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Propolis Action in Controlling Activated T Cell Producing TNF-α and IFN-γ in Diabetic Mice

TNF-α ve IFN-γ Üreten Aktive T Hücrelerinin Kontrolünde Propolisin Etkisi

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Abstract

Objective: This study was conducted to determine the suppressor effect of propolis in controlling activated T cells, also T cells expressing TNF-α and IFN-γ. Those molecules have an important role to generate acute inflammation in diabetes mellitus (DM) condition.

Material and Methods: This study was done by in vivo experiment on streptozotocin-induced diabetic mice. Mice were classified into DM group (diabetic mice model without propolis ethanolic extract); propolis treatment group doses of 50, 100, and 200 mg/kg body weight; normal mice group. Effect of each treatment was observed after 14 days by flow cytometry analysis. The absolute numbers of $CD4^+CD62L^-$, $CD4^+TNF\text{-}\alpha^+$, and $CD4^+IFN\text{-}\gamma^+$ were assessed from splenic cells.

Results: The data showed that administration of propolis ethanolic extract for 14 days significantly decreased the number of activated T cell. Propolis also decreased the expression of TNF-α and IFN-γ by T cells. Administration of all doses of propolis caused decreased blood glucose level compared to baseline levels. Propolis administration at a dose of 100 and 200 mg/kg could suppress the expression of the pro-inflammatory cytokines.

Conclusion: Propolis decreased the number of activated T cells expressing TNF-α and IFN-γ. It also inhibited naive T cells to activate and prevented hyperglycemia.

Keywords: Anti-inflammatory, bee's natural product, hyperglycemic, immunomodulator, pro-inflammatory cytokine

Öz

Amaç: Bu çalışma, propolisin hem aktive hem de TNF-α ve IFN-γ eksprese eden T hücrelerine olan baskılayıcı etkisini araştırmak üzere yapıldı. Bu moleküller, diabetes mellitus (DM) koşullarında oluşan yangıda önemli roller oynar.

Gereç ve Yöntemler: Bu çalışma in vivo olarak, streptozotosin ile oluşturulmuş diabetik farelerde yapıldı. Fareler; DM grubu (propolisin etanolde elde edilmiş ekstresinin verilmediği diabetli fareler), 50, 100 ve 200 mg/kg vücut ağırlığı dozunda propolis verilen diabetli fareler ve kontrol gruplarına ayrıldı. Tedavilerin etkisi 14 gün sonra, akan hücre-ölçer ile irdelendi. Bu yöntem ile dalaktan saptanan $CD4^+CD62L^-$, $CD4^+TNF\text{-}\alpha^+$, ve $CD4^+IFN\text{-}\gamma^+$ hücrelerin sayıları kaydedildi.

Bulgular: Verilere göre, 14 gün boyunca uygulanan propolisin etanolde elde edilmiş ekstresi aktive olmuş T lenfositlerinin sayısını önemli ölçüde azalttı. Propolis ayrıca, T lenfositlerindeki TNF-α ve IFN-γ ekpesyonunu da düşürdü. Propolisin tüm dozları kan glikoz seviyelerini ilk değerlerine göre anlamlı olarak düşürdü. 100 ve 200 mg/kg'lık bir dozda propolis uygulaması, pro-enflamatuvar sitokinlerin ekspresyonunu baskılayabilir.

Sonuç: Propolis, TNF-α ve IFN-γ eksprese eden aktif T hücrelerinin sayısını azalttı. Aynı zamanda, saf T hücrelerinin de aktive olmasını engelledi ve hiperglisemiyi önledi.

Anahtar Kelimeler: Anti-enflamatuvar, arının doğal ürünü, hiperglisemik, immünomodülatör, pro-enflamatuvar sitokin

Introduction

Diabetes mellitus (DM) is known as a metabolic disorder with hyperglycemia.[1] In 2015, the prevalance increased up to 415 million people in the world and expected to increase to 2,1 billion in 2040.[2] Diabetes mellitus may have influences on other diseases, such as obesity, cardiovascular, nephropathy, and the other organs' malfunction. There are so many mechanisms that lead to diabetes. Recent studies have revealed that the diabetes mellitus pathophysiology is related to inflammatory mechanism.[3,4] Inflammation is caused by the

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increase of pro-inflammatory cytokines.[5] Pro-inflammatory cytokines were secreted by immunocompetent cells as responses to infection. Tumor necrosis factor-α and interferon-γ are major pro-inflammatory cytokines that play an important role in DM.[6] Secretion of pro-inflammatory cytokines is one of the cellular responses to antigen or stress condition. In some cases, oxidative stress from intercellular or intracellular, such us ROS, can stimulate pro-inflammatory cytokines' secretion.[7,8] Accumulation of Tumor Necrosis Factor-alpha (TNF-α) and IFN-γ promote inflammatory cells activation such as macrophages, Th cells, and cytotoxic T cells. This activation effects the secretion of more pro-inflammatory cytokines.[1,9]

The accumulation of pro-inflammatory cytokines, in particular for TNF-α does not only promote autoimmune reaction but also induce insulin resistance.[7] Insulin resistance phenomenon is promoted by the accumulation of cytokines in muscle and adipose tissue.[10] TNF-α is a pro-inflammatory cytokine that promotes cell proliferation as responses to infection in tissue.[11] Some studies showed the increase of pro-inflammatory cytokines in the diabetic animals.[10] This complex mechanism involves various pro-inflammatory cytokines that are triggered by innate and adaptive immune system.[6] Diabetes induction in animals can be done by different methods; one of them is diabetic induction using the chemical compound.[12] Streptozotocin (STZ) is one of the various chemical compounds that have diabetogenic effect. This compound can promote insulin regulation failure by DNA alkylation and oxidative stress formation on pancreatic islet cells.[13] A recent study showed that single intraperitoneal injection of 60 mg/kg STZ increases the level of TNF-α, IFN-γ and iNOS significantly in 1 month.[14] Multiple doses of injection of STZ on BALB/c mice could also have increased the level of IFN-γ to become 3 times higher than normal.[15]

Recently, exploration to herbal compounds for disease treatments becomes a great project in medicine and pharmacy.[16] Various herbal compounds known to have anti-inflammation activity are already the focus of research and investigation, especially in diabetes mellitus. Propolis is one of therapeutic natural products that became highlight of discussion because of its bioactivity. Propolis is a resinous substance collected by honey bee from various flowered plants.[17] Propolis contains various substances of phenolic group, essential oil, and di/tri terpenes.[18] These substances also contain some minerals such as Mg, Ca, I, K, Na, Cu, Zn, and Fe.[19,20] Propolis may be seen in various colors, such as dark green or brown. It depends on the botanical origin.[20] Geographical location and bee species influence the chemical composition found in propolis.[17,20]

The compounds in propolis were known to have antibacterial, anti-inflammatory, antitumor, and immunomodulatory activities.[17,18] A recent article indicated that propolis can reduce hyperglycemia in diabetic mice although insulin receptor was blocked.[21] The recent study about propolis and its anti-inflammatory activity constitute the background and idea of advanced research for exploring bioactivity of propolis as an suppressor of inflammation in diabetic mice, suppressing also the secretion of TNF-α and IFN-γ as pro-inflammatory cytokines expressed by Th cells.

Materials and Methods

Ethanolic Extract of Propolis

In this study, we used raw propolis harvested in Lawang, Malang, East Java, Indonesia. Ethanolic extract of propolis was extracted using the method shown by Rifa'i and Widodo.[21] GC-MS analysis was performed by Syamsuddin et al.[22] to analyse the content of ethanolic propolis extract from Lawang. The ethanolic extract of propolis contained (in percentage of total ion current): Benzoic acid 0.41; Phenylic acid 95.62; D-glucofuranuronic acid 0.56; 4-oxo-2-thioxo-3-thiozolidinepropionic acid 0.79; 1-Naphtalenemethanol 95.62; Patchoulene 0.27; D-mannitol 0.51; Threitol 0.86; Glycerol 0.86.

Diabetic Mice Model Preparation

In this study, we used both male and female neonatal BALB/c mice. They were injected intraperitoneally with single dose of *Streptozotocin* 100 mg/kg BW. We used stock concentration of Streptozotocin 7 mg/mL. *Streptozotocin* powder was diluted in 1 mL 0.05 M citric buffer (pH:4.5). Mice were maintained in the pathogen-free animal chamber at Biology Department, Faculty of Mathematic and Natural Sciences, Brawijaya University, Malang, Indonesia. All protocols in this study were approved by University of Brawijaya Ethics Committee (Reg. No. 468-KEP-UB).

Measurement of Blood Glucose Levels and Propolis Treatment

Streptozotocin was injected to 3-weeks old mice; blood glucose level was measured by *OneTouch Ultra*®

Glucometer. The measurement was done by taking blood from mice tail and dropping it into a glucostick. If the blood glucose level was higher than 200 mg/dL, the mice were considered to suffer from diabetes mellitus.[1] This experiment was performed by a completely randomized design method. There were five treatment groups: normal group (healthy control), DM group (diabetic mice model without propolis ethanolic extract); and three propolis treatment groups with doses of 50, 100, and 200 mg/kg body weight. Each treatment group consists of five replications, therefore there were 25 mice examined. Propolis extract was diluted in sterile demineralized water. Propolis with various doses were administered by oral gavage once a day. Blood glucose level was measured until the 14th day. Blood glucose level and body weight were measured in intervals of 3 days. On 15th day the splenic cell was isolated, then cell surface molecules and intracellular cytokines were analyzed by flow cytometry.

Isolation of Spleen Cells, Cell Counting and Flow Cytometry Analysis

Each mouse was sacrificed by neck dislocation method to isolate the spleen. The spleen was isolated and washed twice with sterile phosphate buffer saline (PBS). Spleen was homogenated using a syringe holder in Petri dish containing sterile PBS. Homogenate was then filtered with a sterile filter. PBS was that added to the filtered homogenate and it was centrifuged at 2500 rpm for five minute at a temperature of 10 °C. Then the supernatant was exiled slowly, and the pellet was suspended again in 1 mL of sterile PBS.

Ten µL cell suspension were homogenated in micro-tube contain 90 µL Evans blue. Ten µL homogenates were placed in Haemocytometer counting square. Cells were counted in 5 squares of Haemocytometer central square. Counted cells were calculated using cell counting formula in Equation 1 (dilution factor of 10):

Equation 1: Number of viable cells = Σ cells counted × Σ of squares × 10^4 × dilution factor

Single cell suspension was placed in a micro-tube containing 400 µL PBS and centrifuged in 2500 rpm, 10°C for 5 min. Pellets were stained by FITC-conjugated rat anti-mouse CD4 (Clone: GK1.5, Biologend™) and PE/Cy5-conjugated rat anti-mouse CD62L (Clone: MEL-14, Biologend™). Resuspension was incubated for 20 minutes. Intracellular cytokine staining was performed by adding 200 µL Cytofix/Cytoperm kit (BD-Biosciences Pharmingen). After 20 minutes of incubation, added with 500 µL wash-perm, and centrifuged again. The pellet was stained by PE-conjugated rat anti-mouse TNF-α (Clone: MP6-XT22, Biologend™) and PE/Cy5-conjugated rat anti-mouse IFN-γ (Clone: XMG1.2, Biologend™). The cells that had been stained were suspended again with 500 µL PBS and then transferred to cuvette for flow cytometric analysis. We used BD Biosciences FACS Calibur™ to perform the analysis.

Data Analysis

Data used in this experiment was an absolute numbers obtained from multiple relative numbers of flow cytometric analysis. A relative number was analyzed using BD Cell Quest Pro Software™. Absolute numbers were analyzed using ANOVA (Analysis of Variance) test and tested with Tukey's honest significant difference HSD test.[21] All results were presented as Mean±SD p values<0.05 were considered statistically significant.

Results

Propolis Reduced Blood Glucose Level in 13 days

Propolis administration with various doses affected the blood glucose levels (Fig. 1). The blood glucose level higher than 200 mg/dL is considered to be diabetic levels, based on the protocol of American Diabetes Association.[1] Diabetic mice had blood glucose level higher than 200 mg/dL from day 1 to 13. Diabetic mice had fluctuation in blood glucose level between the 1st day and the 7th, and plateaued until the last day of measurement. Blood glucose levels were significantly higher in treatment groups compared to that of control mice (Fig. 1).

All propolis administered groups of mice had lower blood glucose at day 13 compared to initial levels (Fig. 1). The mean glucose level of mice that were given 50 mg/kg propolis was 168 mg/dL. The mean glucose level of mice that were given 100 mg/kg at 13th day was higher than 200 mg/dL.

Inhibitory Effect of Propolis on T cell Activation in Diabetic Mice

Naive $CD4^+$ T cells are activated when they are bound by antigen molecules[23] or recruited by cytokines signals.[24] Activated T cells are characterized by loss of L-selectin markers or CD62L.[25] Fig. 2a shows that an

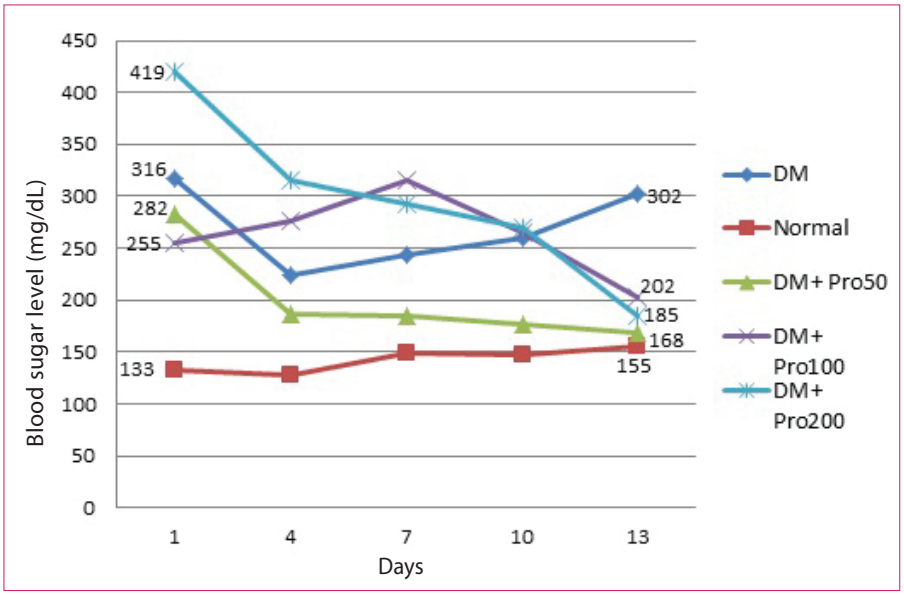
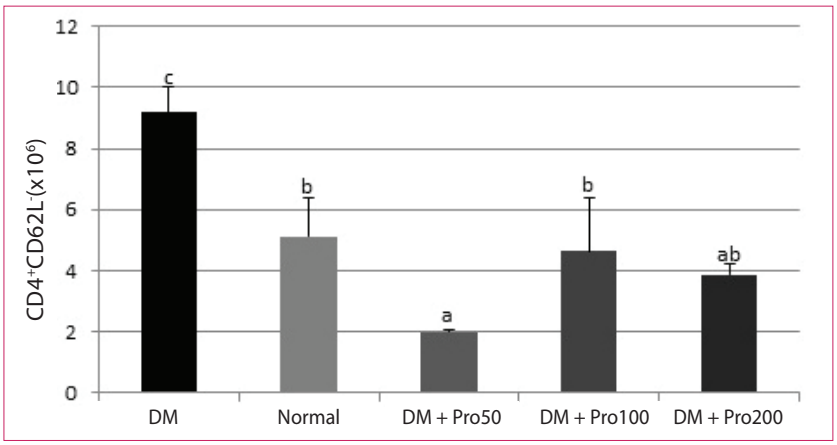


Figure 1. Effect of propolis on blood glucoes levels during 13 days post treatment in different groups of BALB/c mice. Normal: control mice; DM: diabetic mice without propolis treatment; the other groups; DM+Pro50, DM+Pro100 and DM+Pro200: diabetic mice treated with propolis 50, 100 and 200 mg/kg BW, respectively (Mean±SD, N=25, p=0.05).



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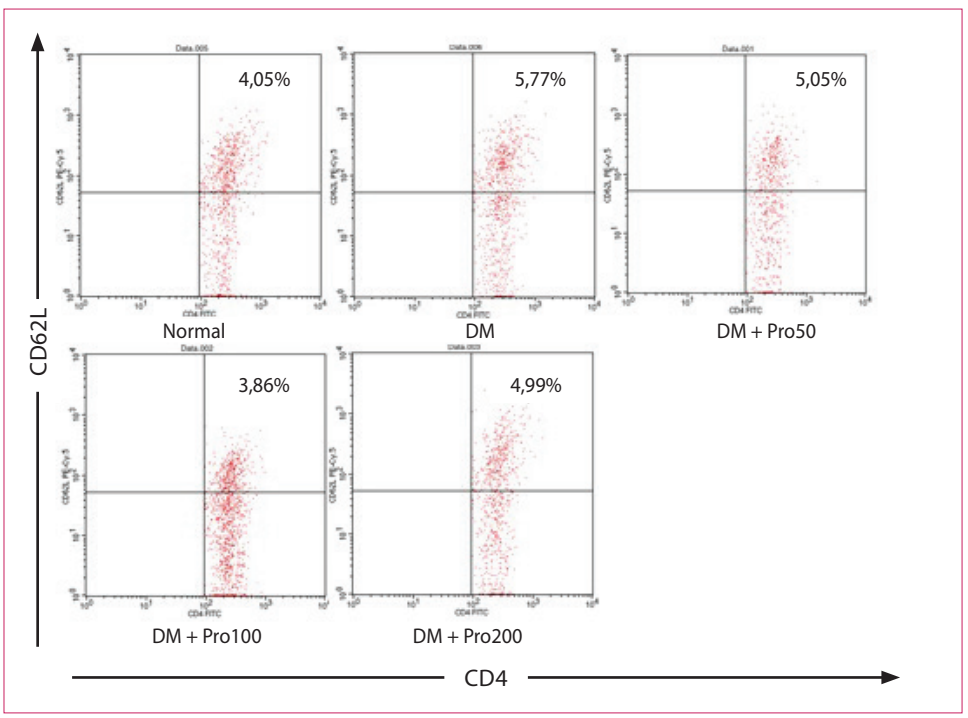

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Figure 2 a, b. Propolis diminished T cells activation during 13 days post treatment. Absolute number of cells (a). Relative number on flow cytometry (b). Normal: control mice; DM: diabetic mice without propolis treatment; the other groups; DM+Pro50, DM+Pro100 and DM+Pro200: diabetic mice treated with propolis 50, 100 and 200 mg/kg BW, respectively (Mean±SD, N=25, p=0.05).

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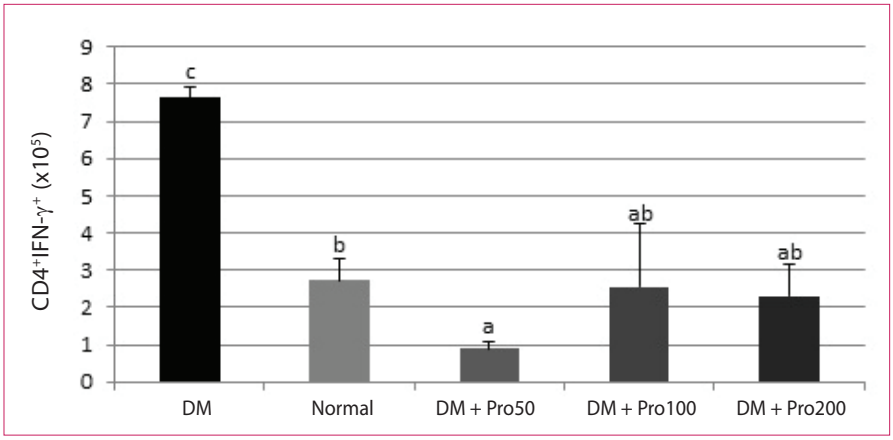

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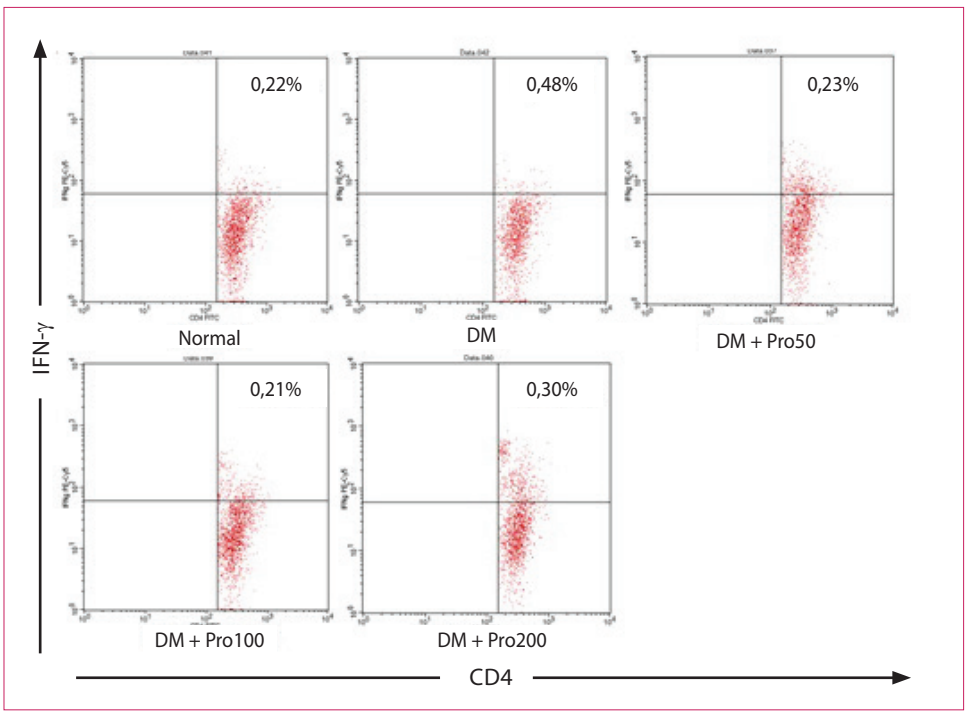

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Figure 3 a, b. Propolis decreased absolute number of IFN-γ expressed by T cells at 13th day of post treatment. Absolute number of cells (a). Relative number of IFN-γ expressed cells (b). Normal: control mice; DM: diabetic mice without propolis treatment; the other groups; DM+Pro50, DM+Pro100 and DM+Pro200: diabetic mice treated with propolis 50, 100 and 200 mg/kg BW, respectively (Mean±SD, N=25, p<0.05).

absolute number of activated T cells of diabetic mice increased compared to that of normal mice; from 5.1×10^6 to 9.1×10^6 cells (p=0.05). Propolis administration on diabetic mice model decreased the absolute number of activated T cells (p=0.05) (Fig 2a).

Doses of 100 and 200 mg/kg decreased activated T cells to the level of those of normal mice.

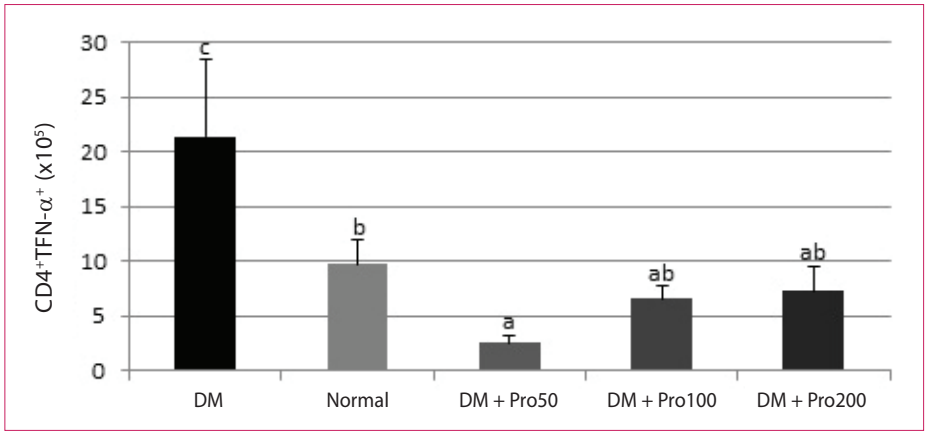
Propolis Decreased the Expression of Interferon-gamma in Diabetic Mice

Interferon-gamma is a pro-inflammatory cytokine which is primarily secreted by Th1. IFN-γ has a role in cytotoxic T cell activation.[25,26] We showed that IFN-γ expressing T cells increased significantly in diabetic mice model (p<0.05) compared to normal mice (Fig. 3a). Streptozotocin injection causes stress on cells and induces immune cells to secrete more pro-inflammatory cytokines. Streptozotocin can penetrate into pancreatic islet cells by glucose transporter GLUT-2 because of glucose functional group in their structure.[27]

The significant effect of propolis administration in diabetic mice model is shown in Fig. 3a. All the doses of propolis decreased IFN-γ expression on T cells significantly (p<0.05).

Propolis Decreased Expression of TNF-α in Diabetic Mice

TNF-α is one of the pro-inflammatory cytokines that contributes to the inflammatory process and insulin resistance mechanism in diabetes mellitus. TNF-α-



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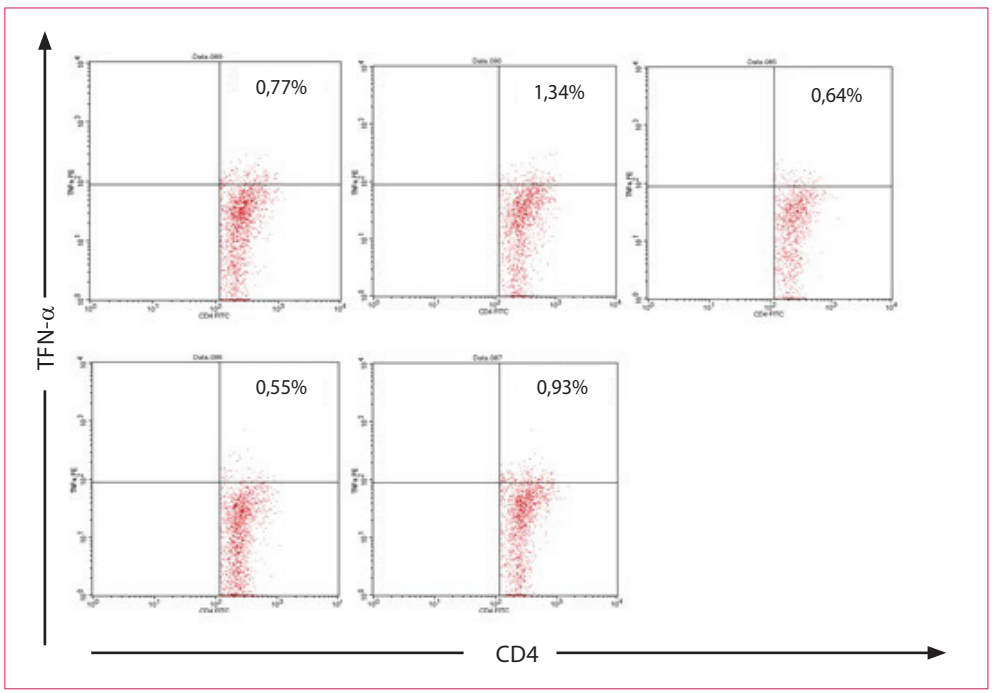

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Figure 4 a, b. Propolis decreased absolute number of TNF-α+ expressed by T cells during 13 days post treatment. Absolute number of cells (a). Relative numbers (b) Normal: control mice; DM: diabetic mice without propolis treatment; the other groups; DM+Pro50, DM+Pro100 and DM+Pro200: diabetic mice treated with propolis 50, 100 and 200 mg/kg BW, respectively (Mean±SD, N=25, p<0.05).

expressing T cells on diabetic mice model were found to be increased in absolute numbers (Fig 4a) (p<0.05). Propolis administration on diabetic mice model decreased the absolute number of TNF-α expressing T cells (p<0.05). The mean number TNF-α expressed T cells decreased to that of control mice.

Discussion

Pro-inflammatory cytokines are important in the pathogenesis of diabetes mellitus. It was shown that immune infiltration diabetic patient finally led to acute hyperglycemia.[8,28] Streptozotocin injection was shown to increase nitric oxide.[13,29] An excessive level of nitric oxide increased NF-kβ activation as a transcription factor of pro-inflammatory cytokines.[30] The insulin level was shown to be reduced in these diabetic mice.[19,21,31] Improvement of insulin regulation was shown to be related to inflammation reduction.[32,33] In this study we have shown that, propolis administration could lower the blood glucose level in mice.

In our study, diabetic mice without propolis administration were found to have increased number of activated T cells. Propolis with a dose of 100 mg/kg was shown to decrease IL-2, IFN-γ, and TNF-α secretion in seven days.[34] Propolis was shown to decrease IFN-γ secretion by regulating IL-2 gene encoded. Interleukin-2 is a cytokine secreted by Th1 cells and it acts as a mediator of T cell proliferation.[35] Secretion of IL-2 induces proliferation and activation of T cells. Activation of T cells leads to IFN-γ secretion. Besides Th1, IFN-γ is also secreted by cytotoxic T cells.[36] Accumulation of IFN-γ increases the macrophage cytotoxicity, so that it induces chemokines secretion in local

tissue such as IL-6 and TNF-α.[37] Increasing the absolute number of IFN-γ expression by CD4 T cells suggests that T cells underwent activation and consequently loss of CD62L molecule on its cell surface. When most T cells undergo activation, homeostatic imbalance occurred and caused physiologically hamperedness.[32,38,39]

TNF-α was secreted by innate and adaptive cells as a response to antigen. Oxidative compounds were shown to induce proinflammatory cytokines. Cells stress caused by oxidative compounds will stimulate local cytokines secretion, such as TNF-α and IL-6.[40] Streptozotocin-induced diabetic mice have an acute inflammation and blood glucose regulation disruption. In STZ-induced diabetic mice, nitric oxide accumulation occurs in peripheral tissue.[12] High nitric oxide was found to induce cellular stress and initiate innate cells to secrete TNF-α on their area.[41] TNF-α could initiate serine/threonine phosphorylation in IR subunit β and IRS-1 on the peripheral tissue so that normally tyrosine phosphorylation will be disrupted.[42] Its phosphorylation could activate *Ikk* complex, especially *Ikk*β. *Ikk*β promotes activation of NF-kβ, transcription factor for various pro-inflammatory cytokines gene. This activation induces the increase of TNF-α expression on effector cells. In another pathway, pro-inflammatory cytokines were able to induce effector cells' activation.[7,43] TNF-α promotes IFN-γ expression, and there are reports showing that TNF-α accelerates T cells proliferation in the presence of IL-2.[37] Serine or threonine phosphorylation of IRS-1 causes an adverse effect on insulin receptor regulation. Insulin receptor disruption causes GLUT-4 translocation failure that has a role in glucose regulation.[7,30,43]

Propolis is composed of the various soluble compounds, such as a flavonoid, phenolic acid, terpenoid, and various aromatic compounds.[44] The main compound which is contained in propolis is caffeic acid phenethyl ester (CAPE), classified into flavonoid. Flavonoid is an antioxidant which is able to avoid free radical effects. Flavonoid decreases oxidative stress by catching anion peroxide and hydroxide radical, and then transfers hydrogen atoms to peroxide radicals.[45] The decrease of oxidative stress level could suppress activation of NF-kβ as a transcription factor for gene encoded pro-inflammatory cytokines, including TNF-α and IFN-γ.[46,47] The geographical origin and bee species determine the compound type, also their level in propolis.[20] The recent studies were focused on African and Brazilian propolis only, such as *Apis mellifera* resin which is commonly used and more widespread.[16] Content compositions of propolis can different (especially CAPE) in different regions.

Various compounds in propolis could have different effects in different ratios. Some authors suggested that reaction between CAPE and the other compounds in propolis could reduce the efficacy of CAPE activity as a suppressor of inflammation.[35,44] Components in propolis that are thought to have an opposite effect on CAPE activity are calcium and glycerol.[19,22] They promote a transcription of the gene encoded IL-2 by NFAT as a transcription factor.[48] This phenomenon could explain clearly how the dose of 100 and 200 mg/kg BW did not show excessive decreases compared to a dose of 50 mg/kg BW.[49]

In summary, propolis decreased an absolute number of activated T cells expressing TNF-α and IFN-γ. Propolis also inhibited naive T cells from activation and prevent hyperglycemia. In diabetic mice model, propolis with the dose of 100 and 200 mg/kg BW could decrease the IFN-γ and TNF-α expression to a normal level. Therefore, these natural products may play a pivotal role to decrease the abnormal activation of T cells in inflammatory diseases, and its application could be an attractive strategy for the prevention or treatment of diabetic patients.

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Statement of potential conflicts of interest
The authors have no conflict of interest to declare.

Ethics
This study has received ethical eligibility certificate (Ethical Clearance) from The Research Ethics Committee (Animal Care and Use Committee) Brawijaya University No. 468-KEP-UB.

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