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Immunomodulator Effect Test of Sungkai Leaves (*Peronema canescsens* Jack.) Ethanol Extract Using Carbon Clearance Method

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ABSTRACT
Sungkai leaf extract *(Peronema canescens* Jack.) has been used traditionally to cure various diseases for humans. This research aimed to determine the immunostimulant effect of sungkai leaf extract using the carbon clearance method, the total of leukocyte cells, and the percentage of leukocyte cell types. 25 male mice were divided into 5 groups. The extract was administered orally for 6 days at a dose of 25; 50; 100 mg/kgbw, Na CMC 0.5% as control and Stimuno 50 mg/kgbw as a comparison. The phagocytic index, total leukocytes, and the percentage of leukocyte cell types are determined on the 7th day. The phagocytosis index value was statistically analyzed by one-way ANOVA followed by Duncan's test. The increase of phagocytosis index showed that the effect of each dose was significantly different ($P < 0.05$) with Na CMC 0.5% as control. The highest phagocytosis index was obtained from a dose of 100 mg/kgbw. Based on the one-way ANOVA test continued by Duncan's test, there was a significant difference ($p<0.05$) in the total leukocyte cell and the percentage of leukocyte cell type. Sungkai leaf extract *(Peronema canescens* Jack.) had an immunostimulant effect on male white mice (*Mus musculus* L.).

Keywords: *Peronema canescens Jack, Immunomodulator, Immunostimulant, Carbon Clearance, Leukocytes*

1. INTRODUCTION

An infectious disease caused by pathogenic microorganisms such as viruses, bacteria, parasites, or fungi that can be transmitted from one person to another or from animals to humans has been one of the health problems that continue to develop. That is, the important role of the immune system in recognizing and destroying pathogenic microorganisms is needed to protect the body [1]. The immune system is divided into the nonspecific immune system and the specific immune system. The nonspecific immune system is the innate body defense that responds quickly in destroys all of the foreign pathogenic microorganisms, it works through the phagocytosis mechanism by white blood cells especially neutrophils, monocytes, and tissue macrophages. While Specific immune systems or adaptive immune that give the specific response towards specific microorganisms [2].

The quality and intensity of the immune system can be increased by giving immunostimulants [3]. Immunostimulants are used as an adjunct therapy in diseases caused by immune response disorders such as immunodeficiency, infections and also as a preventive measure to increase the body's resistance to disease. One of the herbal plants that have potential as immunostimulants are Sungkai *(Peronema canescens* Jack.). Sungkai leaf is used by traditional people as a medicine for fever and malaria Sungkai contains alkaloids, flavonoids, terpenoids-steroids, and tannins [4]. Sungkai has bioactivity as antipyretic, antiplasmodial, antioxidant, antibacterial, and antimicrobial [5,6,7]. Previous research conducted by Yani (2014) reported that sungkai young leaf extract could increase the number of leukocytes in mice [8].

Based on the description above, the research is conducted to determine the immunostimulant activity of sungkai leaf extract. The parameters that will determine

were the phagocytosis index using the carbon clearance method, total leukocyte cells, and the percentage of leukocyte cell types.

2. MATERIALS AND METHODS

2.1. Time and Place

This study was carried out for 4 months at the Research Laboratory and Immunology and Serology Laboratory of the Faculty of Pharmacy of Andalas University.

2.2. Apparatus

Hemocytometer (Assistant), micropipette (Eppendorf), UV-Vis spectrophotometer (Genesys 10S UV-VIS), microscope (Olympus), rotary evaporator (Buchi), sonde instrument, stopwatch, test tube (Pyrex), syringes 1 mL (One Med), leukocyte pipette (Assistant) and animal cages.

2.3. Materials

Sungkai leaf *(Peronema canescens* Jack.), Stimuno (Dexa Medica), quercetin (Sigma-Aldrich), Na CMC, Chinese ink (Yamura), acetic acid 1%, physiological NaCl 0.9%, aqua dest, methanol (Brataco), Giemsa dye (Merck), distilled water, EDTA, Turk reagent (St.Reagensia) and male white mice (*Mus musculus* L.).

2.4. Sungkai Leaf Extract

Extract characterization includes organoleptic examination, drying shrinkage, determination of total ash content, TLC profile test, and phytochemical screening to test the content of alkaloids, flavonoids, phenolics, saponins, terpenoids, and steroids qualitatively.

2.5. Preparation of Experimental Animal

The experimental animals used were 25 male white mice (*Mus musculus* L.) age 2 until 3 months in 20 until 35 g weigh. Animals were randomly divided into 5 groups, each group consisting of 5 mice. Mice were acclimatized for 7 days, sungkai leaf extract was given to the mice at doses of 25, 50, and 100 mg/kgbw, NaCMC 0.5% as control, and Stimuno 50 mg/kgbw as a comparison. The extract was given orally once a day for 6 days with a volume of 0.2 mL/20 g mice. Mice were tested on the seventh day.

2.6. Immunomodulator Activity Test with Carbon Clearance method

On the seventh day after administration, 75 µL of blood was taken from the tail capillary and lysed with 4 mL of acetic acid 1%. This first blood sample is called a sample blank (0th minute). Then 0.1 mL/10 gbw of carbon suspension was injected intravenously. 75 µL of mice blood was taken at the 3, 6, 9, 12, and 15 minutes after carbon injection. Each blood was lysed with 4 mL of acetic acid 1%, then the absorption was measured using a UV-Vis spectrophotometer at a wavelength of 650 nm [10].

2.7. Total Leukocyte Cells with Hemocytometer

Fresh blood dripped by EDTA was sucked with a leukocyte pipette up to the number of 0.5 then sucked up Turk's solution to 11 points, then shake it for 3 minutes, discarded one until three drops, and then enter one drop to the hemocytometer counting chamber. Leave it for 3 minutes. Count the number of leukocytes in the four corners of the counting chamber [11].

2.8. Percentage of Leukocyte Types with Blood Smear

The blood used to count the percentage of leukocyte types is the fresh blood we got from the carbon clearance method procedure. Placed one drop of fresh blood on the slide then mixed it with another slide then dried. After it dried, dripped it with methanol until it coats the entire blood smear, leave it for 5 minutes. Add one drop of Giemsa solution which has been diluted with distilled water (1:20) and leave it for 20 minutes, washed with distilled water, then dried. The number of segmented neutrophils, Banded neutrophils, lymphocytes, monocytes, and eosinophils was counted under a microscope with 1000x magnification [12].

Phagocytosis index, total leukocyte, and percentage of leukocyte type were statistically analyzed by one-way ANOVA and followed by Duncan's test.

2.9. Data analysis

Phagocytosis index, total leukocyte, and percentage of leukocyte type were statistically analyzed by one-way ANOVA and followed by Duncan's test.

3. RESULTS

The sample used in this research was the Sungkai plant with the species *Peronema canescens* Jack from the family of Lamiaceae which has been identified by *Anda* Herbarium, Departement of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Padang.

Sungkai leaf extract was made by 2 kg of a fresh leaf that has been cleaned and air-dried until it becomes dry Simplicia, before being extracted, the sample is processed to make it into powder by blend and sifted it with mesh number 48. The extraction process was

carried out using a cold extraction method, maceration. In the maceration process, as much as 402.35 g of Simplicia powder was soaked with 70% ethanol in a ratio (1:10). The powder is soaked for the first 6 hours while stirring occasionally, then stands for 18 hours [9]. Maceration was carried out three times to make sure the active substances in the sample were maximally attracted. Macerate filtered using cotton and concentrated with a rotary evaporator, then as much as 85,9 g thick extract of sungkai leaf was obtained with the yield percentage of sungkai leaf extract as much as 21.35%.

Based on the organoleptic test of the thick extract of sungkai leaf *(Peronema canescens* Jack.) the extract was thick with blackish-green color and have a characteristic odor with a bitter taste. Based on the phytochemical screening test sungkai leaf extract *(Peronema canescens* Jack.) containing flavonoids, alkaloids, saponins, phenolic, and terpenoids. This is appropriate with the research conducted by Andespal (2020) that Sungkai containing flavonoids compounds, alkaloids, saponins, phenolic, and terpenoids [13]. Drying shrinkage obtained from Sungkai leaf extract *(Peronema canescens* Jack.) was 9.54% and 2.77% for the ash content. TLC profile test is a qualitative test to determine the presence or absence of identity or comparison compounds in sungkai leaf extract. The comparison compound used in the test was quercetin. The mobile phase used was n-hexane: ethyl acetate (2:3). The stationary phase used in the test is silica gel plate F254 (Merck®) because this plate can fluoresce when exposed by the light in the correct wavelength (254 nm) in the ultraviolet (UV) region [9]. From the test, the Rf point for quercetin was 0.72, while it was equal to Rf obtained in sungkai leaf extract. The existence of the Rf point equation from quercetin and sungkai leaf extract indicates that sungkai leaf extract contains quercetin compounds, which can be seen in Figure 1.

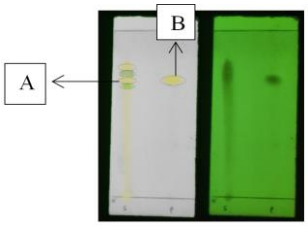

Figure 1. Thin layer chromatography profile of sungkai (*Peronema canescens* Jack.) extract. (A) Sungkai extract, (B) Quercetin.

The carbon clearance method is an immunomodulatory activity test using carbon particles as an antigen which is given intravenously, the speed of carbon clearance must be seen in each interval of 3, 6, 9, 12, and 15 minutes. Carbon is used as a marker because of their advantages such as small and stable particle size so that isn't caused blockage of blood vessels and lungs. Carbon also has characteristics as an antigen because of its isolation which is not normally found in the body [14]. The effect of phagocytosis of sungkai leaf extract can be seen by making a standard curve between the carbon content in the blood and the absorbance value. The maximum wavelength of carbon in the previous research was 650 nm. After repeated measurements, it was found that there was a shift in the maximum wavelength to 636 nm, this shift occurred due to other influences such as the type of solvent, pH of the solvent, temperature, high concentration, and interfering substances [13]. From the results of standard carbon curve, the regression equation is y = 0.0059 x + 0.0048 with R2 = 0.9996 which can be seen in Figure 2. The regression equation shows a linear relationship between the carbon concentration in the mice's blood with absorbance [15].

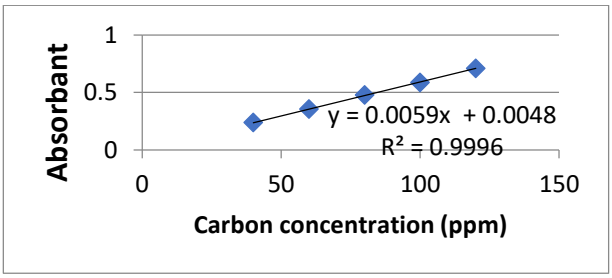

Figure 2. Calibration curve of carbon content in the blood of mice

One of the phagocytosis parameters is the phagocytic constant, where the greater value of the phagocytosis constant, the higher carbon clearance so that the faster the phagocytic cells carry out the phagocytosis process. The phagocytosis index is calculated after the value of the phagocytosis constant is obtained. If the average value of the phagocytic index is greater than 1 (IF > 1), it means the substance has immunostimulants activity [16]. Based on the phagocytic index values, doses of 25, 50, and 100 mg/kgbw were immunostimulants. The highest phagocytic index value was indicated by the group in the dose of 100 mg/kgbw. In Figure 3, phagocytic index value of the control group Na CMC 0.5% is 1.00, 1, 269 for the group with dose of 25 mg/kgbw, 1,548 for the group with dose of 50 mg/kgbw, 1,737 for the group with dose of 100 mg/kg bb and 1,548 for the comparison group Stimuno 50 mg/kgbw. The highest phagocytosis index value indicated by the group with a dose of 100 mg/kgbw, which is 1,74 it means the group with a dose of 100 mg/kg has the best phagocytic ability compared with the control group and the comparison group.

Sungkai extract contains flavonoids. In the previous studies, flavonoids have immunostimulatory effects by increasing the effectiveness of lymphokine proliferation and also increasing the activity of IL-2 (interleukin 2). Activated Th1 cells (T helper 1) will produce IFN-γ

(interferon-gamma) which can activate macrophages. Activated macrophages will produce compounds such as nitric oxide to destroy pathogenic microorganisms [17].

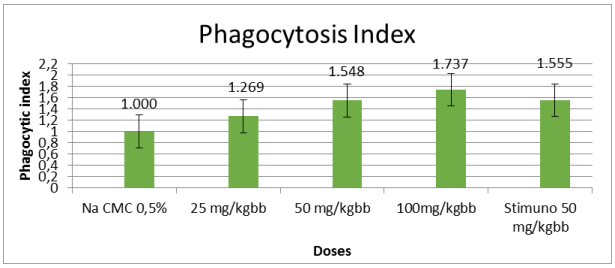

Figure 3. Graphic pahagocytic index values from male white mouse blood after administration of sungkai extract (*Peronema canescens* Jack.)

Hemacytometers are used to count the total leukocyte. Turk solutions are used to dilute and lyse all of the cell's blood except [18]. The administration of sungkai leaf extract can increase total leukocytes as shown in Table 1 and Figure 4. The group were given leaf extract at doses of 25, 50, 100 mg/kgbw showed a significant difference ($P < 0.05$) when compared to the control Na CMC 0,5 %. Sungkai leaf extract at a dose of 100 mg/kgbw showed the highest total leukocytes compared to doses of 25 and 50 mg/kgbw and the comparison group.

Table 1. Mean of total leukocyte after administration of sungkai extract (*Peronema canescens* Jack.)

Total leukocyte (/µL blood)	
Doses	**Mean ± SD**
Na CMC 0,5%	8140 ± 2579.583
25 mg/Kgbb	8170 ± 2762.607
50 mg/Kgbb	10542 ± 1910.385
100 mg/Kgbb	12558 ± 2536.990
Stimuno 50 mg/kgbb	11750 ± 1560.849

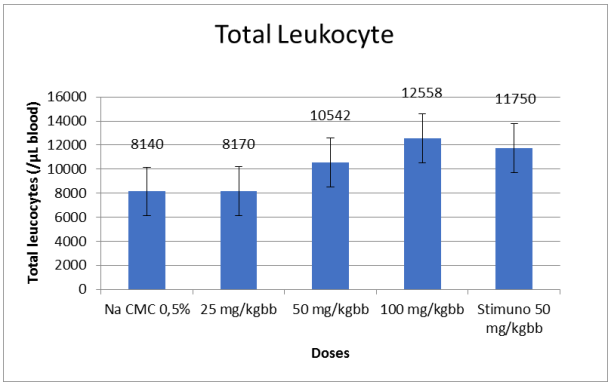

Figure 4. Bar graph of of total leukocyte after administration of sungkai extract (*Peronema canescens* Jack.)

Calculating the percentage of leukocyte cell types using the blood smear method. Blood smears were stained using Giemsa. Giemsa only showed eosinophils, Banded neutrophils, segment neutrophils, lymphocytes, and monocytes. Basophil cells cannot be seen because the cells are soluble in Giemsa stain [19]. Leukocytes calculation can be done with cross-sectioning or counting leukocytes starting from the edge of the object-glass under a microscope until 100 leukocytes are obtained and expressed in percent (%) [20]. The administration of sungkai leaf extract affects the percentage of leukocyte cell types, which can be seen in Table 2 and Figure 5. The group were given leaf extract at doses of 25, 50, 100 mg/kgbw showed a significant difference ($P < 0.05$) when compared with Na CMC 0.5% as control. The result shows that there is an increase in the percentage of segmented neutrophil cell types with increasing doses. The neutrophil is the first line of the body's defense when there is damaged tissue or foreign objects in the body. Neutrophils are highly mobile phagocytic specialists, they eat and destroy unnecessary materials [17].

This research showed that sungkai leaf extract *(Peronema canescens* Jack.) at doses of 25, 50, 100 mg/kgbw had an immunostimulating effect on male white mice (*Mus musculus* L.) through the value of the phagocytosis index (IF>1) which was tested by the carbon clearance method, increases total leukocytes and can increase the percentage of segmented neutrophil cells against male white mice.

Table 2. Percentage of leukocytes after administration of sungkai extract (*Peronema canescens* Jack.)

	NaCMC 0.5%	25 mg/kgbb	50 mg/kgbb	100 mg/kgbb	Stimuno 50 mg/kgbb
Segmented neutrophils	37.4 ± 6.804	32.6 ± 3.647	35.2 ± 4.604	42.6 ± 3.847	18 ± 2.739
Banded neutrophils	9.6 ± 1.949	27 ± 5.958	10.4 ± 1.14	15.4 ± 8.385	16.4 ± 4.099
Lymphocytes	36.4 ± 5.983	29.2 ± 4.266	27.6 ± 5.595	25 ± 8.155	39 ± 2.345
Monocytes	11 ± 3.082	6.2 ± 2.28	19.2 ± 0.837	9.4 ± 2.608	12.2 ± 1.643
Eosinophils	5.6 ± 3.507	5 ± 1.581	7.6 ± 1.817	7.6 ± 2.608	14.4 ± 2.702

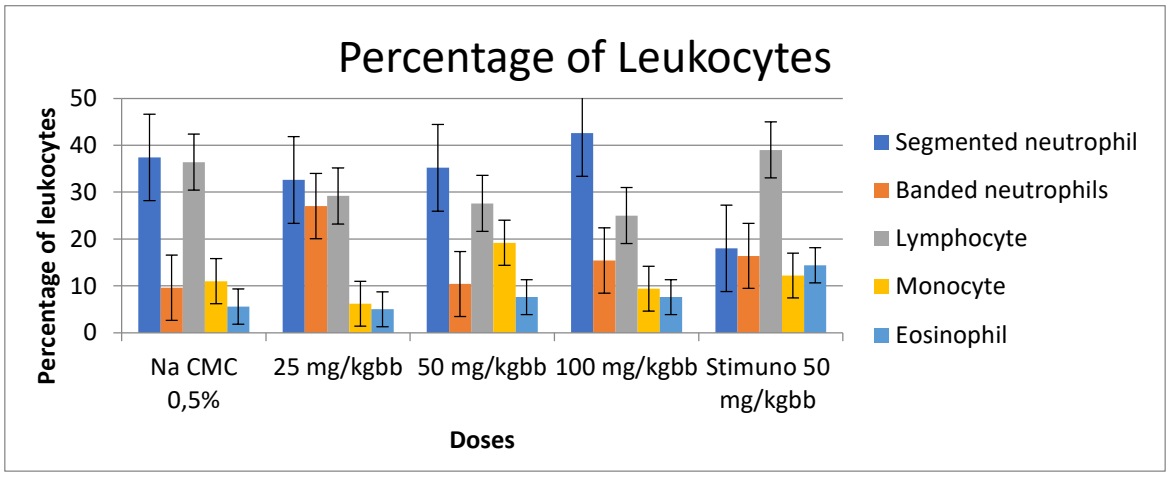

Figure 5. Bar graph of percentage of leukocytes after administration of sungkai extract (*Peronema canescens* Jack.)

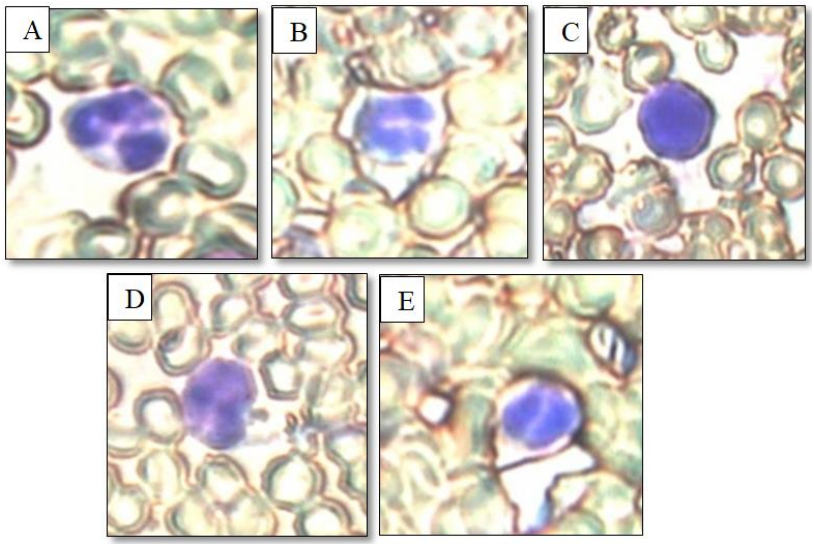

Figure 6. White male mice leukocytes. (A) Segmented neutrophil, (B) Banded neutrophils, (C) Lymphocyte, (D) Monocyte, (E) Eosinophil

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