# Bioactivity of Proplis to the CD4 $^{+}$ and CD8$^{+}$ T cells Producing IFN-γ Cytokines in BALB / C Mice

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## ABSTRACT

**Propolis (bee glue) is a natural resinous product of honey bees which collected from exudates and plant buds, rich in biochemicals constituents including mostly flavonoids, phenols and various acids bond. These compound are believed to be responsible as immunomodulatory agents. The study aims to determine the immunomodulatory activity of ethanolic extract of propolis to the CD4$^{+}$ and CD8$^{+}$ T cells producing IFN-γ cytokines and analyze the differences immune responses between control and treatment group by in vivo. Stages include animal acclimation for ± 1 week, preparation of Ethanolic Extracts of Propolis / EEP, Oral Administration with doses levels of 0 mg / kg BW; 50 mg / kg BW (DI); 100 mg / kg BW( DII); 200 mg / kg BW (DIII) for 2 weeks, isolation of lymphocyte cells from spleen, flowcytometry analysis to asses cell number and surface molecule expression. Data was analyzed using Kruskal Wallis Test with α = 0,05 and followed by Mann Whitney Test by SPSS 16.0 for windows with complete randomized design. The results showed that a dose of 50 mg / kg BW was increases the relative number of CD8$^{+}$ T cells producing IFN-γ cytokines significantly (p <0.05) compared with controls. However, at the same dose the relative number of CD4$^{+}$ T cells producing IFN-γ cytokines was decreased significantly (p<0,05). Based on this case, its dose supposedly that the ethanolic extract of propolis play role in maintaining the balance or homeostatic of IFN-γ cytokines production by T cell subsets. Dose of 100 mg/kg BW and 200 mg / kg BW could decrease the relative number of activated CD4$^{+}$ T cells producing IFN-γ cytokine significantly compared to controls.**

Keywords : CD4$^{+}$ T cells, CD8$^{+}$ T cells, ethanolic extract of propolis, IFN-γ cytokines, in vivo

## INTRODUCTION

Indonesia has great Natural Resources. But, minimum research data for Indonesian natural resources is available yet. The use of herbal plants as health promoters are gaining increasing attention in both consumer and scientific because it has no side effects than drugs made from synthetic. One of Indonesia's natural resources promising as new source of herbal medicine is propolis.

Propolis is a natural resinous product of hooney bees which collected from exudates and plant buds, processed with enzymes secreted by bees and mixed with wax in the hive. Propolis contains a variety of complex chemical compounds, which composition varies depending on the plant source [1]. In general, composition of propolis in nature consisting of 30% wax, 50% resins and balsams, 10% essential and aromatic oils, 5% pollen and other substances [2]. The main compounds of propolis is a resin consisting of flavonoids, phenols and various acids bond [3]. Complex chemical compounds of propolis made them have some benefits to health such as immunomodulatory agents [4].

Immunomodulator through natural or synthetic substance that can modulate the function and activity of the immune system, enabling it to maintain balance (homeostasis) immune system [5]. Chemical compounds of propolis supposed as immunomodulatory agents are flavonoids and caffeic acid phenethyl ester (CAPE) [6].

Based on the previous research conducted by Park *et al*. [7], oral administration of CAPE in propolis at doses of 20 mg / kg BW could increase the production of IL-2, IL-4 and IFN-γ cytokines and ratio of CD4$^{+}$ / CD8$^{+}$ T cells. Other studies have been shown, propolis extract at dose of 100 mg / kg BW could inhibit the production of IL-2, IFN-γ TNF-α cytokines as an anti-inflammatory response [8].

Study about IFN-γ cytokines is critical because these cytokines play a role in the activation of Th1 cells and Tc [9]. IFN-γ cytokines is a pro-inflammatory cytokine produced by various cells of the immune system such as CD4$^{+}$

T cells and a small proportion of $CD8^+$ T cells that play role especially in the non-specific and specific immune system [9].

The last decade, interesting studies about immunomodulatory activity of propolis were performed, but no scientific data for the Indonesian product is available yet. Therefore, purpose of this study was to determine immunomodulator activity of propolis to the $CD4^+$and $CD8^+$ T cells producing IFN-γ cytokines and analyze the differences immune responses between control and treatment groups by in vivo.

## METHODS

### Time and Research Place

The research was conducted on September 2013 to March 2014 in the Laboratory of Animal Physiology, Department of Biology, Faculty of Science; Laboratory Biomedical and Pharmacology, Faculty of Medicine, Brawijaya University, Malang.

### Equipment and Materials

The equipment used are mice cages, husk, spatula, oral administration tool, erlenmeyer glass, surgical scissors & board, petri dish, syringe, wire, micropipette, propylene tubes, eppendorf tubes, yellow and blue tip, centrifugation, flowcytometry cuvets and FACS Calibur™ flowcytometry, while the materials used are BR 1 pellet, mineral water, ethanolic extract of propolis, $Na_2CO_3$, distilled water, alcohol 70%, Pbs sterile, cytofix, wasperm and monoclonal antibody (rat anti-mouse IFN-γ).

### Animal Studies and Experimental Design

Male BALB / c mice (*Mus musculus*), 8 weeks old, approximately 38 gram weight, and healthy condition. The animals were kept in groups of six per cage. Groups consist control and treatment groups (dose of EEP : 50 mg/kg BW, 100 mg/kg BW and 200 mg/kg BW). The animal were maintained on BR 1 pellet diet and mineral water ad libitum. The animal were acclimation 1 weeks before used in experiment.

### Extraction of Propolis

The propolis sample was collected from hives of the *Trigona* sp. bees of Lawang city, East Java. The propolis has characteristics sticky, solid and black colour.

The propolis (200 g), added to 1 L of ethanol absolute and moderately shaken. The extract was filtered then solvent was evaporated at evaporator for ± 1.5-2 hours. The extract approximately 1:10 of the dry natural materials. The extract was filtered and evaporated in a heated dish in the oven. Crude extract of propolis, placed in vials and stored in a refrigerator with temperature of 4 ˚ C.

### Oral Administration of Treatment Group with Ethanolic Extracts of Propolis / EEP

The need of extract each dose depending on the average weight of each groups. Extract dilution with distilled water (1:10). In the process of dissolution was added with $Na_2CO_3$ to make it easy to dissolved. EEP was orally administered to mice at dose levels of 50 mg/ kg BW, 100 mg/ kg BW and 200 mg/ kg BW. Oral administration carried out for 2 weeks.

### Isolation of Lymphocyte Cells

Mice were killed by neck dislocation. Mice were dissected using surgical scissor on a surgical board in the dorsal part, then spleen was taken and washed with Pbs sterile twice.

Spleen was placed in a petri dish containing PBS sterile ± 2 ml, crushed with a syringe base, clockwise until organ was destroyed. The homogenate was filtered with wire and put into 15 ml propylene tube. Pbs was added to the tube until 10 ml. After that, suspension was centrifuged at 2500 rpm, 4 ° C, for 5 min. Supernatant was discarded and pellet was taken. Pellet was resuspended with 1 ml of PBS sterile. It was taken 70µl into eppendorf tube containing 500 mL PBS sterile. Centrifuged at 2500 rpm, 4 ° C, for 5 min

**Antibody Staining**

Intracellular staining process begins with perforation of the cell membrane and specific antibody staining. Pellet from second centrifugation, resuspended with 100µl cytofix, incubation for 20-minutes in dark conditions, resuspended with 1ml washperm. Centrifugation 2500 rpm, 4 ° C, for 5 min. The supernatant was discarded & pellet ready to labelled with 50 µL of specific antibody, pipeting, incubated in an ice box and added 300µl Pbs, then transferred to flowcytometry cuvet.

**Flowcytometry Analysis**

Suspension was prepared in the flowcytometry cuvet ready for analysis according to the parameters that have been set on flowcitometry tool. Flowcytometry used in this research is nozzleBD Biosciences FACS Calibur™ flowcytometry. Then, data analysis using BD Cell Quest Pro software™.

**Statistics Analysis**

The data used was ratio / relative number of $CD4^+$ and $CD8^+$ T cells producing IFN-γ cytokines. Research using completely randomized design. Data were analyzed nonparametric statistics (Kruskal-Wallis test with $\alpha = 0.05$), followed by Mann Whitney test. Data analysis using SPSS 16.0 for Windows.

## RESULTS AND DISCUSSION

The relative number of CD8+ T cells producing IFN-γ cytokines

Extract of Propolis with dose of 50 mg/kg BW gave significant different result ($p<0,05$) on the activation of $CD8^+$ T cells producing IFN-γ cytokine (Fig 1). It have been shown, increase the number of $CD8^+$ T cells producing IFN- γ cytokine by 3.66% compared to 1.61% for the control (Fig 2). Meanwhile, doses of 100 mg / kg BW and 200 mg / kg BW have no significant different with control ($p>0,05$). But, both of doses have significance different. Doses of 100 mg/kg BW and 200 mg/kg BW could decrease the relative number of $CD8^+$ T cells producing IFN-γ cytokine by 1.59% and 1.40% (Fig 2).

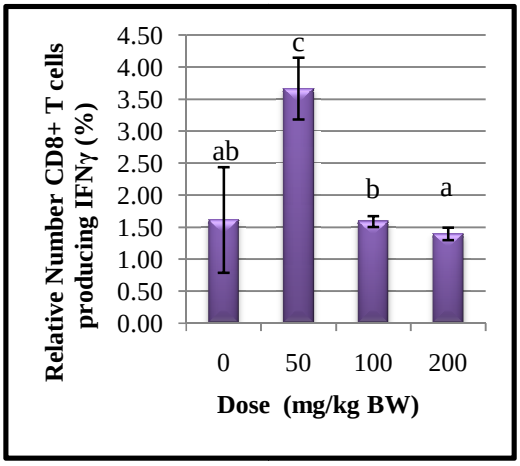


Figure 1. Differences the relative number of $CD8^+$ Tcells producing IFN-γ cytokines between groups.

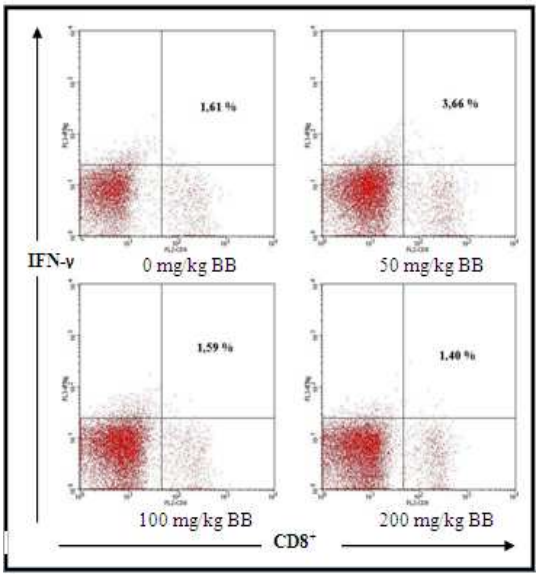


Figure 2. Profile the relative number of $CD8^+$ T cells producing IFN-γ cytokines between groups.

The increase in $CD8^+$ T cells producing IFN-γ cytokines at a dose of 50 mg / kg BW, supposedly induced by CAPE compounds in propolis. This is supported previous research conducted by Park *et al*. [7], CAPE in propolis is able to increase the production of IFN-γ. cytokines. IFN-γ cytokines is a pro-inflammatory cytokine produced by various cells of the immune system such as $CD4^+$ T cells and a small proportion of $CD8^+$ T cells that play role, especially in the non-specific and specific immune system. In the non-specific immunity, this cytokine is the major

cytokine of MAC (Macrophages Activating Cytokine), whereas in the specific immunity plays role in increasing activation of $CD4^+$ and $CD8^+$ T cells [9]. Macrophages have function as a non-specific defense that capture and eliminate foreign antigens through phagocytosis action then present it to T cells [10].

The decrease in the relative number of $CD8^+$ T cells producing IFN-γ cytokines supposedly caused by multicompound properties of propolis which can act antagonists.

Active compounds in plants can be as immunostimulatory that enhance the immune system or suppress the immune system, namely imunosupressor [5]. Simplicia in plants have more than one active compounds or multicompound. In multicompound, exist to synergistically (reinforcing mutually) or antagonistic [11]. Propolis suppresses synthesis of T cells, was mediated by propolis compound namely CAPE, and flavonoid groups quercetin (flavonol) and hesperidin (flavonones) [12]. But, until now molecular mechanism of propolis in IFN-γ cytokine production by T cells subset still unknown.

**The relative number of $CD4^+$ T cells producing IFN-γ cytokines**

Extracts of Propolis were affect the number of $CD4^+$ T cells producing IFN-γ cytokines . It can be seen through the increase of the relative number of $CD4^+$ T cells producing IFN-γ cytokines compared with controls group (Fig 3).

Extracts of Propolis with dose of 50 mg / kg BW, 100 mg / kg BW, and 200 mg / kg BW could decrease the relative number of $CD4^+$ T cells producing IFN-γ cytokine continually (Fig 3). These three different doses have significant difference ($p<0,05$) by 2.20%; 1.75% and 1.57% compared to 3.24% for the control group (Fig 4). However, there were no significant different between treatment groups (Fig 3).

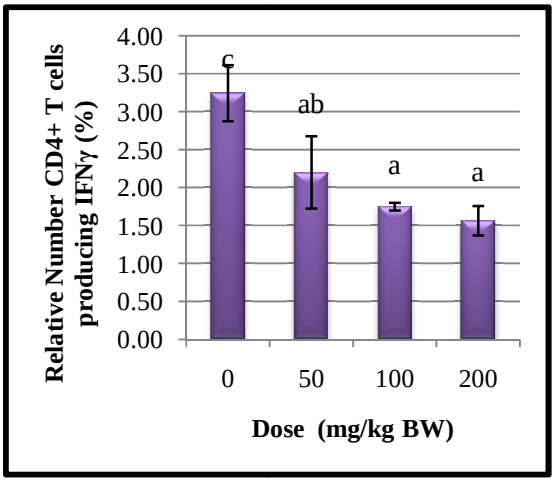


Figure 3. Difference the relative number of $CD4^+$ T cells producing IFN-γ cytokines between groups.

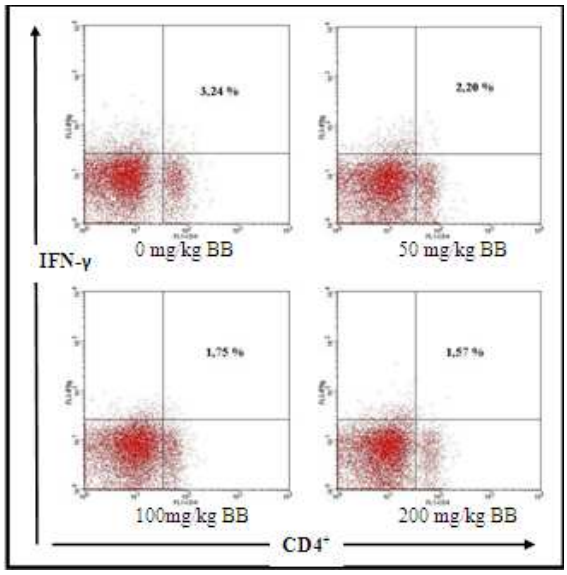


Figure 4. Profile the relative number of CD4+ T cells producing IFN-γ cytokines between groups.

$CD4^+$ T cells that were activated would be differentiated into Th1 that producing IFN--γ cytokines [13]. As described previously, IFN-γ cytokines production by $CD4^+$ T cells play role in the specific immunity by increase activation of $CD4^+$ it self/ autocrine action and $CD8^+$ T cells/ paracrine action [14,9]. But the pattern of $CD4^+$ T cells producing IFN-γ cytokine showed the different trend with the number of $CD8^+$ T cells producing IFN-γ cytokine, especially in dose of 50 mg/kg BW. Extract of propolis in dose of 50 mg/kg BW could decrease the relative number of activated $CD4^+$ T cells producing IFN-γ cytokine

significantly. In this case, dose of 50 mg/kg BW supposedly play role to maintain homeostasis or balance of IFN-γ cytokines in the body.

The existence of IFN-γ cytokines numbers need to be controlled in order to maintain homeostasis or balance immunocompetent cells of the body. This is because cytokines are molecule mediators that plays a critical role in regulating lymphocyte cells, so the cells are maintained in number to keep it balanced. The unbalanced of the immune system components will lead to arising many diseases [15,16,17].

## CONCLUSION

The ethanol extract of propolis dose of 50 mg / kg body weight could increase the relative number of $CD8^+$ T cells producing IFN-γ cytokines significantly compared to controls. However, the relative number of $CD4^+$ T cells producing cytokines IFN-γ were decrease significantly. Based on this, the ethanol extract of propolis dose of 50 mg/kg BW supposedly play role to maintain homeostasis or balance of IFN-γ cytokines in the body. Dose of 100 mg/kg BW and 200 mg / kg BW could decrease the relative number of activated $CD4^+$ T cells producing IFN-γ cytokine significantly compared to controls.

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