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The Study of Trigona *spp* Propolis Against Pro-Inflammatory Cytokine Production in Spraque Dawley Rats Macrophages Infected with *Staphylococcus aureus*

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Abstract

Propolis helps boost the immune system naturally because propolis is rich in bioactive compounds and can help increase the production and activity of immune cells. This product has attracted the interest of researchers in recent decades because of the properties of biological and pharmacological, among others such as antimicrobial, antitumor, anti-inflammatory, antioxidant and immunomodulatory. One of bee species that potentially produce propolis in Indonesia is a local bee species *Trigona* spp. This study aimed to to determine the immunomodulatory effects of ethanol extract of propolis on the Sprague Dawley rats were infected *by Staphylococcus aureus*.contained in propolis liquid. The effects of propolis were analyzed using the macrophage activity as determined by the phagocytic activity and by the production of nitric oxide (NO) in Sprague Dawley rat peritoneal macrophages and the production of antibodies.

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Identification of the phytochemical compounds was undertaken to determine the bioactive compounds contained in propolis liquid. The effects of propolis were analyzed using the macrophage activity as determined by the phagocytic activity and by the production of nitric oxide (NO) in Sprague Dawley rat peritoneal macrophages and the production of antibodies. The results showed that the liquid propolis produces (a) an increase in the phagocytic index, (b) a significant increase in NO production, and (c) an increase over the production of IgG antibodies. This study indicates that the ethanolic extract of propolis of *Trigona* spp. is able to activate macrophages and promote the production of antibodies. The combination of these results suggests that this extract has an immunomodulatory effect and can boost the immune response.

Keywords: propolis *Trigona spp*; pro-inflammatory cytokine; macrophages.

1. Introduction

Immunity is a reaction in the body to foreign substances that enter the body molecularly or cellular. The cells involved in the immune system in the body are T cells produced by thymus and B cells in the spinal cord. B cells and T cells are difficult to distinguish microscopically, to distinguish them from the surface of the molecule. Usually used to distinguish the two cells is a protein marker on the cell surface called Cluster of Differentiation (CD). The protein marker found in all T cells is CD3+, except for suppressor T cells and the cytotoxic protein marker is CD8+, while the T helper marker protein cell is CD4+ [1]. The propolis mechanism as anti-inflammatory has been studied by some researchers where propolis can reduce inflammation due to the presence of CAPE and quertecin content that contribute to suppress T cell activity. CAPE is able to inhibit Nuclear Transcription Kappa B Factor (NF-κB) and IL-2 stimulant Which spur work polifation from the T cell itself, whereas quertecin may affect the cyclooxyginase pathway. Both compounds are equally responsible for blocking lipooksigenase and cyclooxygenase.

The development and activity of T cells can be stimulated by the addition of an immunomodulator. Immunomodulators are substances that can help improve the functioning of the immune system. Clinically an immunomodulator is used in patients with impaired immunity, including in cases of cancer, HIV/AIDS, malnutrition, allergies, and others.

In propolis *Trigona spp*. which contain limonene, is thought to have an immunomodulatory effect which, through in vitro studies, showed that D-limonene increased NO peritoneal macrophage production in tumor-induced mice [2]. According to [3], at low concentrations, CAPE is able to activate T lymphocytes, which exhibit anti-apoptotic effects in B lymphocytes and does not affect NK cells.

Cytokines are a glycoprotein derived from helper T cells, NK cells and macrophages, which play an important role in the body's response to fight infection. Helper T cells consist of 2 subsets each producing a cytokine that regulates the effector effector function and reacts to each other. T helper type 1 (Th1) cells produce TNF-α, IFN-γ, and IL-2. These cytokines activate macrophages, to form proinflammatory cytokines such as TNF-α, IL-1 and IL-6 and induce the immune mechanism of cytotoxic effects of macrophages. In contrast, type 2 helper T cells (Th2) produce IL-4, IL-5, IL-10 and IL-13. This cytokine induces the formation of antibodies but also

inhibits macrophage function and is called anti-inflammatory cytokine [4,5,6].

TNF-α is a pyrogenine-causing cytokine. At low levels can inhibit the growth of blood stages of the parasite by activating the cellular immune system, and can also kill parasites directly but the activity is weak. The dual role of cytokines especially TNF-α is at the right level will provide protection and healing. However, excessive levels that may be a response to hyperparasitemia and excessive parasitic growth will result in severe and fatal tissue damage [5,6].

Propolis has anti-inflammatory activity that can enhance the body's immune system. Based on the above description, further research on immunomodulatory activity of propolis ethanol extract *Trigona spp*. Through the cytokine profile change parameters.

2. Method

2.1. Time and place

This research is part of the main research which runs from January to December 2016. The making of extraction and solution of propolis intervention is done in laboratory of Agricultural Product Technology, Lampung State Polytechnic, animal husbandry is done at Animal Hospital of Education FKH IPB. The analysis of cytokine production (TNF-α, IFN-γ, and IL-2) was carried out at the Immunology Laboratory of Primate Animal Study Center, LPPM IPB, Bogor.

2.2. Materials, Tools and Sample

The research material is from raw propolis from South Sulawesi region obtained from CV Nutrima Sehatalami, Bogor. The ingredients for the extraction are technical grade ethanol, and the filler is propylene glycol. The materials for rodent maintenance are rat feed, liquid propolis, Caffeic Acid Phenethyl Ester (CAPE) C8221-Sigma Aldrich compound, corn oil (SIGMA C8267). Determination of TNF-α levels, IFN-γ, and IL-2 levels, respectively using the TNF-α Mouse ELISA Kit (Legend Max ™-Biolegend), Mouse IFN-γ ELISA Kit (Legend Max ™-Biolegend), Mouse IL-2 ELISA Kit (Legend Max ™-Biolegend),.

Animals in the form of *Sprague Dawley* white mice aged less than 4 months [7] obtained from Animal Laboratory of Food and Drug Supervisory Agency (BPOM), isolate of *Staphylococus aureus* non protein A bacteria obtained from Bacteriology and Imunology laboratory of FKH-IPB.

Equipment used include: 2 μL-1 mL volume pipette, 1-25 mL reagent pipette, 100 mL and 1 liter graduate cylinders, absorbent paper, standard and sample reaction tubes, graph paper logs and ELISA data software, and microplate reader (Bencmark BIO-RAD) λ 450 nm.

This study used a completely random design design with 3 levels of propolis treatment and 2 control treatments. The study used 25 rats, the number of samples was calculated according to formula [Frederer 1967], so each treatment consisted of 5 rats.

2.3. Research Stages

Research begins with the preparation of propolis extracts that have been performed in previous studies, rat maintenance and intervention treatment, and terminal sampling stage.

2.4. Maintenance of rats

Rats are kept in a 50x30x20 cm cage, each cage containing two rats. Prior to use as an experimental animal, all rats were preserved for about 1 week for environmental adjustment, health control, weight gain and uniform feeding and feeding was done ad libitum. The standard rations used are based on AIN-93M [9]. Rats were randomly assigned to 5 groups, group I was given 0.5% (v / b) of CAPE compound 20 mg / kg as a positive control [10]. Group II was given aquades (ad libitum) as a negative control group, group III, IV, and V administration of liquid propolis 0.16%, 0.48%, and 1.44% of body weight/day. Administration of liquid propolis was performed orally for 14 days before the rats were infected with *S. aureus* non protein A. *S. aures* infection was performed on day 14 by injecting bacterial inoculum doses $1x10^9$ cfu intraperitoneally. On the 15th day all the rats in each group were euthanated with ether for blood.

Ethical considerations in the execution of this research were obtained from the Supervisory Committee of Animal Welfare and Veterinary Research, Animal Hospital of Education, Faculty of Veterinary Medicine, Bogor Agricultural University with Number: 2-2016 RSHP FKH-IPB dated January 4, 2016.

2.5. Rat blood collection

After euthanasia was done in mice, it was awaited interval of 5 minutes until the rat fainted calmly and performed blood taking from the heart. Blood sucked out using a capillary pipe then transferred to eppendorf. The blood contained in the eppendorf tube was incubated for 30 min at 37 °C and then centrifuged 13000 rpm, 10 mins 4 °C. After that the supernatant part (plasma) is removed and the serum part is separated and stored in microtube. Then wrapped with fi lms and stored in a cooler with a temperature of -70 ^oC until it will be used.

2.6. The influence of propolis Trigona spp. to cytokine production (TNF-α, IFN-γ, and IL-2)

Determination of the production of cytokines TNF-α, IFN-γ, and IL-2 using the ELISA Kit Mouse, performed according to the protocol.

2.7. Processing and analysis of data

Results are presented in mean ± SD. All statistical analyzes are performed using Microsoft Excel and SAS 9.1. Significant differences between treatments were analyzed using ANOVA, if there was a significant difference between treatment and further testing with DMRT test. The difference is significantly expressed in $p < 0.05$.

3. Results and Discussion

3.1. Identification of phytochemical compounds of ethanol propolis extract

Early stage studies previously reported the identification of phytochemical components of propolis ethanol extract extract *Trigona spp*. Using GC-MS [11]. There are 6 (six) components of identified major compounds such as limonene, 1-heptacosanol, heptacosane, 1-hexadecanol, dioctyl adipate, and hexadecane.

3.2. The influence of propolis Trigona spp. to cytokine production (TNF-α, IFN-γ, and IL-2)

The difference in cytokine production can be seen in Table where propolis is able to increase cytokine production in rat blood serum by different treatments. This shows the provision of propolis extract successfully stimulate the immune response in this study. Propolis 0.16%, significantly increased production of IL-2 cytokines and decreased production of TNF-α and decreased IFN-sit cytokine production, although not significant.

Table 1: Influence of propolis *Trigona spp*. of cytokine production

Kelompok	TNF-α (pg/mL)	IFN-γ (pg/mL)	IL-2 (pg/mL)
CAPE (Positive Control)	4.996±0.51 ^{c}	37.145±1.35 ^{b}	58.813±1.50 ^{a}
Akuades (Negative Control)	12.426±0.68 ^{a}	45.015±2.49 ^{a}	50.741±1.77 ^{b}
Propolis 0.16 %	4.055±1.20 ^{c}	42.865±0.85 ^{a}	56.173±5.74 ^{a}
Propolis 0.48 %	4.724±0.48 ^{c}	35.264±1.49 ^{b}	59.913±2.27 ^{a}
Propolis 1.44 %	9.826±1.41 ^{b}	32.615±0.75 ^{c}	61.017±8.57 ^{a}

Description: the numbers followed by the same letter in the same column states no significant difference at

The figure shows that the the test level of 5%average number of IL-2 due to immunomodulatory activity of propolis ethanol extract extract *Trigona spp*. In Sprague Dawley white rats with 0.16%, 0.48% and 1.44% of the total IL-2 significantly increased compared with the negative control group. While the mean number of IFN-γ and TNF-α was significantly decreased compared with the negative control group.

Activation of macrophages through the release of cytokines by the lymphocyte of the macrophage release pathways in the body's immune system not only through the arachidonic acid pathway but also through the cytokines produced by the lymphocytes. A cytokine is a small protein released by many cells and acts like a hormone, through a receptor on the surface of the target cell. Cytokines have properties, among others: one cytokine has an effect on various cells, various cytokines have the same overlapping effect, 2 or more cytokines show a greater effect than only the additive effect, and one cytokine can prevent the effects of other cytokines [12].

One of the functions of macrophages is as Antigen Presenting Cell (APC). An antigen presentation is a process that allows the antigen to be known by T cells. Some antigens are eaten APC cells in the periphery and transported to secondary lymphoid tissue, while other APC cells are living cells in lymphoid tissue and capture antigens that enter the tissue. In studies conducted by [13], vitamin C can interact with flavonoids that can become immunostimulants by stimulating lymphocyte expenditure. The lymphocyte cells that constitute 20% of all leukocytes in the blood circulation consist of T cells and B cells. T cells when exposed to an antigen develop

into Th0 cells. Then Th0 cells can develop into Th1 and Th2 cells, depending on the induced cytokines. On the influence of IFN-γ and IL-12, Th0 develops into Th1, while the effect of IL-4, IL-5, IL-10, 1L-13, Th0 catalysts develops into Th2. Furthermore, Th1 will produce cytokines IFN-γ which is an important cytokine in macrophage activation and killing microbes in phagolisosomes and stimulate B cells to produce IgG which acts as opsonin and phagocytosis [12].

IL-2 is produced by CD4 + T lymphocytes and in very small amounts by CD8+ T cells. Activation of T cells by antigens and co-stimulators stimulates IL-2 gene cytokines and synthesizes and secretes proteins. The production of IL-2 is temporary with a peak of secretion occurring approximately 8-12 hours after activation. The functional features of the IL-2 receptor are enhanced through antigen stimulation; therefore, T cells familiar with the antigen will activate the biproliferation of IL-2 cytokines during adaptive immune response [14]. The secondary metabolite content of *Trigona spp*. In the form of chemical compounds one of them is monoterpenoid (limonene). The results [15] concluded that D-limonene, limonene-1-2-diol and perillic acid significantly inhibited pro-inflammatory activity of CD4+ and CD8+ T lymphocytes and had cytotoxic potential. [16] D-limonene has the potential to work on lymphokines (IFN-γ) produced by T cells that will stimulate phagocyte cells to perform phagocytic responses and may stimulate lymphocyte proliferation, increase T-cell count and increase secretion of IL- 12. D-limonene may increase the production of IL-2, one of the cytokines essential for lymphocyte proliferation. Based on research [17], D-limonene and its metabolites can increase phagocytes and can significantly increase IL-2. IL-2 is one of the many cytokines that regulate the immune response, functioning as a mitogen for T cells, potentially increasing the proliferation and function of T cells, B cells and NK cells, improving antigen formation and increasing the production and release of other cytokines.

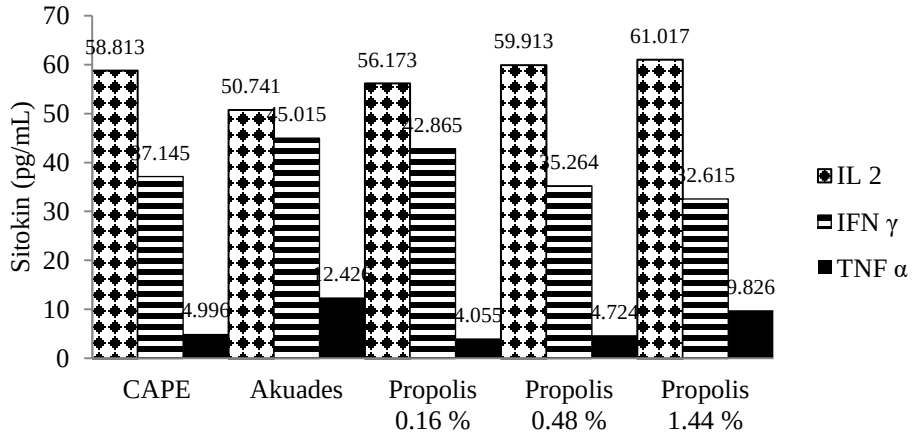

Figure 1: Effect of *Trigona spp*. propolis on cytokine production. Treatment by giving different propolis. 0.5% CAPE is used as a positive control. $p < 0.05$, compared to aquades (negative control) (ANOVA, DMRT test).

Limonene which is one of the active substances contained in propolis *Trigona spp*. can increase the production of NO macrophages through increased secretion of IL-2 which then stimulates Th1 (T helper) to increase IFN-γ secretion [14]. IFN-γ is the potential to induce iNOS which then iNOS will catalyze L-arginine to sitrulin by

freeing NO. In vitro studies have shown that D-limonene increases the production of peritoneal macrophage NO from mice suffering from tumors [2].

Cytokines are immune system proteins that regulate cell-to-cell interactions and trigger immune responses, both in innocent/innate immunity and specific/adaptive immunity. Cytokines are polypeptides produced in response to stimuli of microbes and other antigens and act as mediators in the immune and inflammatory reactions. Cytokines are chemical messenger proteins, or intermediates in intercellular communication. Cytokines play a role in the activation of T cells, B cells, monocytes, macrophages, inflammation and induced cytotoxicity [12, 14]. Cytokines produced macrophages and NK cells that play a role in early inflammation, stimulating the proliferation, differentiation, and activation of specialized effector cells such as macrophages. Whereas in specific immunities, T-cell-produced cytokines activate specific immune cells. Cytokines in non-specific immunity are: TNF, IL-1, IL-6, IL-10, IL-12, IFN type I, IL-15, IL-18, and IL-33. While cytokines in specific immunity are: IL-2, IL-4, IL-5, IFN-γ, TGF-β, Lymphotoxin, IL-13, IL-16, IL-17, IL-23, IL-25, IL -31, IL-9 [14].

The presence of *S. aureus* infection in animals whose macrophages have been activated causes macrophages to move toward the source of infection. Macrophages that have arrived at the site of infection will perform phagocytosis against *S. aureus*. Furthermore, germs are processed in phagolisosomes into peptide fragments. The peptide fragment formed is bound by a major histocampactibility complex (MHC) and brought to the cell surface to be presented to T cells. During phagocytosis and antigen processing, macrophages secrete several secretions, one of which is IL-2.

Helper T cells via T cell receptor (TCR) recognize the *S. aureus* antigen presented by macrophages. Ligands between the MHC-antigen complex with CD3-TCR evoke inositol activity in T cell membranes into inositol triphosphate and glycerol compounds. Inositol triphosphate will increase calcium (Ca ++) ions in the cytoplasm, whereas diacylglycerol will activate the protein-enzyme C-enzyme. Both are signals for activating T cells. T cell activation can be observed with IL-2 secrete. This IL-2 compound is useful for activating B cells into plasma cells [18]. *S. aureus* bacteria are pathogens that are known to have lipoteicoic acid on the bacterial surface and allow these pathogens to be identified by toll-like receptors II (TLR2). Signaling by these receptors further stimulates the production of IL-12 which further stimulates the secretion of IFN-γ [19]. In addition, *S. aureus* bacteria may activate the adaptive immune response via superantigen induction [20]. The interaction between superantigens and cells can induce the production of cytokines and chemokines on a large scale. Cytokines TNF-α and IL-2 have immunostimulatory activity and work synergistically with IFN-γ to enhance immune and inflammatory reactions. However, these cytokines at high concentrations become pathogens and can induce toxic shock [21]. Activated macrophages produce and release products including several cytokines, inorganic reactive radicals, reactive oxygen intermediates, and reactive nitrogen with biological activity. In this study, peritoneal macrophages exposed to active propolis compounds showed the production of cytokines. Proinflammatory cytokines are essential to initiate the inflammatory process that causes tissue damage. These cytokines induce tissue damage and reduce the capacity to repair damaged tissue by stimulating the production of other mediators [22]. Cellular bacteria activate NK cells by inducing expression of NK cell activating ligand on the surface of infected cells, or dendritic cell stimulation and IL-12 production by macrophages which are

strong mecrophage activating cytokines. NK cells produce IFN-γ which in turn activates macrophages and enhances the ability of macrophages to kill the bacteria they swallow [14]. In this study, it was found that propolis *Trigona spp*. capable of reducing pro-inflammatory cytokines such as IFN-γ and TNF-α production and increasing production of anti-inflammatory cytokines, such as IL 2. Thus, it can be suspected that the ethanol extract of propolis has an active compound that acts as an anti-inflammatory agent in response to superantigen which results in toxic shock in mice infected with *S. aureus* bacteria. The production of IL-2 can be stimulated by the presence of immunomodulatory compounds and involves the role of a differentiated T cell into Th cells capable of producing IL-2 that can activate other immune systems. The combined results show the immunomodulator activity of propolis extract.

4. Conclusions

1. This study shows that administration of *Trigona* spp. In rats try to indicate that macrophages have been activated through the administration of different propolis treatments.

2. Provision of 0.16% propolis, was able to decrease TNF-α production (4,055 pg/mL) and production of IFN-sit cytokines (35.264 pg/mL) although not significant and significantly increased production of IL-2 cytokines (56.173 pg/mL). The combination of these results indicates that the propolis ethanol extract has an immunomodulatory effect and is capable of enhancing the immune response.

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