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Effectiveness of Andrographis paniculata Nanoparticle on The Expression of CD4⁺ and CD8⁺ of Rats Listeriosis

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Abstract

Listeria monocytogenes is a Gram-positive bacterium that causes listeriosis which is known as foodborne disease. L. monocytogenes infection will activate both innate and adaptive immune responses. Androghrapis paniculata has an effect as an immunostimulant. Nanocapsulation-chitosan can increase solubility and reduce the particle size making it easier for compounds to enter the cell. This research focuses on the utilization of nanoparticle A. paniculata as an immunomodulator against CD4⁺ and CD8⁺. The study design was an experimental study with post only control group trial. A total of 30 male Wistar rats were divided into 6 groups (Normal; K-; EAP (extract of A. paniculata); nEAP 100 (nanoparticles extract of A. paniculata); nEAP 200 and nEAP 400), with 5 rats in each group. All groups were injected with L. monocytogenes intravenous except the Normal Group. EAP 200 group was administered oral crude extract of A. paniculata at a dose of 200 mg/kg BW for 7 days. nEAP 100, nEAP 200 and nEAP 400 groups were administered oral Nanoparticle-Extract of A. paniculata at a doses of 100 mg/kg BW, 200 mg/kg BW, and 400 mg/kg BW respectively for 7 days. CD4⁺ and CD8⁺ cells were detected by isolating PBMC cells then analyzed using flow cytometry. Statistical analysis applied ANOVA. Both crude extract of EAP 200 and nanoparticles nEAP 400 significantly increase the number of CD4⁺ (P= 0.050) and CD8⁺ (P= 0.010). Crude and nanoparticles extract of A. paniculata increase the production of CD4⁺ and CD8⁺ cells in rats infected with L. monocytogenes. In addition, crude and nanoparticles extract of A. paniculata also has the potential as a therapy for intracellular bacterial infections (Listeriosis) by increasing the number of CD4⁺ and CD8⁺ cells.

1. INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium and commonly known as pathogenic bacteria that is transmitted through food. These bacteria can cause listeriosis, which may develop into a severe infection in animals and humans [1]. *L. monocytogenes* is resistant to disinfectants and can multiply at extreme temperatures. Listeriosis can cause dangerous infections in humans, especially in the elderly, pregnant women and immunodeficiency patients are at risk for developing meningitis, septicemia, neonatal death, and premature death [1], [2].

Infection of *Listeria monocytogenes* will induce an adaptive immune response through $CD4^+$ and $CD8^+$ cells that cause *L. monocytogenes* elimination and the formation of T cells memory [3]. MHC class I and II will efficiently synthesize antigenic peptides *L. monocytogenes* that are associated with $CD4^+$ or $CD8^+$ cell receptors [4]. Cassidy *et al.* (2023) reported that the brain of mice treated with antibiotics derived from *Listeria monocytogenes* infection showed an increase in the number of T lymphocytes, namely $CD8^+$ and $CD4^+$ [5]. The innate immune response is important for the early recognition and control of bacteria, while the adaptive immune response by $CD4^+$ and $CD8^+$ T cells serves for the elimination of bacteria and mediates long-term protection against re-infection with pathogens [6].

Listeriosis can be treated with antibiotics depending on the severity of the infection [7]. Antibiotics used for the treatment of listeriosis are ampicillin, penicillin, and cephalosporins [8]. However, long-term use of antibiotics will cause side effects in the body including liver and kidney damage [7]. In addition, the presence of resistance to antibiotics and the absence of prophylactic vaccines in hypervirulent of *L. monocytogenes* cause difficulty in controlling the infection [4]. Therefore, other alternatives are needed to treat *Listeria monocytogenes* infections, such as by using herbal plants.

Andrograhpis paniculata or sambiloto belongs to the *Acanthaceae* family and it is widely spread in South Asia and Southeast Asia [9]. *A. paniculata* has a potential as an antibacterial against Gram-positive and Gram-negative bacteria [10]. Crude extract of *A. paniculata* has a wide range of pharmacological activities, such as analgesic, anticancer, antidibetic, antimalarial, antimicrobial, antivenom, hepatoprotective, and cardioprotective [11]. In addition, *A. paniculata* also has anti-inflammatory and immunostimulatory effects [11], [12].

Andrographis paniculata has many therapeutic benefits such as anti-inflammatory, antioxidant, antiviral, and antibacterial activity [13]. However, it has limitations such as low water solubility, short lifetime and low permeability [13]. Nanoparticles can be used to develop therapies with special properties such as smaller size, limited plant availability, biocompatibility, and penetration capacity into cells [14].

The use of nanoencapsulation drugs can improve solubility as well as increase the rate of drug release at the targeted location [13]. The benefits contained in the plant are concerning to $CD4^+$ and $CD8^+$ ultimately led to this study. The purpose of this study was to determine the effect of giving crude and nanoparticle extract of *A. paniculata* as an immunostimulant to increase $CD4^+$ and $CD8^+$ T cells in rats infected with *Listeria monocytogenes*.

2. MATERIALS AND METHODS

Ethical Clearance

This was an experimental study with post only control group trial design. This research has obtained ethical approval from the Ethics Committee of the Faculty of Medicine, Diponegoro University. No. 055/EC-H/KEPK/FK-UNDIP/VI/2024.

Extract Preparation

Simplicia powder of *A. paniculata* obtained from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional, Tawangmangu, Central Java was extracted using 70% methanol solvent. 20 grams of simplicia was dissolved in 200 ml of methanol 70% for 1x24 hours at a temperature of 37°C. The rendemen is filtered using Whatman No.1 Filter Paper. Then, the filtrate is evaporated using a rotary evaporator at 50°C to a paste [15].

Nanoparticles-Chitosan Preparation

Nanoparticles were made by ionic gelation and modified by ultrasonification. 1% of chitosan dissolved into 1% of acetic acid (w/v). 0.15 grams of *A. paniculata* were diluted with 70% of methanol of 5 drops. The solution was mixed into chitosan, and then sodium triphosphate was dissolved into aquades up to 1.5% concentration. STPP was dripped into a solution of chitosan extract using a syringe and stirrer. 250 ml solution was reduced its size using a sonicator at a frequency of 20 kHz for 60 minutes (20°C). Furthermore, the mixed solution was centrifuged at a speed of 10,000 rpm for 10 minutes to separate nanoparticles (pellet) and supernatant [16].

Listeria monocytogenes (IV) Preparation

Isolate *L. monocytogenes ATCC* 7644 was taken as much as 1 ose and then streaked on a petri dish containing brain heart infusion agar medium then incubated at a temperature of 37° C for 24 hours [17]. Suspension of *L. monocytogenes* was made in PBS with a concentration of 5 *Mcfarland* equivalent to 10^9 which then infected to each rat as much as 0.5 mL intravenously.

Treatment Administration

A total of 30 Wistar rats weighing 200-300 g, aged 8-12 obtained from the University of Muhammadiyah, Semarang were acclimatized for 7 days. During adaptation and treatment, the rats were fed with a standard diet ad libitum. The rats were divided into six groups of 5 rats each group. All groups except the Normal group were infected with *L. monocytogenes* 0.5 cc intravenously. EAP 200 group was received *A. paniculata* crude extract at the dose of 200 mg/kg BW. The nanoparticle groups were administrated with *A. paniculata* Nanoparticles-extract at doses of 100 mg/kg BW (nEAP 100), 200 mg/kg BW (nEAP 200), and 400 mg/kg BW (nEAP 400). Respectively crude extract and nanoparticle extract at various doses was administrated 3 hours after the infection *L. monocytogenes* intravenous. After 7 days, all rats were euthanized. The blood was collected in an EDTA tube then prepared for PBMC isolation.

PBMC Cells Isolation and FCM

Animals were euthanized with chloroform, the blood was taken with microhematocrite. The blood sample was given the heparin so that the blood does not clot formation. Isolation of PBMC begins with a blood sample of 1 ml added PBS (1:1). Samples were taken and transferred to a tube that had been filled with Ficoll-Hipaque d=1.077 g/mL and centrifuged at 1600 rpm for 30 minutes (room temperature). PBMC ring was transferred to a 15ml *polypropylene* tube and washed with PBS and centrifuged at 2500 rpm for 5 minutes. Pellets were washed with 1ml PBS and re-centrifuged then the PBMC cells formed at the bottom of the tube. 50 µL of anti-CD4 and anti-CD8 solutions were added to each tube containing PBMC cell pellets, and then incubated in a dark room for 20 minutes (4°C). After incubation, PBS 400µL was added then transferred to the FCM cuvette for flow cytometry analysis pellets [18].

Data Analysis

Data analysis using One Way ANOVA followed by post-Hoc LSD test to determine the differences between treatments.

3. RESULTS and DISCUSSION

The study showed that the number of CD4⁺ was higher in the EAP 200, nEAP 200 and nEAP 400 treatment groups than in the negative control group (Figure 1).

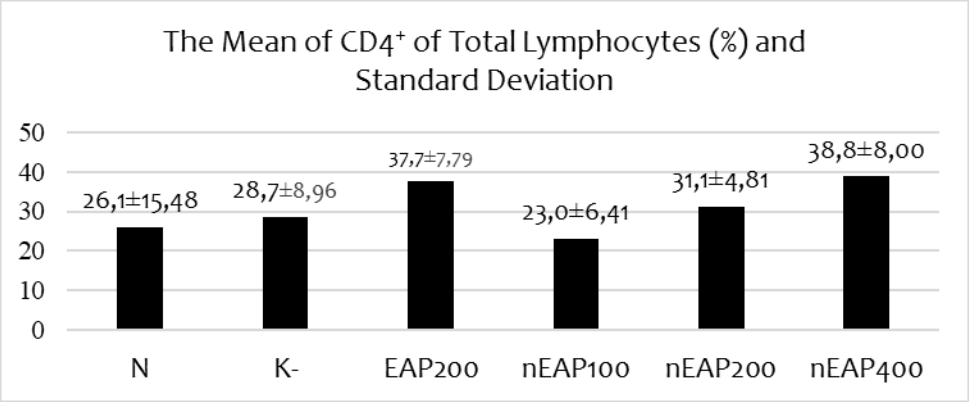

Figure 1. The mean of CD4⁺ T Lymphocyte of Each Group Results

Furthermore, the number of CD4+ was significantly higher in the EAP 200 and nEAP 400 treatment groups than in the Normal group (Figure 2). The number of CD4+ in the nEAP 200 group was higher but not significant (Figure 2). This means the administration of EAP 200 and nEAP 400 showed significantly higher number of CD4+ in the group of rats that were infected L. monocytogenes

	N	K-	EAP200	nEAP100	nEAP200	nEAP400
N		0.637	0.046*	0.588	0.365	0.029*
K-	0.637		0.116	0.315	0.660	0.078
EAP200	0.046*	0.116		0.014*	0.248	0.835
nEAP100	0.588	0.315	0.014*		0.154	0.008*
nEAP200	0.365	0.660	0.248	0.154		0.176
nEAP400	0.029*	0.078	0.835	0.008*	0.176	

Figure 2. The comparison of CD4⁺ T Lymphocyte of Each Group Results
Notes: (*) = P < 0.05; based on Post-Hoc LSD test.

The mean of CD8+ T lymphocytes of group EAP 200, nEAP 100, nEAP 200 and nEAP 400 was found to be higher compared to the negative control group (Figure 3). The number of CD8⁺ was significantly higher in the EAP 200 and nEAP 400 treatments compared to the Normal group (Figure 4). In addition, the number of CD8⁺ showed significantly higher in the EAP 200 and nEAP 400 compared to the negative control group. The number of CD8⁺ was not significantly higher in nEAP 100 and nEAP 200. This means that only EAP 200 and nEAP 400 showed a significantly higher of the number of CD8⁺ in the group of rats that were infected *L. monocytogenes*

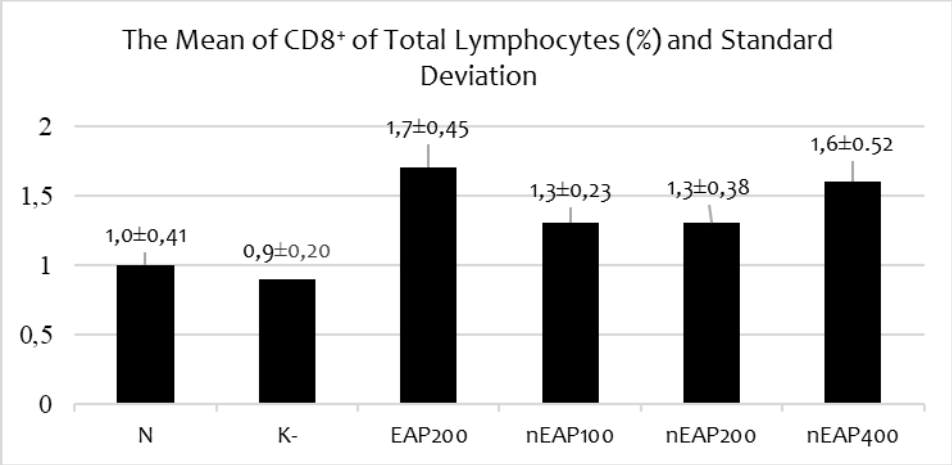

Figure 3. The mean of CD8⁺ T Lymphocyte of Each Group Results

	N	K-	EAP200	nEAP100	nEAP200	nEAP400
N		0.656	0.006**	0.162	0.144	0.014*
K-	0.656		0.002**	0.070	0.062	0.005**
EAP200	0.006**	0.002**		0.122	0.138	0.689
nEAP100	0.162	0.070	0.122		0.948	0.243
nEAP200	0.144	0.062	0.138	0.948		0.269
nEAP400	0.014*	0.005**	0.689	0.243	0.269	

Figure 4. The comparison of CD8⁺ T Lymphocyte of Each Group Results
Notes: (*) = P < 0.05; (**) = P < 0.01; based on Post-Hoc LSD

4. DISCUSSION

This study showed that the crude EAP 200 mg/BW and nanoparticles EAP 400 mg/BW increase the number of $CD4^+$. This means *A. paniculata* can stimulate $CD4^+$ proliferation and has the potential to enhance immunity. Rajanna et al. (2021) reported that the research in healthy adults who get EAP also increased the absolute number of $CD4^+$ T cells on Day 7 of EAP administration[19]. Jannat et al. (2021) declared the nanoparticle A. paniculata (AgNP) can prevent CD4 damage in HIV patients by inhibiting the binding between the HIV virus and CD4 [20]. The increase in the number of $CD4^+$ in the study could be due to *A. paniculata* which contains andrographolide compounds that provide immunostimulatory effects in the form of increasing the number of $CD4^+$ T cell proliferation [21].

The number of $CD8^+$ was also significantly higher after the administration of crude EAP 200 mg/BW and nanoparticle EAP 400 mg/BW. This means the compounds contained in *Andrographis paniculata* have the potential as immunomodulators by increasing the number of CD8+ T cells. The results are in accordance with the research of Wang et al. (2022) which reported that administration of andrographolide compounds contained in *A. paniculata* increased the number of $CD8^+$ as well as stimulating $CD8^+$ infiltration in mice lung tumors (NSCLC) [22]. The presence of exposure to antigens produced by *L. monocytogenes* triggers interactions between $CD4^+$ and $CD8^+$, $CD8^+$ will recognize antigens in the cytoplasm of infected cells and kill infected cells. $CD8^+$ is necessary for the regulation of pro-inflammatory cytokines and AIDS $CD4^+$ T

cell performance [23]. $CD8^+$ also secrete cytokine IFN-γ which will activate naive macrophages increasing in the process of phagocytosis in intracellular microorganisms [24]

The increase of the number of $CD4^+$ and $CD8^+$ did not differ significantly between the crude EAP 200 group and the nanoparticle group n. This may be caused by several factors, one of which is the bias due to the addition of 1% chitosan in the EAP 200 group treatment because chitosan itself is an immunomodulator and this is a weakness in this research. Wang et al., (2020) stated chitosan and its derivatives have immunomodulatory effects and are used as stimulators on the immune response to enhance immunity [25]. As for the nanoparticle extract Group *A. paniculata* the polymer chitosan already binds to STPP so that chitosan has no mass and is neutral [26]

This study suggests that crude EAP and nanoparticle EAP can be used as supporting therapy in patients with immunodeficiency by triggering $CD4^+$ proliferation for instance in HIV patient, however, this requires further HIV research. Crude EAP and nanoparticle EAP also have a potential as therapy in intracellular bacterial infections (Listeriosis) since they increase the number of $CD8^+$. It is necessary to conduct further research related to histopathology and toxicity effects to determine the effects of multi-level of dosage.

5. CONCLUSION

Both crude EAP and nanoparticle EAP increase the number of $CD4^+$ and $CD8^+$ proliferation in rats infected with *L. monocytogenes.* It has a potential to be used as a support for immunodeficiency patients and intracellular bacterial infections. Further research is needed regarding the addition of variations in the dose of *A. paniculata* nanoparticles to find the optimal dose and addition of groups with antibiotic or anti-inflammatory administration as positive control groups.

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