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EXPRESSION OF IL-6 ON BREAST CANCER MICE TREATED BY COMBINATION OF *Phyllanthus urinaria* AND *Catharanthus roseus* EXTRACT

EKSPRESI IL-6 PADA MENCIT MODEL KANKER PAYUDARA DENGAN PEMBERIAN KOMBINASI EKSTRAK Phyllanthus urinaria DAN Catharanthus roseus

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ABSTRACT

Breast cancer is one kind of cancer that can cause death in women. Although many types of treatments have been done to suppress the growth of breast cancer cases, the number of issues still increased. An increase in the relative amount of IL-6 indicates a poor prognosis for breast cancer mice. Chemotherapy is one kind of treatment used to suppress breast cancer cases. But it is known that it can give many poor effects. So it needs to get an alternative treatment such as using a herbal plant as medicine like Phyllanthus urinaria and Catharanthus roseus which are both of them known to have several potential contain as anticancer such as alkaloid, antiviral, anti-inflammatory, and antioxidant. This research aimed to study the effect of P. urinaria and C. roseus on IL-6 expressed by CD11b in a breast cancer model. This research using RAL was divided into six groups namely normal group (N), cancer group (K), cisplatin treatment (C), dose 1 (D1) (P. urinaria 500 mg/kg weight and C. roseus 15 mg/kg weight), dose 2 (D2) (P. urinaria 1000 mg/kg weight and C. roseus 75 mg/kg weight), and dose 3 (D3) (P. urinaria 2000 mg/kg weight and C. roseus 375 mg/kg weight). The mice were injected with DMBA in 0.015 mg/kg weight for six weeks. The combination of P. urinaria and C. roseus was administrated orally for two weeks. The expression of IL-6 expressed by CD11b was analysed by flow cytometry. Data were analysed by SPSS in one-way ANOVA and Tukey HSD test. The result showed that combination can suppress the expression of IL-6 which down-regulation of IL-6 indicated a good prognosis for the breast cancer mice. It showed that giving combination extract orally to breast cancer mice can suppress IL-6 expression in 33.80% to 9.97% in dose 3 treatment. Based on the result, it showed that the combination can use as an immunomodulatory agent in humoral immunity through the IL-6 regulation.

Keywords: *breast cancer, Catharanthus roseus, IL-6, macrophage, Phyllanthus urinaria*

ABSTRAK

Kanker payudara merupakan salah satu jenis kanker penyebab kematian pada wanita. Meskipun telah banyak dilakukan pengobatan untuk menekan pertumbuhan kasus kanker payudara namun diketahui bahwa jumlah kasus kanker payudara masih meningkat. Diketahui bahwa peningkatan jumlah relative IL-6 menunjukkan prognosis yang buruk terhadap mencit kanker payudara. Kemoterapi merupakan salah satu pengobatan yang digunakan untuk menekan peningkatan kasus kanker payudara. Namun, kemoterapi diketahui memiliki dampak buruk terhadap penderita kanker payudara. Sehingga dibutuhkan pengobatan alternative seperti pemanfaatan tanaman yang berpotensi sebagai agen antikanker seperti tanaman Phyllanthus urinaria dan Catharanthus roseus. Tujuan penelitian ini yaitu untuk megetahui pengaruh Phyllanthus urinaria dan Catharanthus roseus terhadap ekspresi IL-6 dari CD11b pada mencit model kanker payudara. Rancangan penelitian menggunakan rancangan acak lengkap (RAL) yang dibagi menjadi enam kelompok perlakuan yaitu kelompok Normal (N), kelompok kanker (K), kelompok kanker dengan perlakuan cisplatin (C), Dosis 1 (D1) (Phyllanthus urinaria 500 mg/kg BB dan Catharanthus roseus 15 mg/kg BB), dosis 2 (D2) (Phyllanthus urinaria 1000 mg/kg BB dan Catharanthus roseus 75 mg/kg BB) and dosis 3 (D3) (Phyllanthus urinaria 2000 mg/kg BB dan Catharanthus roseus 375 mg/kg BB). Mencit diinjeksikan dengan DMBA sebanyak 0,015 mg/kg BB enam kali selama enam minggu. Pemberian kombinasi ekstrak pada mencit dilakukan dengan metode sonde setiap hari selama dua minggu. Ekspresi sitokin IL-6 yang diekspresikan oleh CD11b dianalisis dengan flow cytometry. Data dianalisis dengan SPSS untuk uji one-way ANOVA dan uji Tukey HSD. Berdasarkan hasil penelitian diketahui bahwa kombinasi ekstrak mampu menekan ekspresi IL-6 pada mencit model kanker payudara dengan jumlah relative IL-6 33,80% menjadi 9,97% pada mencit pemberian ekstrak dosis 3. Sehingga berdasarkan hal tersebut, diketahui bahwa kombinasi ekstrak P. urinaria dan C. roseus berpotensi sebagai agen immunomodulator dalam imunitas humoral melalui regulasi sitokin IL-6.

Kata kunci: *Catharanthus roseus, IL-6, kanker payudara, makrofag, Phyllanthus urinaria*

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INTRODUCTION

Breast cancer (BC) is one type of cancer that has been considered the leading cause of cancer-related death in women globally [1]. Breast cancer is common, with 1.7 million cases worldwide. In 2012, it was known that BC is the leading cause of death due to cancer, the number of cases for 521,900 cases in women [2]. Cancer is a disease characterized by abnormal cell growth that continues to increase, spreads to various tissues, and causes death [3]. Most cancer deaths are known to be due to a link to metastasis mediated by many cytokines [4].

Breast cancer treatment is divided into two treatment types: locally and systemically. Local therapy is therapy such as radiation, surgery, and photodynamics. While systemic therapy is chemotherapy both hormonally and biologically [5]. Although many cancer therapies are available, cancer patients who develop metastasis are still found in large numbers. Metastasis is spreading cancer cells from the main organs to other organs through lymphatic and vascular pathways through various molecules in the body. The molecule can be used as a target in breast cancer treatment through immunotherapy, such as the interaction between the receptor of PD-1 and its ligand (PD-L1), which allows it to trigger apoptosis in cancer cells. IL-6 can be one of the target molecules in breast cancer [6].

IL-6 is a cytokine produced by activated B cells, T cells, fibroblasts, active macrophage cells, and monocytes. IL-6 is a pleiotropic cytokine that has several functions. IL-6 is known to be a proinflammatory cytokine that has an important effect on adaptive immunity, especially after the activation of T cells and B cells [7]. IL 6 is one of the important cytokines in the inflammatory process of hypoxia conditions. Tumor cells can also express IL6 as one of the triggers for inflammatory factors. IL-6 is a cytokine produced by tumor cells and immune cells such as $CD4^+$ [8].

Chemotherapy treatment has many negative impacts on breast cancer patients, so safer alternative treatment is needed. In recent years, several studies have shown anticancer effects on melanoma, prostate cancer cells, breast cancer, osteosarcoma, and prostate cancer cells [5]. *Phyllanthus urinaria* is one of the traditional medicinal plants known to have various biological properties and potential as drugs such as antivirals, immunoregulation, antibacterial, and anti-inflammatory [9, 10]. *Catharanthus roseus* is a traditional Indian and Chinese plant whose extract can be used to fight various diseases such as Hodgkin's disease, malaria, and diabetes. *Catharanthus roseus* has also been reported to contain more than 70 chemical compounds such as alkaloids indole, ajmalicine, serpentine, and reserpine [12]. In addition, *C. roseus* also includes vincristine, and vinblastine is also known to have anticancer properties [11]. Both plants have the role of anticancer, so it is expected to be able to suppress IL-6 cytokines expressed by CD11b. This research aimed to study the effect of *P. urinaria* and *C. roseus* on IL-6 expressed by CD11b.

METHODS

Experimental design. Twenty-four female mice (*Mus musculus*) BALB/c strain were used as an experiment with the age of 6-7 weeks. The mice got from Gadjah Mada University, Yogyakarta, Indonesia. The group of mice was divided into six groups as normal group as N, cancer group as K, Cisplatin treatment as C, and the cancer group treated with the combination of *P. urinaria* and *C. roseus* in three different doses are dose 1 as D1 (*P. urinaria* 500 mg/kg weight and *C. roseus* 15 mg/kg weight), dose 2 as D2 (*P. urinaria* 1000 mg/kg weight and *C. roseus* 75 mg/kg weight) and dose 3 as D3 (*P. urinaria* 2000 mg/kg weight and *C. roseus* 375 mg/kg weight). Every group in the experiment consist of four replications. This experiment has been approved by ethical clearance by the Ethical Committee of Brawijaya University (Reg. No. 125-KEP-UB-2021).

Induction of DMBA. 7,12-Dimethylbenz(α)anthracene (DMBA) was induced in every mouse except the normal group with 1.5 mg/kg weight. The dose was induced in the mice using a conversion of the FDA table. Corn oil using to dissolve DMBA. The mice were subcutaneously injected with DMBA as their weight every week for six weeks.

Oral administration of combination extract and cisplatin. The combination extract that was administrated to the mice was dissolved in water. The combined extract was administered orally to breast cancer mice based on weight. Whereas cisplatin was injected into the mice intraperitoneally 5 mg/kg weight dose. Both of them were done in two weeks every two days.

Spleen isolation. After 14 days of the cheral treatment, spleen isolation was carried out after mice were sacrificed by the cervical dislocation technique. The spleen was isolated and washed with phosphate-buffered saline (PBS) and mashed. The homogenate was moved to a polypropylene tube 15 mL and added with the PBS until 10 mL volume. After that, 10 mL homogenate was centrifuged on 2500 rpm at 10ºC for 5 minutes. The pellet was then suspended with 1 mL PBS and prepared for antibody staining.

Antibody staining and flow cytometry analysis. Antibody staining was done by two kinds

of staining, namely membrane staining, and intracellular staining. Membrane staining used FITC anti-mouse $CD4^+$, and intracellular staining used anti-IL-17. Extracellular staining was done early than intracellular staining to know the specific location of the cytokine source. Extracellular staining was done in the sample by adding 50 µL extracellular antibody FITC anti-mouse $CD4^+$. Then the sample was incubated for 20 minutes in an icebox (4°C). Intracellular staining was carried out by adding 50 µL Cytofix (BioLegend, USA) and incubated for 20 minutes in an icebox. The sample was added 400 µL Wash Perm Solution (WPS) and centrifuged on 2500 rpm at 10°C for 5 minutes. Then, the supernatant was discarded, and the antibody intracellular of anti-IL-17 was added to the pellet and incubated for 20 minutes in the icebox. Then, 400-500µL PBS was added, and the sample was entered into a cuvette for flow cytometry analysis.

Data analysis. Data were analysed using a one-way ANOVA test with a 95% confidence level in the IBM SPSS Statistics 26. A further test was carried out with the Tukey HSD test to confirm the differences between treatments statistically.

RESULTS AND DISCUSSION

Expression of $IL\text{-}6^+$ expressed CD11b on breast cancer mice treated by *Phyllanthus urinaria* and *Catharanthus roseus*. Based on the results of flow cytometry analysis (Figure 1), it is known that the relative amount of $CD11b^+IL\text{-}6^+$ in each treatment decreased significantly ($p<0.05$) when compared to cancer treatment. Where the amount is in the treatment of healthy control, cancer, cisplatin, dose 1, dose 2, and dose 3 in a row are 5.47%, 33.80%, 19.15%, 16.33%, 13.22%, and 9.97%. Mice with cancer control treatment increased the relative amount of IL-6 expressed by CD11b significantly ($p<0.05$) when compared to normal control treatments. The data showed that breast cancer mice have high expression of IL-6. Interleukin–6 (IL-6) is one of the pleiotropic cytokines that have multifunctional roles such as immune response, non-immune cell proliferation, inflammation, inducing acute phase protein synthesis, hematopoiesis, and cellular metabolism [13]. Several studies have shown a dual role of IL-6 in both inducing adaptive immunity and promoting tumor growth in the tumor microenvironment [14]. A study has reported that overexpression of IL-6 is found in some cancers, including breast cancer cases [15]. Increasing levels of IL-6 are known to be a poor prognosis and indicate low survival rates in breast cancer patients [16]. In addition, more than 50% of cases of breast cancer showed activation of STAT3, which IL-6 is its main activator [15]. Some types of cells which have been considered the main sources of IL-6 growth triggers in the microtumor environment are cancer cells, helper T cells, fibroblasts, tumor-associated macrophages (TAMs), and myeloid-derived suppressive cells (MDSCs) [17].

The results showed that cisplatin treatment could decrease IL-6 expression significantly compared to cancer control, from 33.80% to 19.15%. However, it can be known that the decrease in cisplatin treatment is not better than the decrease in other doses. The decrease in IL-6 levels can be suspected due to the influence of the provision of the therapy drug cisplatin. Cisplatin is one type of therapeutic drug that is often used in chemotherapy. Cisplatin will provide its anti-tumor activity by covalently binding to DNA to form an adduct so that it will trigger apoptosis [18]. Once entered the bloodstream, cisplatin will show high affinity in the sulfhydryl group (protein) and nitrogen donor atoms (nucleic acids). It will form an adduct causing aquation, which can form a strong electrophile [19], although many repair DNA mechanism has been done. Still, sometimes the cell can not do apoptosis caused of residual RNA, DNA, and protein [20].

Based on the result showed that every dose in this research can suppress the expression of IL-6 expressed by CD11b. But we can conclude that dose 3 is the optimal dose that can suppress the growth of IL-6 expressed by CD11b. Dose 3 is the highest dosage treatment in this research. The suppression of cytokine IL-6 expressed by CD11b in dose 1, and dose 2 is not better than dose 3 in suppression caused by the effect of *P. urinaria* and *C. roseus* compound. The experimental and epidemiological study showed that herbal plants have a big potential in managing the growth of cancer such as breast, prostate, colon, ovarian carcinomas, and liver. Based on the plant's phytochemistry, the plant has the potential to suppress the growth of any cancer cell in vivo and in vitro [21]. *Phyllanthus urinaria* is a plant that has the potential as an anticancer. Research has shown that *P. urinaria* is a plant that can suppress cancer cells by inducing apoptosis. A study by Geethangili & Ding [22] showed that *P. urinaria* can suppress any cancer cell without affecting normal cells. Extraction of *P. urinaria* using water or methanol showed the ability to suppress the metastasis, proliferation, and angiogenesis in human melanoma cancer (MeWo) through MAPKs, NFkB, Myc/Mac, and hypoxia. *Phyllanthus urinaria* has proven to suppress the metastasis of A549 cells by suppressing the invasion and migration of A549 cells by ERK1/2 and the signaling pathway of hypoxia [23].

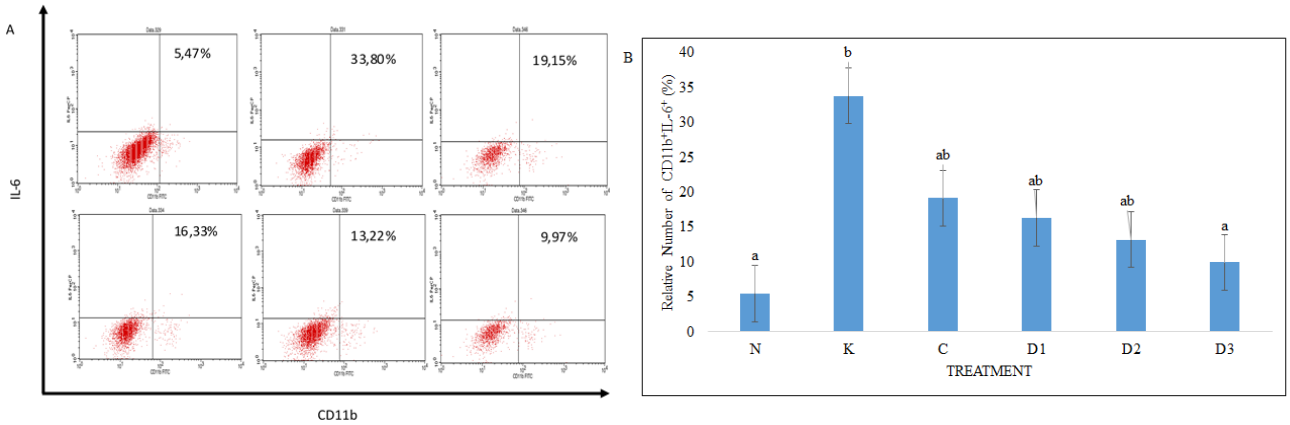

Figure 1. Relative number of $CD11b^{+}IL\text{-}6^{+}$ (%) in mice with a wide variety of treatments. N = Healthy control (Normal); K = DMBA injection (cancer control); C = DMBA and Cisplatin injected mice treatment; D1 (dose 1) = DMBA injection and 500 mg/kg weight *P. urinaria* and 15 mg/kg weight *C. roseus*; D2 = injection of DMBA and 1000 mg/kg weight *P. urinaria* and 75 mg/kg weight *C. roseus*; D3 = injection of DMBA and 2000 mg/kg weight *P. urinaria* and 375 mg/kg weight *C. roseus*, line on bar = standard deviation. A. Results of T Cell flow cytometry analysis (%); B. $CD11b^{+}IL\text{-}6^{+}$ (%) graph results.

Inflammatory cytokines have a role in tumor initiation, and invasive growth will stimulate cell proliferation and reduce apoptosis [24]. During chronic inflammatory conditions, inflammatory cytokines such as IL-6 will cause DNA damage that can result in tumor initiation. So that this statement is in line with the results of research that has been done, when mice have breast cancer, the relative amount of IL-6 expressed by macrophages increases when compared to the relative amount in healthy control mice.

In addition to playing a role in proinflammatory cytokines as a cause of increased proliferation of tumor cells, IL-6 also has a role in increasing angiogenesis [25]. Angiogenesis caused chronic inflammation in cancer mice which affected tumor-infiltrating lymphocytes (TILS) to produce cytokine proinflammatory excessively like IL-6, IL-1α, IL-1β, and TNF-α. This condition could enhance the COX-2 expression in the body. So that it could increase the result of vascular endothelial growth factor (VEGF) expression as well [24].

IL-6 is one kind of cytokine proinflammatory that can be found in any cell in the tumor microenvironment, including cancer. IL-6 has a main role in expansion and differentiation in tumor cells [15]. The increasing number of IL-6 found in serum and tumor located has shown in many cases of cancer, including breast cancer [26]. Increasing the level of IL-6 showed a poor prognosis and low life in breast cancer patients, and decreasing the level of IL-6 showed a good prognosis in treating breast cancer [27]. The influence of IL-6 can affect all aspects of tumorigenesis by managing proliferation, metabolism, angiogenesis, apoptosis, and metastasis [26]. IL-6 can also modulate the resistance in the treatment process, such as in multidrug resistance (MDR) [16]. This research is in line with previous research [15] that the increase in IL-6 level showed a poor prognosis, in this research, the increase of IL-6 is shown in cancer conditions.

CONCLUSION

The result showed that *P. urinaria* and *C. roseus* could suppress the expression of IL-6 expressed by CD11b in any dose. Combining 2000 mg/kg weight of *P. urinaria* and 375 mg/kg weight of *C. roseus* (Dose 3) could suppress IL-6 expression. It showed that in breast cancer, the number of IL-6 expressions was 33.80%, and the oral administration of *P. urinaria* and *C. roseus* combination could suppress until 9.97%, which is a number relatively with normal conditions.

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