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Immune-Enhancing Effect of Ethanol Extract of Pegagan Herb (*Centella Asiatica* Urban) on Rat

(Efek Peningkat Respon Imun dari Ekstrak Etanol Pegagan (*Centella Asiatica* Urban) pada Tikus)

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Abstract: This research had been conducted to verify the activity of ethanolic extract of Pegagan or *Centella asiatica* Urban (CA) from Indonesia as immune enhancing with the activity and capacity of peritoneal macrophages as parameters tested. *Sprague Dawley* male rats were divided into 4 groups of 5 rats each. Dose 1, dose 2 and dose 3 groups were treated with extract at the dose of 10, 20 and 40 mg/200 g b.w. p.o. for 15 consecutive days, respectively, and the control group was supplemented with carrier. One day after the last treatment, all rats were sacrificed after induced by Staphylococcus epidermidis (10^9 CFU per rat) i.p. and then the activity and capacity of peritoneal macrophages were examined with giemsa staining microscopically. The results showed that the immune-enhancing effect of the extract was a dose dependent manner. Based on statistical analysis (ANOVA, $p < 0.05$), the administration of CA extract at the dose of 40 mg/200 g bw demonstrated the highest result and differed from control group significantly (96% of macrophage activity and 61 bacteria of macrophage capacity). From these experiments could be concluded that the ethanolic extract of CA had potential to be developed as immune-enhancing agent. However, it was still needed to conduct further study for elaborating the mechanism of immune enhancer completely.

Keywords: activity and capacity macrophage, *Centella asiatica* Urban, *Staphylococcus epidermidis,* rat.

Abstrak: Penelitian ini bertujuan memferifikasi aktivitas ekstrak etanol pegagan atau *Centella asiatica* Urban (CA) local sebagai peningkat imunitas tubuh dengan parameter uji kapasitas dan aktivitas fagositosis makrofag peritoneum secara in vivo. Tikus jantan galur *Sprague Dawley* dibagi menjadi 4 kelompok, masing-masing terdiri dari 5 ekor. Kelompok dosis 1, dosis 2 dan dosis 3 diberi ekstrak 10, 20 dan 40 mg/200 g BB. secara oral selama 15 hari berturut-turut, dan kelompok kontrol mendapat pembawa. Satu hari setelah pemberian ekstrak yang terakhir, semua hewan coba dibunuh setelah diinduksi dengan bakteri *Staphylococcus epidermidis* (10^9 CFU per ekor) i.p. Aktivitas makrofag dan kapasitas makrofag peritoneum dihitung dengan dengan pewarnaan giemsa menggunakan mikroskop. Hasil menunjukkan bahwa terdapat korelasi positif antara peningkatan dosis dan aktivitas maupun kapasitas makrofag. Nilai aktivitas dan kapsitas makrofag pada kelompok yang mendapat ekstrak 40 mg/200 g BB secara statistik berbeda secara bermakna dibanding kelompok control (96% aktivitas makrofag dan 61 bakteri untuk kapasitas makrofag). Berdasarkan penelitian ini dapat ditarik kesimpulan bahwa ekstrak etanol CA berpotensi untuk dikembangkan sebagai bahan peningkat daya imunitas tubuh. Namun demikian, masih perlu dilakukan pengujian lebih lanjut untuk mengelaborasi kemampuan peningkat system imun secara lengkap dan detail.

Kata kunci: aktivitas dan kapasitas makrofag, *Centella asiatica* Urban, *Staphylococcus epidermidis*, tikus.

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INTRODUCTION

INGREDIENTS of plant origin have long been used by Indonesian for disease treatment. Some plants are believed to promote health and to protect body against infectious agents by maintaining body equilibrium. It has been believed that the protective and restorative properties of these plants are concerned with their role in enhancing immune response. The concept of protective is started from external body (physical and mechanical protection) such as skin, mucosa membrane, cilia respiratory tract, coughing and sneezing. Further, the cellular (granulocyte, macrophage, complement system, natural killer and monocyte) and molecular (antibody) functions will be activated after infectious agents enter into the body[1].

Macrophages are one type of white blood cell that engulfs foreign materials entering the body. They are the first agents that against invader materials. Monocytes are the previous state of macrophage cells that circulate in the blood only. When a foreign material invades to the body, monocyte cells migrate immediately to the body tissue and differentiate into macrophage. Macrophages easily move either from blood circulation to tissue or between tissues. Besides ingesting, macrophages also carry antigens into their surface and present it to T and B cells known as antigen-presenting cell (APC). The other way to stimulate the immune system, macrophage will release chemical substances, known as cytokines, which attract other immune cells to destroy antigens[2,3].

Recently, the interenst in herbal medicine (natural product) is increasing not only in Indonesia but also in various regions of the world in which it is in line with the development of (back to nature) culture. One of well-known medicinal plants is *Centella asiatica* Urban (CA) or pegagan. CA that belong to the Umbeliferae, has long beenused for treatment of disease[4]. Some research had stated that CA showed effectivity for leprosy, lumps, syphilis and TBC treatments[5]. Patil and team also reported that dried powder of CA at dose of 100 mg/kg bw in rat gave a positive respond in increasing of antibody titer and cell-mediated respond at[6]. It was also claimed that the aqueous extract of whole CA plant produced an effect on classical and alternative human complement systems in an in vitro model[1].

Basically, the pharmacological effect of plant is due to the chemical compound containing in it. The former research had found some chemical compounds of CA such as asiatic acid, asiaticoside, madecassice acid and madecassoside. Either as a single compound or a whole extract, scientists had proven their pharmacological and biological activities[7,8]. The quality and quantity of the compounds are affected by the location of planting. Hence, the present studies were, therefore, aimed to assess the potency of CA collected from Indonesia as immune-enhancing agent in vivo with macrophage capacity and activity as parameters.

MATERIALS AND METHODS

MATERIALS. *Centella asiatica* (collected from Balitro Bogor), object and cover glass, sodium carboximethyl cellulose (CMC-Na) (Sigma), Giemsa (Sigma), paraffin, disodium hydrogen phosphate, potasium hydrogen phosphate, Brain Heart Infusion (BHI) culture media, NaCl 0.9%, sulphuric acid, hydrochloric acid, ethanol, methanol, Entellan®.

Animals. *Sprague dawley* rats (2-2.5 months) 110-140 g body weight (purchased from BPOM-The Ministry of Health- -RI) were housed in polycarbonate cages. Each cage was contained 2-3 rats with bedding of husk and 12 dark/light cycles were provided. Feed and water were given ad libitum. The environmental condition was maintained at a temperature of 210 ± 2 ^{o}C and a humidity of 30-70%. Before being used, rats were acclimatized to laboratory condition for seven days prior to initiate of dosing

METHODS. Preparing test sampel. *Centella asiatica* dry powder was macerated with distillated ethanol at room temperature with agitation for 24 hours. The filtrate was collected and dried under vacuum using vacuum rotavapour machine until semisolid mass was obtained. The extract was stored in the clean dark bottle and ready to be used. The extract was resuspended in CMC-Na 0.5% solution prior to administration into rats.

Preparing *Staphylococcus epidermidis* suspension [9]. Bacteria preparation was based on Aly R. *et al.* with a little modification. Briefly, from the stock culture, the bacteria, one ose, was cultured in a sterile BHI media and incubated for 18-24 hours at 37 ^{o}C temperature. The pure bacteria was collected by centrifugation at 5000 rpm for 15 minutes and then washed with NaCl 0.9% solution three times. Finally, the bacteria sediment was suspended and diluted with sterile NaCl 0.9% solution until a transmitant value of 10% (≈ 10^{9}CFU/ml) was observed.

Animal Treatment[10]. Twenty female rats were randomly separated into 4 groups of 5 rats each group. Individual rat was tail marked using ink marker. Group D1, D2 and D3 were treated with 10, 20 and 40 mg/200 g bw, respectively, while control group was given carrier, a CMC-Na 0.5% solution. Extract was gavaged orally everyday for 15 consecutive days using ball-tipped intubation needle fitted onto syringe. One day after the last treatment, without fasting, each rat was inducted by suspension of *Staphylococcus epidermidis* (10^{9} CFU per rat) intraperitoneally and followed by free access of food and tape water. Two hours next, all of rats were

sacrificed by dislocation technique and the peritoneal fluid was collected using a 1mL Terumo® syringe without needle.

Peritoneal fluid smear preparation were made by putting a drop of peritoneal fluid on an object glass, smearing with another object glass, then fixating in methanol for 10 minutes and drying at room temperature. These slides were processed with giemsa stain and observed using conventional light microscope (Olympus microscope®) with 100x magnification. The number of capacity and activity of macrophages were calculated from 4-5 random insights and repeated for 3 times. Active macrophage is characterized by the formation of "pseudo feet" like a pink-coloring thin membrane surrounding it with blue-coloring small bullets (*Staphylococcus epidermidis*) in it. Non active macrophage was a blue-coloring bullet (Figure 1).

The macrophage activity is the number of active macrophages per 100 macrophages, stated in % and calculated by equation: Macrophage activity = (the number of active macrophages out of 100 macrophages/ 100) x 100%.

The macrophage capacity is the average number of bacteria that are ingested by each macrophage, as many as of the 25 active macrophages are accounted for and counted with this equation: Macrophage capacity = the total number of bacteria of 25 active macrophages /25.

Statistical analysis. Data were repesented as means±SEM and analized statistically with ANOVA method for parametric or Kruskall Wallis method for non parametric followed by Duncan method for determinating value difference between groups using SPPS 15 program. $\alpha < 0.05$ was considered statistically significant.

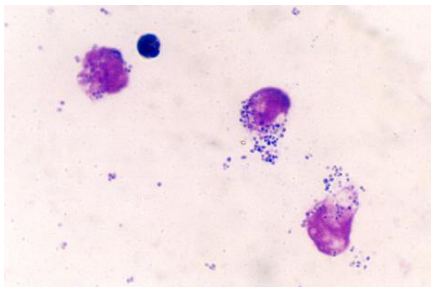

Figure 1. Photo of active (A) and non-active (B) peritoneal macrophages, (C) *Staphylococcus epidermidis* with giemsa staining under 100x magnification microscopically.

RESULTS AND DISCUSSION

The efforts of finding immune-enhancing agents from plant sources increased significantly. These agents gave benefits as adjuvant for cancer treatment and also for the prevention and management of infection caused

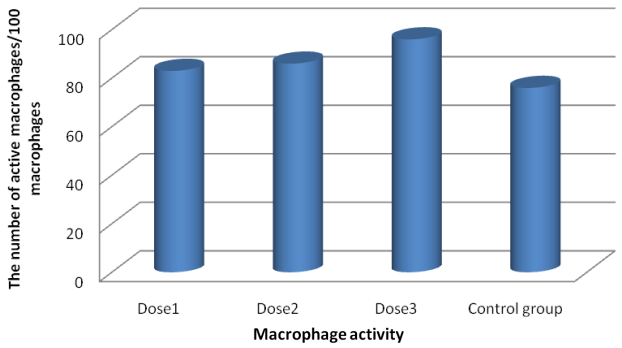

Figure 2. Diagram of macrophage activity from each group. Dose 1, Dose 2, and Dose 3 groups were treated with CA ethanolic extract at dose of 10, 20 and 40 mg/200 g bw, respectively, while control group was given carrier, a CMC-Na 0.5% solution. Extract was gavaged p.o. everyday for 15 consecutive days. Macrophage activity was counted as (The number of active macrophages of 100 macrophages)/100 x 100%. Values were means from 5 rats each group. *: differ from control group ($\alpha < 0.05$).

by virus, bacteria and foreign materials. Asiaticoside acid from CA has been known having biological activities and become a therapeutically active marker compound[7]. The ethanol extract of CA was tested at this present study to determine its effective dosage as immune-enhancing agent. The result showed that there was a positive correlation between regiment dosages and macrophage activity. The average numbers of macrophage activities were 83% of dose 1 group, 86% of dose 2 group and 96% of dose 3 group, respectively, while the control group was 76% (Figure 2). When the statistical analyses (ANOVA and DUNCAN, with $p < 0.05$) were applied to these groups, a significant result was observed between dosage and control groups. The macrophage activities of the two highest dosages (dose 2 and dose 3) were different significantly from the control group while the lowest dosage (dose 1) did not show difference significantly.

The same result was also showed on the observation of the value of macrophage capacity. All of the dosages demonstrated a tendency of augmentation of the macrophage capacity. The higher the dosage, the more the bacteria could be ingested. The macrophage capacities of each dosage were 47 (dose 1), 53 (dose 2), 61 (dose 3), while the control group was 45 bacteria per macrophage (Figure 3). However, statistically, CA extract just at dose of 40 mg/200 bw could increase the macrophage capacity that differed from control group significantly.

Peritoneal macrophage is one kind of non-specific cellular immune response against bacteria (body foreign substances). The mechanisms of bacteria destruction known as macrophage phagocytosis are started with bacteria attachment to macrophage surface receptor, C3b. Using the long elongated membrane, called pseudopodia, bacteria were engulfed into

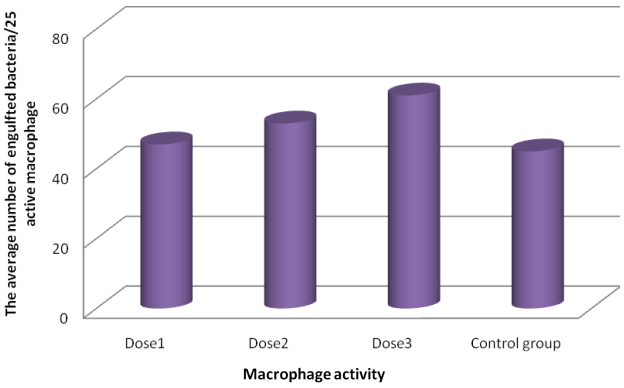

Figure 2. Diagram of the average number of macrophage capacity from each group. Dose 1, Dose 2, and Dose 3 were treated with CA ethanol extract at dose of 10, 20 and 40 mg/200 g bw, respectively, while control group was given carrier, a CMC-Na 0.5% solution. Extract was gavaged orally everyday for 15 consecutive days. Macrophage capacity was determined as (the total number of bacteria in 25 active macrophages)/25. Values were means from 5 rats each group. *: differ from control group ($\alpha < 0.05$).

phagosome and it will fuse with the lysosome to become phagolysosome. Bacteria are destroyed enzimatically by lysosomal enzyme and eliminated from the macrophage[11]. These experimental results in agreement with the claim by Mali RG and team. They found ethanol extract of CA could stimulate cell-mediated immune responses by increasing human-neutrofil phagocytic function at the concentration range of 25-100 mg/mL in vitro[12]. The effectivity of ethanol extract of CA on non-spesific cellular immune response showed that CA extract had immune-enhancing properties. Wang XS and team claimed that pectin compounds containing in the CA extract played an important role in immunostimulant activity in vitro[13]. Punturee K *et al*. had also confirmed that ethanol extract of CA decreased the production of IL2 and TNF-alpha. Over production of these agents is associated with various diseases such as cancer, infection and autoimmune diseases[4]. Finally, from this research could be drawn a conclusion that treatment with *Centella asiatica* ethanol extract collected from Indonesia at the dose of 40 mg/200 bw orally everyday for 15 consecutive days could increase the capacity and activity of rat peritoneal macrophages after challenged by *Staphylococcus epidermidis* compared with control group.

CONCLUSION

It was concluded that ethanol extract of *Centella asiatica* had a potential as an immune-enhancing agent at dose of 40 mg/200 g bw *in vivo*.

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