunomodulator Testing on Ethanol Extract of *Gynura procumbens* Leaves to *Mus musculus* Adaptive Immune System: in Vitro Study

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Abstract

Immunomodulator is a substance that has an ability to modulate the activity and function of immune system. *Gynura procumbens* supposed to has benefit as an immunomodulator because of it afficacy to cure many diseases. The aim of this study is to determine the effect and dose variations of *G. procumbens* extract on biological aspect of $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T cells. *G. procumbens* extract concentrations that used in this experiment were 0 µg /ml, 0.1 µg/ml, 1 µg/ml, and 10 µg/ml. Spleen cells were cultured for 4 days in 5% CO_2 incubator at a temperature of 37˚C. Cultured cells were harvested and analyzed by flowcytometry to asses cell surface molecule expression. The resulting data were tabulated and analyzed using ANOVA analysis with a significance of 0.05% on SPSS version 16. Results showed that the extract of *G. procumbens* can increase the proliferation of $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T cells compared to the control. Dose of 1 µg/ml showed the highest effect to promote cell activation compared with the dose of 0.1 µg/ml and 10 µg/ml. Dose of 10 µg/ml could suppress $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T cells development. This study suggests that the ethanol extract of *G. procumbens* has benefit as an immunomodulator and involved in the immune system.

Keywords: *Gynura procumbens*, immunity, immunomodulator, in vitro, lymphocytes.

INTRODUCTION

Immunomodulator is a substance that has an ability to modulate the activity and function of the immune system. Immunomodulator has ability to influence the number of cells that play role in the adaptive immune system such as $CD4^+$ and $CD8^+$ T cells. Research on immunomodulator that has been done used the recombinant cytokines. Recombinant cytokines that were used were interferon-γ (IFN-γ) in combination with vaccines [1,2]. The weakness of this substance are unstable, easily degraded, and have negative effects such as neutrophilia or defective neutrophil function, lymphophenia and monocytophenia [3]. Based on that case, we need another alternative such as active compound in a plant that has immunomodulator activity. One of them is *Gynura procumbens* or *Sambung Nyawa*.

G. procumbens has been long used by people in Java as a traditional medicine. Based on recent studies, leaves of *G. procumbens* have a lot functions such as anti-cancer, anti-inflammation of the kidneys and as anti-diabetic [4]. The immunological side *G. procumbens* such as immunomodulator activity has not been studied yet. In fact, with so many functions of *G. procumbens*, it is possible that these plants have immunomodulator capabilities that can affect the activity of immunocompetent cells such as $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T cells. This study aimed to determine the effect of *G. procumbens* and dose variations of *G. procumbens* extract on the number of $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T cells.

MATERIALS AND METHODS

Medium Preparation

Culture medium that was used in this study was RPMI medium with antibiotics penicillin and streptomycin. The medium were put in 2 propylene tubes, each as much as 5 ml and used as control medium. *G. procumbens* extract powder weighed 0.2 gr and diluted with 200 ml of sterile water and then homogenized. Then, a stock solution with a concentration of 1mg/ml. 100 µl was diluted with 9900 µl control medium to obtain a dose of 3 medium with a concentration 10 µg/ml. 500 µl dose in 3 medium was diluted with of 4500 µl control medium to obtain a dose 2 medium with a concentration 1 µg/ml. 500 µl dose 2 medium was diluted with of 4500 µl control medium to obtain a dose 1

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medium with a concentration 0.1 µg/ml. Dose 1, 2, and 3 medium filtered with cell strainer (Millipore membrane) and transferred into a new propylene tube. After filtered, 4 types of medium were added with 1 µl of 2-mercaptoethanol, 10% Fetal Bovine Serum (FBS), and 1% α-CD3.

Cell Isolation and Counting

Spleen organ was isolated from mice. Spleen was washed in petri dish that contained PBS. The spleen was transferred to another petri dish which also contained PBS, and then crushed. The suspension was filtered and transferred into propylene tube, then added with PBS until 12 ml suspension. Suspension of cell was centrifuged with a speed of 2500 rpm for 5 minutes at 4˚C. Pellet resuspended in 1 ml of control medium. This suspension taken 20 µl and added with 80 µl evans blue and then homogenized by pipetting. The number of cells was counted with haemocytometer.

Cell culture

Each medium, i.e. control, dose 10 µg/ml, 1 µg/ml, and 0.1 µg/ml were added with ± 122 µl of cell suspension and mixed gentle. Each of these medium which contain cell was inserted into the well in plate. The cells were incubated in a 5% CO_2 incubator at 37˚C for 4 days. After 4 days, the cells were harvested and then centrifuge with a speed of 2500 rpm for 5 min at 4˚C. Pellet was resuspended in 1 ml PBS. Each sample was taken 20 µl and added with 80 µl evans blue after that was homogenized by pipetting to count the number of cells. Each sample was also taken 300 µl and transferred into micro tube containing 500 µl PBS. A sample in 3 micro tubes was centrifuged with a speed of 2500 rpm for 5 minutes at 4˚C. Pellet stained with antibodies with extracellular staining.

Flowcytometry Test

Pellet in micro tube was added with 50 µl antibody. Antibodies that were used were FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD8, and PE/Cy5-conjugated rat anti-mouse CD62L. After that, the samples were homogenized by pipetting and incubated in the ice box for 20 minutes. Each sample in a micro tube was transferred to the flowcytometry cuvettes and added with 300 µl PBS. Next, the samples were ready for running with flowcytometer.

Experimental Design and Data Analysis

The design of this study was experimental research with a completely randomized design consist of 4 treatments (control, dose 10 µg/ml, 1 µg/ml, and 0.1 µg/ml). Flowcytometry results were visualized using BD CellQuest PRO™ software then the resulting data were tabulated and analyzed using ANOVA analysis with a significance of 0.05% on SPSS version 16.

RESULT AND DISCUSSION

Population of $CD4^+CD62L^-$ and $CD4^+CD62L^+$ T Cells

Extract of *G. procumbens* with 4 different doses gave significant different results on the activation of $CD4^+$ T lymphocytes (Fig. 1). Control treatment 0 µg/ml indicated the relative numbers of $CD4^+CD62L^-$ T cells were 23.91%, and the absolute numbers of cells were $5376x10^3$ cells. Dose of 0.1 µg/ml in cell culture showed greater cell numbers compared with control with relative number 29.54% as many $17015.1x10^3$ cells. Dose of 1 µg/ml showed the highest absolute number of 4 treatments given with the absolute number $30272.5x10^3$ cells (39.41%). The absolute numbers of cells in a 10 µg/ml dose were $9301x10^3$ cells (37.67%). This number was decreased compared to dose 0.1 µg/ml and 1 µg/ml, but still higher than the control.

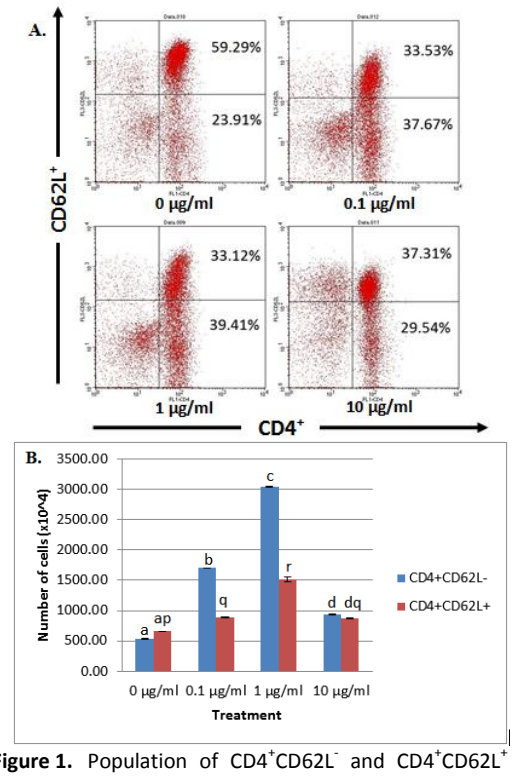

Figure 1. Population of $CD4^+CD62L^-$ and $CD4^+CD62L^+$ T Cells: A. Relative number; B. Absolute number.

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Ethanol extract of *G. procumbens* was also affected the proliferation of $CD4^+CD62L^+$ T cells. The relative numbers of the cells in the control treatment were 52.29% and the absolute numbers were $6551.2x10^3$ cells. Relative numbers of $CD4^+CD62L^+$ T cells in the 0.1 µg/ml dose of the extract were 37.31% and the absolute numbers were $8822.5x10^3$ cells. The relative numbers of cells in the 1 µg/ml dose treatment were 33.12% with the absolute numbers $15164.6x10^3$ cells. Treatment dose of 10 µg/ml showed the relative number of 33.53% with the absolute number $8733.7x10^3$ cells.

The pattern of $CD4^+CD62L^-$ T cell proliferation showed the same trend with the proliferation of $CD4^+CD62L^+$T cells. The number of cells increased at treatment doses 0.1 µg/ml, 1 µg/ml and 10 µg/ml compared to the control treatment. Dose 1 µg/ml showed the highest cell number compared to other treatments. The number of cells decreased in a dose of 10 µg/ml when compared to dose 0.1 µg/ml and 1 µg/ml. Comparison of the number of $CD4^+CD62L^-$ T cells and $CD4^+CD62L^+$ T cell can be seen clearly that in the control treatment $CD4^+CD62L^+$ T cell count more than $CD4^+CD62L^-$ T cells, while cells after treatment with the *G. procumbens* extract given, the number of $CD4^+CD62L^+$ T cells tend to decrease compared to $CD4^+CD62L^-$ T cells. CD62L was a molecule that is owned by the naive cells [5]. It showed that extracts of *G. procumbens* has the ability to activate $CD4^+$ T cells so $CD4^+CD62L^+$ T cells lose CD62L molecule into $CD4^+CD62L^-$ T cells.

The increase of activated $CD4^+$ T cells number after the administration of extracts *G. procumbens* supposedly because the content of flavonoids and saponin in *G. procumbens* as an immunostimulant. According to Middleton *et al.* [6], flavonoids have the ability to trigger the activity of MAP Kinase. Mitogen can stimulate the increase of IL-2. IL-2 is a proliferation and differentiation factor of immunocompetent cells. Concentration of cyclin D2 and cyclin E would have increased when the cells were exposed to IL-2. IL-2 was also served to inhibit the p27 concentration. Under these conditions, IL-2 was able to induce cell cycle continuation of the G1 phase to the S phase of the cell cycle so it proliferated actively [7]. Saponin has the ability to increase cytokine IFNγ [8]. Lee *et al*. [9] and Shi *et al*. [10] mentioned that IFNγ can stimulate the up-regulation of MHC-II expression so that more T cells differentiate into $CD4^+$ T cells.

The decrease of the number of cells in the dose 3 treatment showed that *G. procumbens* at a dose of 10 µg/ml have immunosuppressive effects on $CD4^+$ T cells. This immunosuppressive effect was also obtained from the ethanol extract of leaf flavonoids in *G. procumbens*. According to Schroeter *et al.* [11], the actions of flavonoids were very complex, sometimes synergistic and antagonistic at times depend on the specific components, cell type, concentration, and experimental design. Based on this statement, it can be seen that the concentration of flavonoids in the given doses influenced the cells. Immunosuppressant tends to inhibit the transcription of cytokines so the numbers of cytokines such as IL-2 and IFNγ that play role in cell activation were decreasing [5].

Population of $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T Cells

Extract of *G. procumbens* in cell cultures influence the activation of $CD8^+$ T lymphocytes (Fig. 2). The relative numbers of $CD8^+CD62L^-$ T cells in a control treatment were 4.04% and absolute number of cells $2421.1x10^3$ cells. Treatment with the *G. procumbens* ethanol extract showed the higher proliferation compared with control treatment. The numbers of cells in dose 1 treatment were $4584.6x10^3$ cells (4.70%), dose 2 were $7767.5x10^3$ cells (3.38%), and dose 3 were $3518.2x10^3$ cells (2.93%). Same with $CD4^+$ T cells, a dose 2 also showed the highest proliferation ability of the $CD8^+$ T cells.

Extract of G. procumbens cell culture also affect proliferation of $CD8^+CD62L^+$ T cell. The relative number of $CD8^+CD62L^+$ T cells in the control treatment were 2.07% and the absolute number were $404x10^3$ cells. The dose 0.1 µg/ml treatment showed the relative number of cells 3.31% and the absolute number were $615.5x10^3$ cells. At a dose 1 µg/ml, the relative number of cells 2.63% was as many $1029.1x10^3$ cells. While at a dose 10 µg/ml, the relative number of cells 1.95% was as many $292.8x10^3$ cells. These results indicated that proliferation of $CD8^+CD62L^+$ T cell was higher in the treatment with the *G. procumbens* extract dose 0.1 µg/ml and dose 1 µg/ml, while the dose 10 µg/ml cell number decreased compared to the control.

The increase in $CD8^+$ T cells was affected by the increase in cytokines IL-2 and IFNγ that

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induced flavonoid and saponin in *G. procumbens* extracts as described previously. The increase of $CD8^+$ T cells were also influenced by $CD4^+$ T cells that activated because $CD4^+$T cells that were activated would be differentiated into Th1 that producing IL 2 and IFN-γ [12]. Dose 10 µg/ml provides suppressive effect on $CD8^+$ T cells because this dose also has suppressive effects on $CD4^+$ T cells. IL-2 that produced by $CD4^+$T cells as addition used for the up-regulation itself and itwas also used by $CD8^+$T cells as a stimulant for proliferation. It is explained further that the $CD8^+$ T cells have a higher affinity than the affinity of $CD4^+$T cells in the use of IL-2 [12].

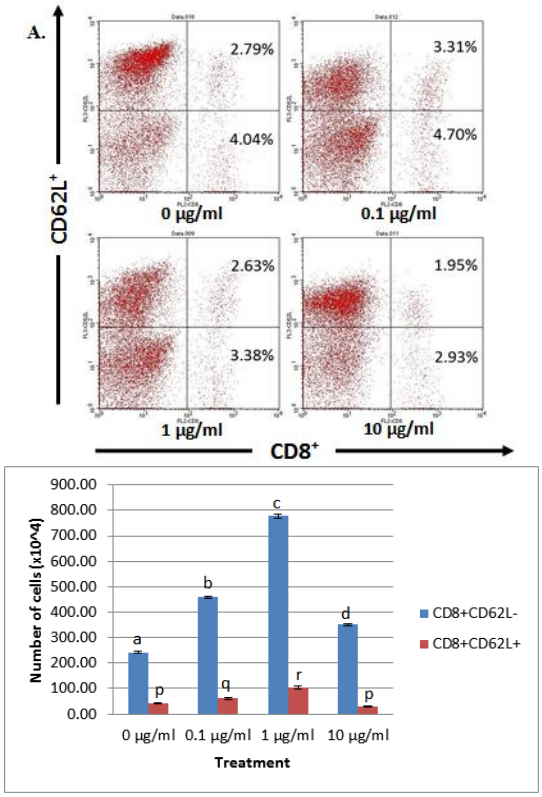

Figure 2. Population of CD8+CD62L- and CD8+CD62L+ T Cells: A. Relative number; B. Absolute number.

CONCLUSION

We concluded that the extract of *G. procumbens* can increase the proliferation of $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$, and $CD8^+CD62L^+$ T cells. Dose of 1 µg/ml showed the highest cell activation capability compared to the dose of 0.1 µg/ml and 10µg/ml. Dose of 10 µg/ml was given suppressants effect to $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$, and $CD8^+CD62L^+$ T cells because at that dose the number of cells was decreasing.

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