Turkish Journal of Immunology - Open Access
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Turk J Immunol 2017; 5(3)69–76

Immunomodulatory Effect of Propolis Extract on Population of IL-10 and TGF-β Expression in $CD4^+CD25^+$ Regulatory T Cells in DMBA-induced Breast Cancer in Female Sprague-Dawley Rats

DMBA ile Oluşturulmuş Meme Kanserli Sıçanlarda $CD4^+CD25^+$ Regülatuvar T Hücre IL-10 ve TGF-beta içeriklerine Propolis Ekstresinin Düzenleyici Etkisi

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Received: Apr 21, 2017
Accepted: Sep 16, 2017

doi: 10.25002/tji.2017.573

Abstract

Introduction: The aim of this study was to investigate the effect of propolis extract to the population of regulatory T cells ($CD4^+CD25^+FoxP3^+$) expressing IL-10 and TGF-β in vivo (DMBA-induced breast cancer in Female Sprague-Dawley Rats).

Materials and Methods: Thirty virgin female Sprague-Dawley rats, aged 45–50 days-old were injected subcutaneously with 2x10 mg of 7.12-dimethylbenz(a)anthracene (DMBA) for one-week intervals. A week later each rat was administered orally with 5 mg DMBA. Ten rats without DMBA served as negative control. To ensure the development of breast cancer before starting treatment, at week 8, five rats that received DMBA and five negative controls were randomly selected and euthanized. Their breast tissues were then dissected for histological analysis by hematoxylin-eosin staining. Breast ductal cell morphological changes were apparent in DMBA-treated rats. The remaining rats were divided into 5 groups (6 rats per group). Group I served as a negative control, and Groups II–V were animals treated with DMBA, where Group II served as positive control, and Group III, IV, and V were animals treated with ethanolic extract of propolis (EEP) at doses of 50, 100, and 200 mg/kg body weight (BW), respectively, for 4 weeks. The animals were sacrificed by cervical decapitation. Breast and spleen tissues were subjected to histological and flow cytometric analysis respectively.

Results: Statistical analysis by linear regression showed a significant decline in the relative number of $CD4^+CD25^+FoxP3^+$ regulatory T cells expressing IL-10 or TGF-β after treatments with propolis extracts.

Conclusion: In summary, propolis may act as immunomodulatory agent that can be useful for curing cancer patients.

Keywords: Breast cancer, propolis, regulatory T cells, IL-10, TGF-β, in vivo

Öz

Giriş: Bu çalışmada DMBA ile oluşturulmuş meme kanserli dişi Sprague Dawley sıçanlarda regülatuvar T Hücrelerindeki ($CD4^+CD25^+FoxP3^+$)IL-10 ve TGF-beta ifadelerine propolis ekstresinin bağışıklık düzenleyici etkisinin araştırılması amaçlandı.

Gereçler ve Yöntemler: Daha önce çiftleşmemiş 45-50 günlük 30 dişi Sprague-Dawley sıçana bir haftalık aralıklar ile subkutan olarak 2 kez 10 mg 7.12-dimetilbenzantrasen (DMBA) uygulandı. Bir hafta sonra ise, 5 mg DMBA ağız yoluyla tekrar verildi. DMBA verilmeyen 10 sıçan ise kontrol grubu olarak belirlendi. Meme kanserinin oluştuğundan emin olmak için, tedaviye başlamadan önce, 8. haftada DMBA verilen 5 sıçan ile verilmeyen 5 sıçan rastgele olarak seçilip ötanazi uygulandı ve bu deneklerin meme dokularının kesitleri hematoksilen-eozin boyası ile boyanarak irdelendi. Meme dokularındaki duktal hücrelerdeki morfolojik değişiklikler belirgin idi. Kalan sıçanlar, her grupta 6'şar denek olacak şekilde 5 gruba ayrıldı. 1. Grup negatif kontrol olarak belirlendi. İkinci, 3., 4. ve 5. gruplara ise DMBA verilirken, 2. grup pozitif kontrol grubu olarak isimlendirildi. İkinci, 4. ve 5. grup deneklere ise, propolisin etanol ile elde edilen ekstresinden sıra ile her bir gruba vücud ağrılığına göre 4 hafta boyunca 50, 100 ve 200 mg/kg dozunda verildi. Hayvanlar, servikal dekapitasyon yöntemi ile öldürüldü.

Bulgular: Lineer regresyon yöntemi ile yapılan istatistiksel irdelemede IL-10 ve TGF-beta ifade eden $CD4^+CD25^+FoxP3^+$ T lenfositlerinin görece sayısında istatistiksel olarak anlamlı bir düşme izlendi.

Sonuç: Propolis, bağışıklığı düzenleyen bir madde olarak davranmaktadır ve kanser hastalarının tedavisinde kullanılabileceği düşünülebilir.

Anahtar Kelimeler: Meme kanseri, propolis, regülatuvar T hücreleri, IL-10, TGF-beta, in vivo

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Introduction

The immune system is an integrated part of the study of new cancer therapies. Research shows that the immune system plays an important role in mitigating the progress and recurrence of cancer as well as therapeutic efficacy. Cytotoxic T cell activation is an important part of the success of the adaptive immune response against cancer cells. However, various peripheral immunotolerant mechanisms may decrease the effectiveness of the cytotoxic T lymphocytes (CTLs), among which is the mechanism of immunosuppression induced by regulatory T cells ($CD4^+$ $CD25^+$ FoxP3).[1] Regulatory T (Treg) cells are a subset of T cells that are known to have immunoregulation and immunosuppression activity. Treg cell activity in cancer cases is linked to a poor prognosis.[2–4]

The population of Treg cells is higher than normal breast tissue in invasive breast cancer and ductal carcinoma in situ (DCIS); in invasive breast cancer, the number of Treg cells is more than in DCIS. High Treg cell populations were also reported to be associated with increased risk of recurrence in patients with DCIS and lower disease free survival (DFS) and overall survival (OS) patients with invasive breast cancer.[2] Many studies have reported that Treg cells begin to infiltrate tumor lesions at a very early stage[5], and FoxP3 expression in breast tumor tissue was higher than normal breast tissue.[4]

Activated Treg cells suppress the innate and adaptive immune responses through several mechanisms, including producing immunosuppressive cytokines interleukin-10 (IL-10) and Transforming Growth Factor-β (TGF-β).[6,7] TGF-β can suppress the systemic immune system, inhibit immune surveillance and affect immune cell populations.[7] TGF-β also inhibits the activity of CTL and encourages the formation of Treg cells and Th17 cells.[8] IL-10 is known as a cytokine synthesis inhibitory factor (CSIF), a cytokine anti-inflammatory that inhibits gene expression and synthesis of cytokines such as Interferon Gamma (IFN-γ), Interleukin-2 (IL-2), IL-4, IL-5, IL-13 and TNF-α by T cells and macrophages, inhibiting the ability of antigen presentation by lowering the expression of MHC and B7 molecules, a co-stimulator on antigen presenting cells (APCs) that also inhibits the synthesis of IFN-γ by activated T cells and peripheral blood mononuclear cells (PBMC).[9,10]

DMBA (7.12 dimethylbenz(a)anthracene) is a type of polycyclic aromatic hydrocarbons (PAH) and is widely used to induce breast cancer in research laboratories. DMBA has a strong carcinogenic effect especially against Sprague-Dawley rats.[11] The histology of DMBA-induced breast cancer tissue in Sprague-Dawley (SD) rats is generally recognized to be similar to that of human breast cancer, making it an ideal model for breast cancer research.[11,12]

Propolis is a natural substance produced by bees from plant resins that are collected and combined with wax and secretions from the salivary glands of bees and is rich in various enzymes.[13,14] Bee propolis is used to coat the walls of the nest to protect the animals from the entry of the attacker from the outside, inhibit bacterial and fungal growth, strengthen the hive cell walls and also maintain an aseptic environment in a nest.[15]

Propolis has activity as an immunomodulator, which reportedly comes from a variety of active compounds; among these are CAPE, artepilin C, cinnamic acid, quercetin, and hesperidin. In a previous study[16], we identified the active compounds (caffeic acid phenethyl ester (CAPE), artepilin C, cinnamic acid, and quercetin) in the propolis extracts used in the present study. This study reports on the effect of propolis extracts on the population as well as the functional activity of Treg cells ($CD4^+CD25^+FoxP3^+$) in Sprague-Dawley rats induced with DMBA. Propolis extract and its active compounds (quercetin, hesperidine, CAPE) have an influence on the production of inflammatory cytokines IL-2, IL-4, IL-10, IL-12, and TGF-β1. Histologically, tissue from DMBA-induced breast cancer in Sprague-Dawley rats (SD) is generally recognized to have similarities to human breast cancer, making it the ideal model for breast cancer research.[17,18] Decrease in IL-10 also occurs in treatment with propolis extract or its active compounds quercetin and hesperidin.[19]

Materials and Methods

Propolis extraction

The propolis extraction process was performed based on published method with some modifications.[17] A total of 100 g of propolis was cut into small pieces and frozen at -80°C. After that, propolis was ground and extracted with 70% ethanol at a ratio of 1:10. The solution was sonicated using an ultrasonic bath at 25°C for 30 minutes in the dark. After sonication, the solution was filtered through Whatmann No. 1 filter paper. Sonication using new solvent was repeated in order to obtain the active compounds.

The total solution was then kept in the refrigerator for wax removal overnight. The solution was filtered through Whatman No. 1 filter paper and evaporated to remove the organic solvent.

Animals

A total of 40 female Sprague-Dawley rats aged 45–50 days-old with a weight of 100–150 grams were acclimatized in pathogen-free facility for 1 week. A total of 30 rats were then injected subcutaneously twice with 10 mg of 7.12-dimethylbenz(a)anthracene (DMBA)/rat at an interval of one week. A week later the rats were also administrated orally with 5 mg DMBA/rat. DMBA was obtained from TCI Chemicals, Japan. The animals were maintained under the controlled environmental condition with 12 hr dark/light cycle. Commercial pelleted feed and water were given to animals ad libitum. All experiments were approved and conducted in accordance with the guidelines of the Research Ethic Committee of Brawijaya University, Malang Indonesia (No. 379/EC/KEPK-S3/10/2016).

To ensure the development of breast cancer before starting treatment, at week 8, five rats that received DMBA and 5 rats of the negative control were randomly sampled for biopsies of breast tissue; sampled rats were euthanized then breast tissue dissected for histological analysis by hematoxylin-eosin staining. The remaining rats were divided into 5 groups with 6 rats per group. Group I served as a negative control; and Groups II–V were animals treated with DMBA, where Group II served as positive control, and Group III, IV, and V were animals treated with ethanolic extract of propolis (EEP) at doses of 50 mg/kgBW, 100 mg/kgBW, and 200 mg/kgBW, respectively, for 4 weeks. At the end of the experimental period, the animals were sacrificed via cervical decapitation. Breast and spleen tissues were subjected to histological and flow cytometric analysis respectively.

Histological analysis of breast cross sections

Sections were obtained from the excised breast tissue and stained with haematoxylin-eosin. Briefly, 5 µm of thick tissue sections were deparaffinized and stained with hematoxylin for 1–5 minutes which will stained staining the nucleus and cytoplasm blue. Sections were then treated with 0.6% HCl to reduce the blue color of the nucleus and remove the blue stain from the cytoplasm. To clarify the blue color in the cell nucleus, a bluing process was carried out with 0.5% lithium carbonate, followed by eosin staining II to give the red color in the cytoplasm of the cells. This was followed by dehydration with a graded alcohol solution (80%, 90%, and 100%). The histological preparations were then mounted by using Entellan and observed by using microscope BX51 (Olympus, UK).

Lymphocytes isolation

T lymphocytes were isolated from the excised spleen of the rats. The excised spleen was washed with sterile PBS 2x and put on a petri dish containing sterile PBS. The spleen organ was pressed by using syringe holder. The single cell solution was filtered with a sterile wire and put into a 15 mL polypropylene tube. To the cell suspension in polypropylene, PBS was added up to 10 mL and then put in a centrifuge (2500 rpm, 4°C for 5 min). The supernatant was discarded and the obtained pellet was resuspended in 1 mL of sterile PBS and subjected for flow cytometric analysis.[18]

Flow cytometry analysis

In order to assess the population and activity of immune cells, we use specific markers such as antibodies anti-CD4-FITC (Biolegend, Japan), anti-CD25-PE (Biolegend, Japan), anti-FoxP3-alexa Fluor 647 (Biolegend, Japan), anti-IL10-Cy5 (Bioss USA), anti-TGFβ-Cy3 (Bioss, USA), and anti-CD152/CTLA-4-PE (Biolegend, Japan), for the detection of $CD4^+CD25^+FoxP3^+IL\text{-}10^+$, 100 µl of single cell suspension containing $2\text{-}3\times10^6$ cells was washed with PBS and stained with FITC-conjugated anti-rat CD4, PE-conjugated anti-rat CD25, by adding 5 mL of the monoclonal antibody anti-rat CD4-FITC (Biologend, Japan) and anti-rat CD25-PE (Biologend, Japan) to the cell suspension and incubated for 30 min at room temperature in the dark. The solution was centrifuged and the cells were washed with the washing solution. The samples were then fixed with 300 mL of 1% formaldehyde and permeabilyzed using 300 mL of 0.1% saponin (Sigma-Germany). Anti-rat FoxP3-PE (Biologend, Japan) and IL-10-Cy5 (Bioss, USA) were then added and the suspension incubated for 30 minutes in a dark room. Next, 0.4 ml of Cell Staining Buffer (Biolegend. Japan) was added to the cell suspension and analyzed by flowcytometer (BD FACS Calibur, USA) using CellQuest software for analysis. The total percentage of $CD4^+CD25^+$ T cells were first calculated in a lymphocyte gate. To determine the percentage of $FoxP3^+$ Treg cells from a population of $CD4^+CD25^+$, $CD4^+$ lymphocyte gate finally applied to the dot plot of $CD25^+/FoxP3^+$, and CD4 lymphocyte percentage of $CD^+CD25^+FoxP3^+$

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cells is Treg population.[19] To obtain the percentage of $CD4^+CD25^+FoxP3^+IL\text{-}10^+$, $CD4^+$ lymphocytes gate was applied to the dot plot FoxP3/IL-10. The same procedure was performed to detect $CD4^+CD25^+FoxP3^+TGF\text{-}\beta^+$.

Statistical analysis

All data were analyzed statistically with significance level $p \leq 0.05$ and confidence level 95% with SPSS 18.00 software. The normality and homogeneity of data were tested with Kolmogorov-Smirnov test and Levena test, respectively and followed by Nested ANOVA and Tukey (Post Hoc Test) parametric test.

Results

Histological pattern of breast cancer in rats eight weeks after DMBA induction

As noted above, eight weeks after the initial DMBA injection, we chose five rats randomly from the control group and DMBA-administered group to evaluate breast cancer in rat models via hematoxylin-eosin staining of tissue sections. We found that breast ductal cell morphological changes were apparent in all rats that received DMBA (Figure 1).

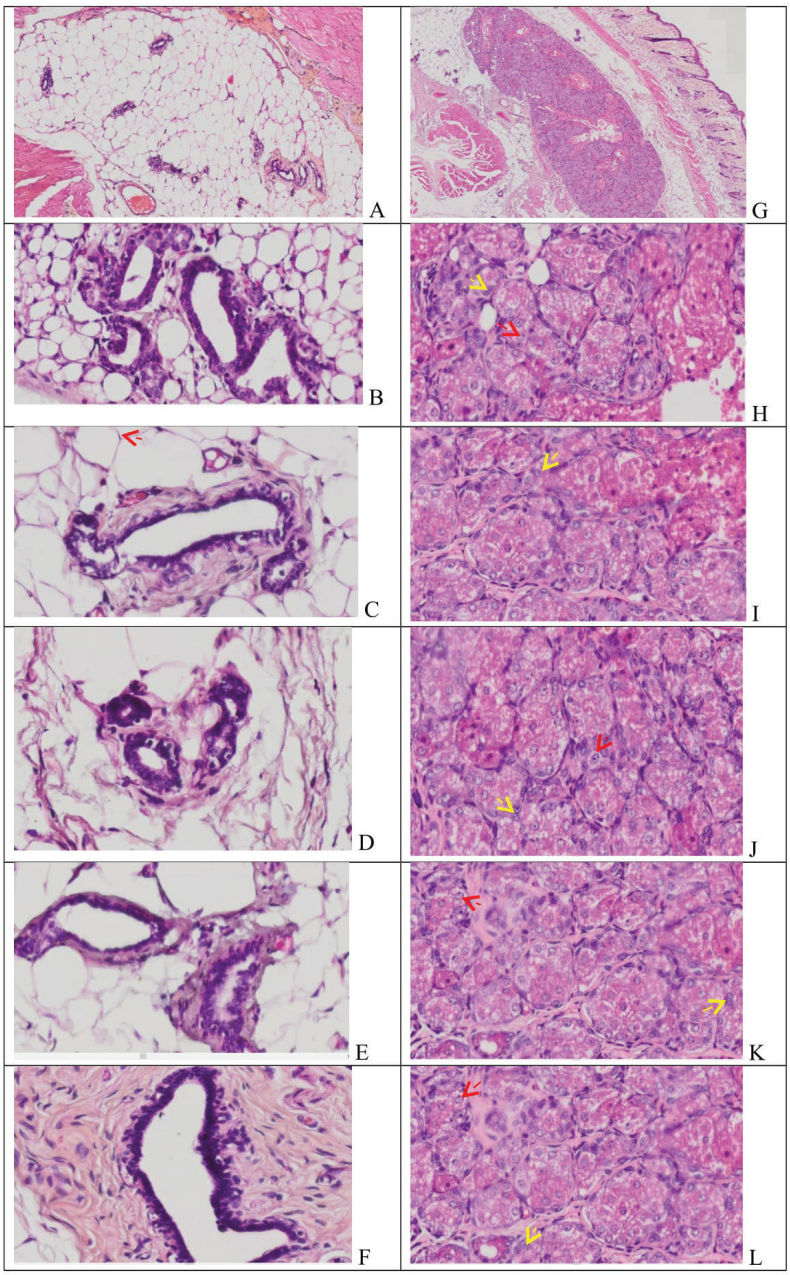


Figure 1. Rat's breast duct histology, A-F negative control (healthy female rats without induction of DMBA), A. observed with a magnification of 40x, B-F. Magnification of 200x. G-L show treatment group that received DMBA injection at a dose of 2x10 mg/rat and oral administration of 5 mg/rat. G. Magnification of 40x, H-L were observed at 200x magnification. The red arrow shows the cell with a big nucleus, yellow arrows show mitotic cells.

To determine the degree of differentiation of breast cancer, we used a histological pattern of the cells. In terms of cellular characteristics of the ductal epithelium, the nucleus to cytoplasm ratio ranged from 1:4 to 1:6. The morphology of the ductal epithelium in DMBA-treated rats showed a very clear change compared with normal rat duct, showing pleomorphic cells undergoing cell division, the nuclei of the cells looked bigger with an abnormal shape, Furthermore, the nucleus and the cytoplasm ratio was 1:2 to 1:1. In addition, tubules were no longer clearly seen in cells undergoing mitosis (Figure 1, G-L). These observations assured us that the DBMA-treated animals actually had breast cancer.

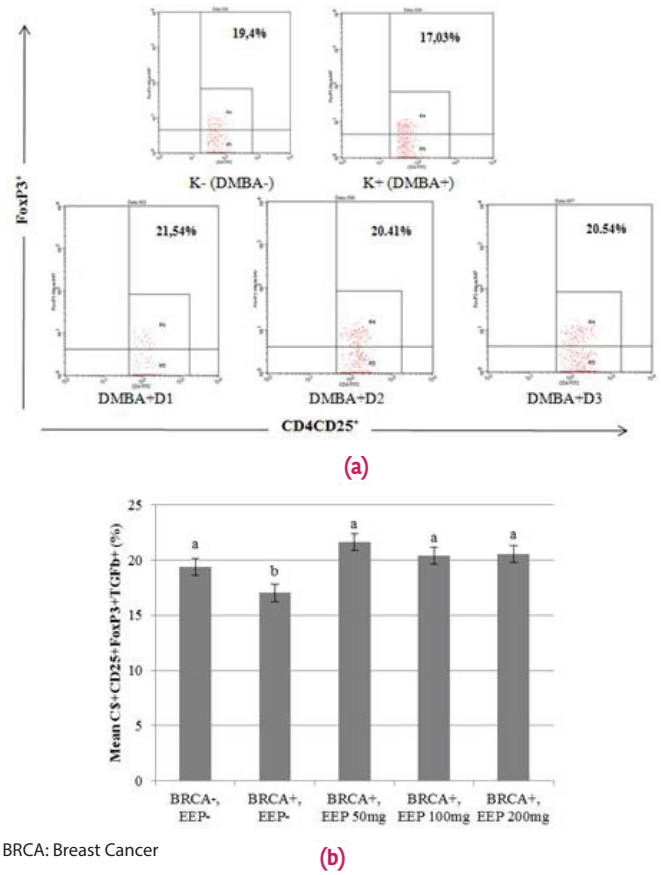
Relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing FoxP3

Treatment with propolis extract showed no significant effect on the relative number of CD4⁺CD25⁺FoxP3⁺, in all groups the relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cell were almost equal (Figure 1). Positive control group had 17.03% CD4⁺CD25⁺FoxP3⁺ regulatory T cell, while those dosed with 50, 100 and 200 mg propolis extract/kgBW were 21.63%, 20.41% and 20.54% respectively (Figure 2).

These data provide information that propolis extract treatment did not affect the population of regulatory T cells (CD4⁺CD25⁺FoxP3⁺) in the rat model of breast cancer in quantity. We suspect the immunomodulatory activity of propolis extract may be through other pathways. We assessed the effect of propolis extracts on the functional activity of regulatory T cells via the expression of IL-10 and TGFβ.

Relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing IL-10

Treatment with propolis extracts caused a decrease in the relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing IL-10 at all doses of 50, 100 or 200 mg/kgBW (Figure 3). To determine whether the decrease in the relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing IL-10 was the effect of propolis extract treatment, we performed a linear regression analysis using SPSS software version 18. The results showed a p value <0.0001, an R-square of 0.343 and a regression coefficient of -5.85. This means the provision of propolis extract treatment had a significant influence on the decline in the relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing IL-10. Propolis extract treatment to decrease the



BRCA: Breast Cancer

Figure 2. The Relative Number of CD4⁺CD25⁺FoxP3⁺ between Group. Group I (BRCA-, EEP-) served as a negative control, Group II until V (BRCA⁺) were animals treated with subcutaneous injection 2x10 mg of DMBA in 0.5 ml corn oil in one-week interval and 5 mg oral administration of DMBA. After 11 weeks of tumor induction Group III-V (BRCA⁺, EEP⁺) animals were treated with ethanolic extract of propolis (EEP; 50.100 and 200 mg/kgBW) for 4 weeks. Rats were sacrificed by cervical dislocation and analyzed using flow cytometry (a) and tabulated into Microsoft Excel (b). Data are mean ± standard deviation values of six rats in each group with p-value <0.05. Different letters (a and b) indicate significant difference based on Tukey's high significant differences test at a 95% significance level.

relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing IL-10 was 34.3%. The regression coefficient of -5.85 (negative) showed the opposite effect on the treatment on the relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing IL-10, in which the increase of propolis dose causes a decrease in the relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing IL-10.

Relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing TGF-β

Treatment with propolis extract caused a decrease in the relative number of CD4⁺CD25⁺FOXP3⁺ regulatory T cells expressing TGF-β at all doses of 50, 100 or 200 mg/

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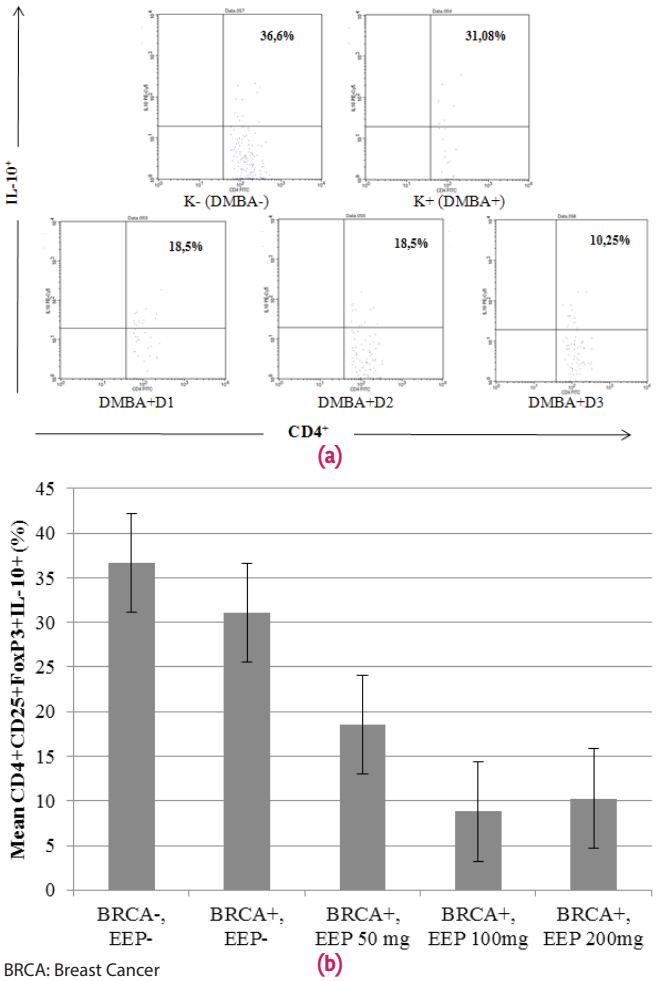

Figure 3. a, b. Ethanolic Extract of Propolis is able to decrease the percentage of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing IL-10 compared to control group. One-way ANOVA with LSD post hoc test showed that the treatment of propolis extract at a dose of 50, 100, and 200 mg / kgBW had significantly different of relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing IL-10 compared to positive control. Rats were terminated and analyzed using flow cytometry (a) and tabulated into Microsoft Excel (b). Data are mean of ± standard deviation values of six rats in each group with p-value <0.05. Different letters indicate significant difference based on Tukey's high significant differences test at a 95% significance level.

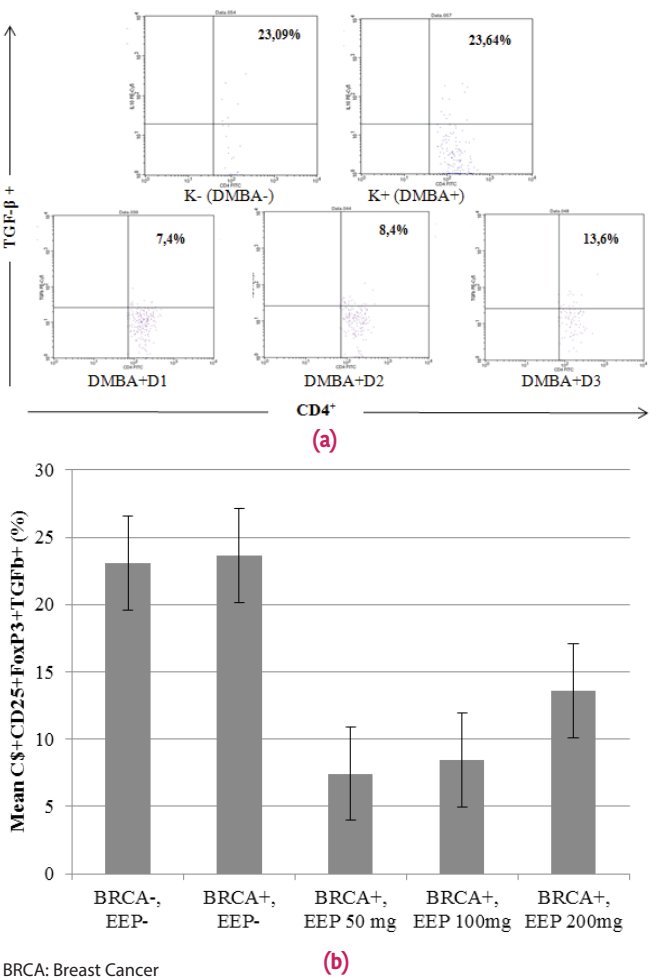

Figure 4. a, b. The percentage of CD4⁺CD25⁺FoxP3⁺ Regulatory T Cells Expressing TGF-β decrease in ethanolic extract of propolis (EEP) group compared to control. One-way ANOVA with LSD post hoc test showed that the treatment of propolis extract dose 50, 100, and 200 mg / kgBW had significantly different of relative number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells expressing TGF-β compare with positive control. Rats were terminated and analyzed using flow cytometry (a) and tabulated into Microsoft Excel (b). Data are mean ± standard deviation values of six rats in each group with p-value <0.05. Different letters indicate significant difference based on Tukey's high significant differences test at a 95% significance level.

kgBW (Figure 4). Linear regression analysis assessing the treatment effect of propolis extract on the relative number of CD4⁺CD25⁺FoxP3 regulatory T cells expressing TGF-β shows the significance value equal to 0.016, R-square of 0.144 and a regression coefficient of -2.8. This means that the treatment of propolis extract gives significant influence on the decline in the relative number of CD4⁺CD25⁺FoxP3 regulatory T cells expressing TGFβ. R-square value of 0.144 means the contribution of propolis extract treatment to decrease the relative number of CD4⁺CD25⁺FoxP3 regulatory T cells expressing TGF-β is 14.4% ; the regression coefficient of -2.8 indicates the opposite effect of the treatment on the relative number of CD4⁺CD25⁺FoxP3 regulatory T cells expressing TGFβ, where increasing doses of the treatment leads to a decrease in the relative number of CD4⁺CD25⁺FoxP3 regulatory T cells expressing TGF-β.

The decline in Treg cell populations expressing both IL-10 and TGF-β can be assumed to be a decrease in the expression activity of IL-10 and TGF-β by Treg cells (CD4⁺CD25⁺FoxP3⁺), not because of a decrease in T-cell

populations. This is based on data that the treatment of propolis extract does not decrease the population of Treg cells (Figure 2).

Discussion

The therapeutic effect of propolis extract could probably attributable from its active compounds. Our previous study showed that the propolis used in this study contains caffeic acid phenethyl ester (CAPE), quercetin, hesperidin and artepillinC.[14] Thus it is strongly suspected that these active compounds affect a decrease in the relative number of $CD4^+CD25^+FoxP3^+$ regulatory T cells expressing IL-10 and TGF-β. The result of this research is similar to a previous study where PBMCs and T cells from a healthy donor were induced to produce the cytokines IL-2, IL-4, IL-10, IL-12 and TGF-β1 with PWM stimulation (pokewees mitogen) and given various concentrations of propolis extract and the active compound (quercetin, hesperidin, CAPE). Cytokine production was measured by ELISA. Their results showed that propolis extracts or the active compounds were able to reduce cytokine production, namely IL-2, IL-4, IL-10, and IL-12, both in PBMCs and T cells, with a stronger decreasing effect on T cells.[20] These data indicate that propolis extract and quercetin, hesperidin, and CAPE have the effect of lowering the activity of Treg cells in which IL-10 is one of the immunosuppressive cytokine produced by Treg cells.[6] Other studies have reported that the decrease in IL-10 also occurs in the treatment with propolis extract and the active compound; quercetin and hesperidin.[21] There is also a study that has found that another active compound in propolis, cinnamic acid, also has immunomodulating activity, affecting the expression of TLR2, TLR4, HLA-DR, CD80, TNF-α and IL-10.[22]

In the case of cancer, a decrease of IL-10 and TGF-β is immunologically beneficial, because IL-10 is an immunosuppressive cytokine synthesized by Treg cells. IL-10 affects mechanisms related to activation of the immune system, such as inhibiting the function of dendritic cells by suppressing the production of inflammatory cytokines, inhibits MHC II, and expression of co-stimulatory molecules.[6,23] Some of this immunosuppressive activity supports the role of IL-10 as an immunosuppressive cytokine that supports the development of cancer.

TGF-β can suppress the systemic immune system, inhibiting immunosurveillance and can affect immune cell populations.[7] TGF-β also inhibits the activity of CTL, $CD4^+$ T cells, macrophages, dendritic cells, and natural killer cells that have an important role in tumor progression, and also encourages the formation of Treg cells and Th17 cells.[8] TGF-β lowered cytolytic and pro-apoptotic transcription factors such as Granzyme A and B, perforin, interferon-γ and FAS ligand at either the gene or protein level, and also inhibits the function of APC (antigen presenting cells), which causes a decrease in T cell activation.[24] Our findings show the potential of propolis to be further developed as an immunomodulatory agent that can be useful for cancer patients. These results are important in order to understand how propolis acts on immunity and shows a dual effect on lymphocyte activation, and does not affect lymphocyte proliferation and decreasing IL-10 and TGF-β production. Further research is needed to investigate the effect of propolis on the immune system for a better understanding of its mechanism of action.

Peer-review
Externally peer-reviewed.

Conflict of Interest
No conflict of interest was declared by the authors.

Acknowledgements
We would like to thank Directorate General of Higher Education, Ministry technology research and higher education of Republic Indonesia for providing grant for this research.

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