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Editors :

Dwi Utami M.Si.,Apt

Iis Wahyuningsih M.Si.,Apt

Wahyu Widyaningsih, M.Si., Apt

Nanik Sulistyani, M.Si., Apt

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WATER FRACTION OF SAMBILOTO (ANDROGRAPHIS PANICULATA NEES) ETHANOL EXTRACT EFFICACY IN INDUCING THE NUMBER OF MACROPHAGE, NEUTROPHIL, AND THE LEVEL OF TNF-α ON WISTAR RATS

Wahyu Dewi Tamayanti, Lidwina Tri Kristanti, Hendra Kurniawan, Martha Ervina, Lannie Hadisoewignyo, Lisa Soegianto, Ratna Megawati Widharna

Faculty of Pharmacy, Widya Mandala Catholic University Surabaya

Email : dewffua@gmail.com

Abstract

Background. *The study of the effect of water fraction of Sambiloto (Andrographis paniculata Nees) ethanol extract on the number of macrophage, neutrophil, and level of TNF-α in the body fluid of male Wistar rats after Staphylococcus aureus induction.*

Methods. *Animals were divided into 3 groups, namely: negative control (NC), treatment (T), and positive control (PC) group. The NC, T and PC groups were administered by 0.9% NaCl, water fraction of Sambiloto ethanol extract of 40mg/200g BW dose, and ibuprofen of 7.2 mg/200g BW dose, respectively. The treatment was performed for 7 consecutive days. The number of macrophages, neutrophils, was identified using light microscope whereas the TNF-α level was identified using ELISA.*

Outcome Measured. *The number of macrophage, neutrophil, and level of TNF-α in the body fluid of male Wistar rats after Staphylococcus aureus induction.*

Results. *The number of macrophages in the KC, the T, and the PC groups were 2.33 ± 1.58; 3.80 ± 4.06; and 2.67 ± 1.55 cells, respectively. The number of neutrophils were 0.83 ± 0.33; 0.58 ± 0.32; and 1.33 ± 0.38 cells respective to the KN, T, and PC groups. The level of TNF-α were 17.78 ± 11.67; 23.48 ± 15.95; and 27.90 ± 30.54 pg/ml respective to the KN, the T, and PC groups.*

Conclusion. *This study indicated that water fraction of Sambiloto ethanol extract able to increase the number of macrophage and TNF-α level yet decrease the number of neutrophil in the body fluids of male Wistar rats after Staphylococcus aureus induction.*

Keywords : *macrophages, neutrophils, TNF-α, Herba Sambiloto, ibuprofen*

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INTRODUCTION

Inflammation is a local response to infection or tissue injury. It may occur as a local, systemic, acute, and chronic disorder that lead to the pathologic condition. Inflammatory response precedence by the activation of cell mediated immune system. The cells of immune system respond to the foreign substances that invade the body by several mechanisms, such as chemotaxis, in which the immune cells move to the site of infection, followed by an increase of vascular permeability, and subsequently changes of blood osmotic pressure proceed. Those mechanisms facilitate leucocytes migration to the site of infection (Abbas *et al* 2007).

Development of the inflammatory response plays an important role in initiating pathological condition. Therapeutic approaches to reduce the inflammatory response caused (one of them) by the *Staphylococcus aureus* need to be conducted (Baratawidjaja and Rengganis 2012). Medication choice of inflammation may utilize synthetic drugs and/or natural ingredients. The long term uses of synthetic drugs may initiate unwanted effects on the human body. Therefore, natural ingredients are now becoming chosen alternatives as a healing therapy of inflammatory condition. Some examples are the leaves of Salam (*Syzygium polyanthum*), herbs of Sambiloto (*Andrographis paniculata* Nees.), leaves of Cassava (*Manihot utilissima* Pohl), leaves of Red Betel (*Piper crocatum* Ruiz & Pav), leaves of breadfruit (*Artocarpus altilis*), and others.

Sambiloto (*Andrographis paniculata Nees*) is a natural ingredient which is widely studied because of its efficacy as medicine.Sambiloto (AP) herbs have several active compounds. One of the compounds is known as Andrographolide which posses anti-inflammatory activity (Tewari et al 2010). Previously, it was identified that Andrographolide in ethanol extract of AP herbs inhibited inflammation in Wistar rats (Evacuansiany and Soebiantoro, no date). Other studies mentioned that AP herbs active as anti-inflammation by inhibiting the production of radical oxygen of the neutrophil. Moreover, AP inhibited macrophage migration, and production of TNF-α and IL-12 (Lin and Chao 2010) in the animal serum that treated with AP herbs water fraction containing 10% Andrographolide (Raharjo *et al* 2009).

Among the previous studies of AP anti-inflammatory activity, study of AP especially on phagocytic cells including: monocyte, macrophage, neutrophil, and eosinophil have not yet been conducted. We, therefore, identified the AP anti-inflammatory activity in male Wistar rats.

The objective of this study is to conduct a local inflammation by infecting rats with *Staphylococcus aureus* and identify the response of immune system cells that react on the infected bacteria after an hour. The number of phagocytic cells, namely macrophages and neutrophils were measured as parameter. Since the cells will release cytokines following their activation, therefore, levels of cytokines (TNF-α) also be counted. TNF-α is the main cytokine that is released during acute inflammation against bacteria and other microbes by phagocytic cells (Baratawidjaja and Rengganis 2012). The level of TNF-α measured by ELISA (Enzyme Linked Immunosorbent Assay) method. This study identified the effect of AP herb water fraction of ethanol extract compared to ibuprofen on alleviating local acute inflammation after *Staphylococcus aureus* infection.

METHODS

The apparatuses: distillation equipment, percolator, rotary evaporator (Buchi Rotavapor R-124); separating funnel, chamber, ose, tube, centrifuge apparatus (Hettich Zentrifugen), Eppendorf, syringe, pipette volume, light microscope (Olympus); microplate reader (Thermo Scientific Multiskan-Go, USA).

The materials: Andrographidis Herbs that were collected and determined in the Materia Medica, Batu, East Java; ibuprofen; distilled water; ethanol 96%; ammonia; $CHCl_3$;

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HCl 10% v / v; klorhidrik alcohol; $FeCl_3$; gelatin solution; Steasny reagent; Na acetate; Na hydroxide; ether; acetic acid glacial; Mayer reagent; Dragendorf solution, n-hexane, ethyl acetate; methanol; butanol, acetic acid; kuercetin; ; *Staphylococcus aureus* (ATCC 25923); MSA medium; BaCl2 1%; concentrated H2SO4; 0.9% NaCl; Giemsa dye; TNF-α ELISA kit (Abcam, USA).

Animal handling

Animal used were 18 male Wistar rats 2 - 3 months of age and approximately 150-200 g of weight with healthy as well as normal activities. Prior to use, rats were adapted for a week to the new environment. The inclusive criteria of animals were: no symptoms of illness and uniform body weight. Prior to the experiment, rats were fasted for 18 hours (Winter in Hadisoewignyo 2010). Rats were divided into 3 groups of 6 animals. Group I was the negative control (NC), group II was the AP water fraction treated (AP), and group III was treated by ibuprofen (PC).

RESEARCH METHODS

Preparation and Fractionation of AP Crude Extracts

The study was preceded by maceration of crude AP powder by 96% ethanol for 24 hours at room temperature. The obtained extract was filtered and evaporated with a rotary evaporator. The condensed extract was dissolved in 50 ml of methanol:water (7:3). Subsequently, the solution was separated by adding 100 ml of n-hexane and shook in a separator flask for 60 minutes. The formed second layer was separated. The n-hexane addition was repeated for three times. The methanol layer was further separated by ethyl acetate. Ethyl acetate was added to the water layers and shook in a separator flask for 60 minutes. Then, the formed second layer was separated. This process was repeated for three times.

Anti-inflammatory Activity

In order to conduct anti-inflammatory activity, the rats were injected with *Staphyloccocus aureus* (SA). The SA preparation preceded with the rejuvenation of SA in MSA medium and incubation at 37°C for 24 hours. The rejuvenated SA, was mixed to 0.9% NaCl. Subsequently, the turbidity of SA-NaCl suspension was compared to Mc Farland I, in which contained 3 × 108 cfu/ ml of bacteria. Before injected to the rats, the suspension was incubated for 2 hour at 37° C (Wasito et al 2008).

Rats were allowed to stand for an hour after SA injection before conducting dissection. Subsequently, the rats were allowed to stand for 5 minutes after 0.9% NaCl injection. Rats that were ready for dissection were anesthetized with ether. Peritoneal fluid was taken from the abdominal cavity. The peritoneal fluid was subsequently smeared and fixed by absolute methanol for 5 minutes. Finally, the fixed peritoneal fluid was stained with Giemsa for 20 minutes, rinsed and dried. The stained peritoneal fluid were identified under a light microscope (400x magnification) and counted for the number of macrophages (Kusmardi et al 2006).

To study the number of the neutrophiles, the blood was taken from the heart, then disposed in a tubes contained EDTA. Prior to centrifugation, part of the blood was taken and subjected to be smeared, and stained as the peritoneal fluid. Subsequently, the stained blood was identified under light microscope (magnification of 400x) to count the number of neutrophil cells. The remaining of the blood was centrifugated at 2,000 rpm for 20 minutes. After centrifugation, serum was taken to be stored at -20° C for measuring the level of of TNF-α by ELISA method (Abcam, 2012).

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Table I Standardisation of AP Herbs

No.	Parameter	Qualifications (IHD 1979)	Test results
1	Ashes	< 12%	11.13 ± 0.35
2	Water soluble extract	≥ 18 %	17.03 ± 0.38
3	Ethanol soluble extract	≥ 9.7%	13.53 ± 0.10

IHD : Indonesian Health Departemen

Table II. Rf of AP Herbs Extract and Water Fraction under UV (254 and 366 nm) and UV Visible

Extract	UV λ 254 nm		UV λ 366 nm			UV visible		
	Rf	Compounds	Rf	Color	Compounds	Rf	Color	Compounds
AP Exstract	0.77	Flavon	0.60	Blue	Flavanon	0.56	Yellow	Flavanon
	0.87	Isoflavon	0.79	Blue	Flavanon	0.69	Yellow	Flavanon
			0.87	Red	Isoflavon	0.87	Brown	Isoflavon
AP-water Fraction	0.62	Flavanonol	0.6	Grey	Flavanonol	0.56	Brown	Flavanonol
	0.75	Flavon	0.81	Blue	5-deoksiisoflavon	0.69	Brown	Flavanonol
	0.87	Isoflavon						
Andrografolide	0.81	Andrographolide	0.25	Blue	Andrographolide			

Table III. The Number of Macrophage, Neutrophil, and TNF-α Leve

Groups	Macropage	Neutrophil	TNF-α
Negative control	2.33 ± 1.58	0.83 ± 0.33	17.7 ± 11.67
AP Herbs	3.80 ± 4.06	0.58 ± 0.32	23.48 ± 15.96
Positive control	2.67 ±.1.54	1.34 ± 0.38	27.90 ± 30.54

AP Herbs: AP Water fraction

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Table IV. *One Way Anova Analysis* (α=0,05) of Macrophage, Neutrophil, and TNF-α level Between Groups

Note	F	F table	Significancy
Macrophage	0.42	3.59	0.67
Neutrophil	4.85	4.07	0.04
TNF-α	0.07	3.34	0.94

Table V. LSD Analysis of the Number of Neutrophil

Groups	Compared to	Sig.	Note
Negative control	AP Herbs	0.34	NS
	Positive control	0.07	NS
AP Herbs	Negative control	0.34	NS
	Positive control	0.01	S
Posjtive control	Negative control	0.07	NS
	AP Herbs	0.02	S

NS: not significant S: significant AP Herbs: AP Water fraction

Results

Before used, several parameter of AP water fraction (AP WF) was standardized including level of ashes, level of water soluble extract, and level of ethanol soluble extracts (Table I). The Indonesian Health Department (1979) qualified that level of AP WF ashes, water soluble extract, and etanol soluble extract were less than 12%, more than 18%, and more than 9.7%, respectively. The levels of crude AP herbs water-soluble extract (17.03 ± 0.38) were not reach the qualification, therefore, the study was continuously undergone subsequent extraction steps used ethanol as the solvent.

This study used 1 kg of dried AP herbs that macerated by ethanol. The macerated extract obtained was 245 g (24.5%). The extract was subsequently undergone organoleptic test for the color, taste, and smell parameter. The ethanol extract of dried AP herbs was dark green in color, bitter in taste and specific in odor which were qualified according to the previous mentioned data (Indonesian Health Departement 1979).

Following the organoleptic test, the ethanol extract was fractionated extracts by water. The fractionated extract was then subjected to Thin Layer Chromatography (TLC) with butanol: acetic acid: water (3: 1: 1) as mobile phase. The TLC plate was dotted and eluated until reacted with ammonium and so produced a yellowish brown color which was a specific indication of the presence of flavonoids (Harborne 1987) (Table II).

Table II showed the results of Rf level of AP extract and water fraction compared to Andrographolide. When observed at λ 254 and 366 nm, the Rf level of AP extracts and water fractions were different to Andrographolide. It was predicted due to the flavonoids remained in both of AP extracts and water fraction, thus the Andrographolide was not clearly stained. The Rf following observation under the UV visible showed no Rf of andrographolide. It was due to the ammonium vapor was reacted solely to the flavonoids while Andrographolide is belongs to the diterpenoid classes (Lin and Chao 2010).

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Table III showed the number of macrophages which was higher than the negative and positive control group. It was probably caused by the injected *Staphylococcus aureus* (SA) were produced an exfoliative which was able to activated macrophages, therefore macrophages were stimulated to release TNF-α, IL-6 and pyrogenic toxin superantigens that subsequently induce the activation of T cells and other macrophages to move to the site of infection (Hidayani no date). The result indicated that AP WF treatment may reduce nitric oxide production and increase macrophage activity (Guan, *et al.* 2012). Moreover, the increased number of macrophages was not different statistically compared to the negative control group that may be caused by the AP WF bacteriostatic and bactericidal activity (Anonymous 2005) which may encounter SA following injection, thus prevent the movement of macrophages to the site of infection. The number of macrophages in the positive control group, that contained ibuprofen, was lower than the AP WF group. This was an indication of less anti-inflammatory activity conducted by ibuprofen since approximately 90% of ibuprofen was bound to protein serum. Therefore, it would be more difficult for ibuprofen to penetrate to the cell membrane to produce anti-inflammatory effects (Wilmana and Gan, 2007).

The number of neutrophil of the AP WF group was lower compared to the negative and positive control group which can be explained due to the antioxidant activity of AP WF that may suppress the synthesis of nitric oxide (NO) and thus less NO will be produced and neutrophil adhesion will be inhibited, consequently (Ezeamuzie, *et al.* 2009). In the positive control group, the number of neutrophil was higher than the negative control and the AP WF group. This was explained due to the action of ibuprofen that affect the biosynthesis of several inflammatory mediators such as prostaglandins and leukotrienes (Wilmana and Gan 2007) that stimulate the increased number of neutrophils (Baratawidjaja and Rengganis 2012).

The levels of TNF-α of the AP WF and the positive control groups showed higher level than the negative control group. This is may be caused by macrophages that activated and released TNF-α into the blood circulation (Abbas, et al. 2007) since TNF-α is known as the major cytokine in the acute inflammatory response to bacteria and microbes. In acute inflammation condition, the TNF-α and endothelial leukocytes are working in coordination. Thus, the increased of macrophages is accompanied by elevated level of TNF-α (Baratawidjaja and Rengganis 2012).

CONCLUSION

Conclusively, this study indicate that AP WF may increase the number of macrophages in the peritoneal fluid that had been infected by *Staphylococcus aureus* and consequently increase the level of TNF-α in the serum of the Wistar rats. However, decrease the number of netrophils in the seum of the Wistar rats.

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