

## 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 efficacy 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<sup>+</sup>CD62L<sup>-</sup>, CD4<sup>+</sup>CD62L<sup>+</sup>, CD8<sup>+</sup>CD62L<sup>-</sup> and CD8<sup>+</sup>CD62L<sup>+</sup> 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<sub>2</sub> 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<sup>+</sup>CD62L<sup>-</sup>, CD4<sup>+</sup>CD62L<sup>+</sup>, CD8<sup>+</sup>CD62L<sup>-</sup> and CD8<sup>+</sup>CD62L<sup>+</sup> 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<sup>+</sup>CD62L<sup>-</sup>, CD4<sup>+</sup>CD62L<sup>+</sup>, CD8<sup>+</sup>CD62L<sup>-</sup> and CD8<sup>+</sup>CD62L<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> 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, lymphopenia 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<sup>+</sup>CD62L<sup>-</sup>, CD4<sup>+</sup>CD62L<sup>+</sup>, CD8<sup>+</sup>CD62L<sup>-</sup> and CD8<sup>+</sup>CD62L<sup>+</sup> T cells. This study aimed to determine the effect of *G. procumbens* and dose variations of *G. procumbens* extract on the number of CD4<sup>+</sup>CD62L<sup>-</sup>, CD4<sup>+</sup>CD62L<sup>+</sup>, CD8<sup>+</sup>CD62L<sup>-</sup> and CD8<sup>+</sup>CD62L<sup>+</sup> 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  $\mu$ g/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  $\mu$ l of 2-mercaptoethanol, 10% Fetal Bovine Serum (FBS), and 1%  $\alpha$ -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  $\mu$ l and added with 80  $\mu$ l evans blue and then homogenized by pipetting. The number of cells was counted with haemocytometer.

### Cell culture

Each medium, i.e. control, dose 10  $\mu$ g/ml, 1  $\mu$ g/ml, and 0.1  $\mu$ g/ml were added with  $\pm$  122  $\mu$ l 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<sub>2</sub> 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  $\mu$ l and added with 80  $\mu$ l evans blue after that was homogenized by pipetting to count the number of cells. Each sample was also taken 300  $\mu$ l and transferred into micro tube containing 500  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ g/ml, 1  $\mu$ g/ml, and 0.1  $\mu$ g/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<sup>+</sup>CD62L<sup>-</sup> and CD4<sup>+</sup>CD62L<sup>+</sup> T Cells

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

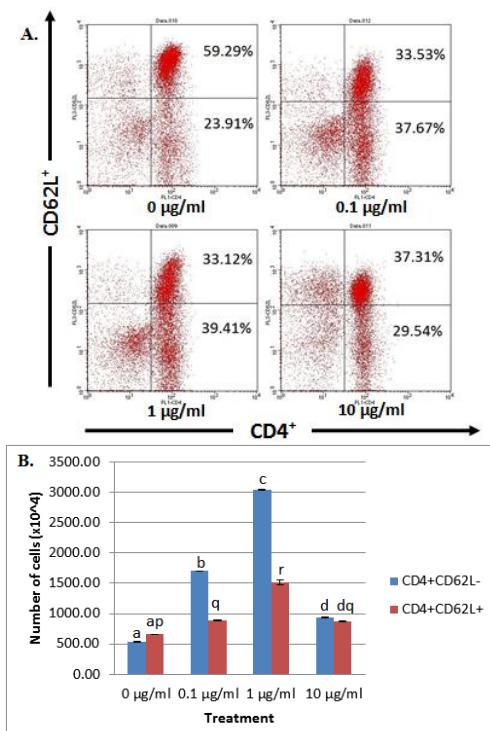


Figure 1. Population of CD4<sup>+</sup>CD62L<sup>-</sup> and CD4<sup>+</sup>CD62L<sup>+</sup> T Cells: A. Relative number; B. Absolute number.

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

The pattern of CD4<sup>+</sup>CD62L<sup>+</sup> T cell proliferation showed the same trend with the proliferation of CD4<sup>+</sup>CD62L<sup>+</sup> 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<sup>+</sup>CD62L<sup>+</sup> T cells and CD4<sup>+</sup>CD62L<sup>+</sup> T cell can be seen clearly that in the control treatment CD4<sup>+</sup>CD62L<sup>+</sup> T cell count more than CD4<sup>+</sup>CD62L<sup>+</sup> T cells, while cells after treatment with the *G. procumbens* extract given, the number of CD4<sup>+</sup>CD62L<sup>+</sup> T cells tend to decrease compared to CD4<sup>+</sup>CD62L<sup>+</sup> 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<sup>+</sup> T cells so CD4<sup>+</sup>CD62L<sup>+</sup> T cells lose CD62L molecule into CD4<sup>+</sup>CD62L<sup>+</sup> T cells.

The increase of activated CD4<sup>+</sup> 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 IFN $\gamma$  [8]. Lee *et al.* [9] and Shi *et al.* [10] mentioned that IFN $\gamma$  can stimulate the

up-regulation of MHC-II expression so that more T cells differentiate into CD4<sup>+</sup> 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<sup>+</sup> 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 IFN $\gamma$  that play role in cell activation were decreasing [5].

#### Population of CD8<sup>+</sup>CD62L<sup>+</sup> and CD8<sup>+</sup>CD62L<sup>+</sup> T Cells

Extract of *G. procumbens* in cell cultures influence the activation of CD8<sup>+</sup> T lymphocytes (Fig. 2). The relative numbers of CD8<sup>+</sup>CD62L<sup>+</sup> T cells in a control treatment were 4.04% and absolute number of cells 2421.1x10<sup>3</sup> 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<sup>3</sup> cells (4.70%), dose 2 were 7767.5x10<sup>3</sup> cells (3.38%), and dose 3 were 3518.2x10<sup>3</sup> cells (2.93%). Same with CD4<sup>+</sup> T cells, a dose 2 also showed the highest proliferation ability of the CD8<sup>+</sup> T cells.

Extract of *G. procumbens* cell culture also affect proliferation of CD8<sup>+</sup>CD62L<sup>+</sup> T cell. The relative number of CD8<sup>+</sup>CD62L<sup>+</sup> T cells in the control treatment were 2.07% and the absolute number were 404x10<sup>3</sup> cells. The dose 0.1 µg/ml treatment showed the relative number of cells 3.31% and the absolute number were 615.5x10<sup>3</sup> cells. At a dose 1 µg/ml, the relative number of cells 2.63% was as many 1029.1x10<sup>3</sup> cells. While at a dose 10 µg/ml, the relative number of cells 1.95% was as many 292.8x10<sup>3</sup> cells. These results indicated that proliferation of CD8<sup>+</sup>CD62L<sup>+</sup> 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<sup>+</sup> T cells was affected by the increase in cytokines IL-2 and IFN $\gamma$  that

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

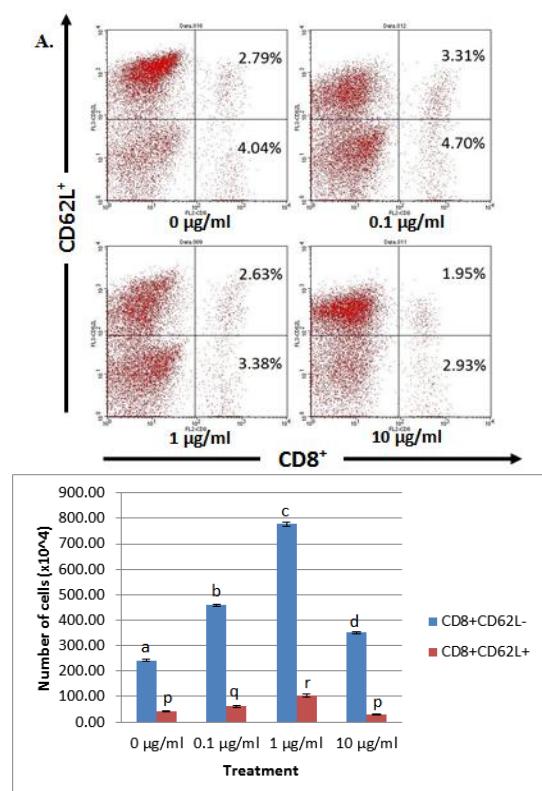


Figure 2. Population of CD8+CD62L- and CD8+CD62L+ T Cells: A. Relative number; B. Absolute number.

## CONCLUSION

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

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