Advances in Health Sciences Research, volume 40
2nd International Conference on Contemporary Science and Clinical Pharmacy 2021 (ICCSCP 2021)

Immunostimulant Effect of Peronema canescens. Jack Leaves Extract and Propolis in Male White Mice

Dwisari Dillasamola[1*], Syofyan[1], Almahdy[1], Fitri Rachmaini[1], Mila Yultri[1], Hanifatul Larisa[1], Elsya Gustia[1], Skunda Diliarosta[2], Biomechy Oktomalioputri[3]

[1]*Faculty of Pharmacy, Universitas Andalas, Padang 25163, Indonesia*
[2]*Faculty of Mathematics and Science, Universitas Negeri Padang, Padang 25173, Indonesia*
[3]*Faculty of Medicine, Universitas Andalas, Padang 25163, Indonesia*
[*]*Corresponding author. Email: dwisaridillasamola@phar.unand.ac.id*

ABSTRACT
Peronema canescens leaves are one of the natural ingredients that have the potential to increase the immune system. This study determine the immunostimulant effect which identify the effectiveness of phagocytosis in killing pathogens using the carbon clearance method. The parameters that used in this study were the number of leukocyte cells. Thirty male mice used in this study which randomly divided into six groups. First group is given Na CMC 0.5% as negative control. The second group is given propolis 195 mg/kgBW as comparison group. The third group is given suspension extract 10 mg/kg BW. The fourth group is given extract suspension 50 mg/kg BW. The fifth group is given extract suspension 100 mg/kg BW. Whereas, the sixth group is given extract suspension 200 mg/kg BW. All of groups were treated for seven days and on the 8th day the mice were euthanized and their blood was taken to observe the leukocyte cells and then the tails were injected with carbon intravenously. The data were analyzed statistically with ANOVA. The result shows a significant difference ($p<0.05$) against the negative control group in carbon clereance and the total number of leukocytes in male white mice. It can be concluded that there is an immunostimulatory effect on the ethanol extract of sungkai leaves (*Peronema canescens. Jack*) in male white mice.

Keywords: *immunostimulant, Peronema canescens, carbon clearance, leucocyte cells*

1. INTRODUCTION

The immune system is the body's defense mechanism to against all of foreign substances that enter in the body including pathogenic microorganisms [1]. The defense consists of the non-specific (innate) and specific (adaptive) immune system. The non-specific immune system is the body's first defense and provides a direct response to antigens. Meanwhile, the specific immune system takes time to recognize the antigen before it can respond [2]. Indonesia has plenty of plants that can be used as raw materials for traditional medicine. Natural substances that are derived from plants have immunomodulator effects to control immune responses certainly [3]. Peronema canescens. Jack, as known as Sungkai, is a plant that has the potential of immunomodulator effects. It can be seen in Figure 1.

Figure 1. Sungkai (Peronema canescens. Jack)

Sungkai is one of the specific export commodities in Sumatra and Kalimantan island. The active ingredients of sungkai leaves consist of flavonoids, alkaloids, steroids, phenolics, tannins, and saponins which have the main activity as antibacterial [4]. Groups of polysaccharides, terpenoids, alkaloids and polyphenols have bioactivity as immunostimulant agents [5]. The carbon clearance method

aims to evaluate the effect of drugs on the reticuloendothelial system (RES). This system consists of fixed tissue macrophages and cellular macrophages which phagocytocyte the foreign substances that enter in the body [6].

2. METHODS

2.1. Tools and materials

Maceration bottle, vial, rotary evaporator (Buchi®), separating funnel (Pyrex®), filtering paper, aluminum foil, analytical balance (Ohaus®), spatel, mouse scale, slide, watch glass, drip plate, capillary tube, vaporizer , scissors, sonde, syringe, micro pipette, measuring pipette, volumetric flask (Iwaki®), measuring cup (Pyrex®), erlenmeyer (Pyrex®), UV-Vis spectrophotometry, haemocytometer (Nauber®), test tube (Iwaki®) , microscope (Olympus CX43®), blood tube (Vaculab®), thoma pipette, and dropper pipette.

The materials in this study were fresh Sungkai leaves (Peronema canescens) 1.5 Kg, Propolis (melia propolis®), Aquadest, Na CMC 0.5%, Ethanol 70% (Dwipraga®), Giemsa dye, Chinese ink, Asetic acid 1%, physiological NaCl.

2.2. Sampling

Sungkai is obtained at Jl. Rasyidin Hospital, Padang City. Sample identification was carried out at Herbarium of University Andalas. The leaves had been separated from the stems, then were washed and dried at room temperature for three days. The dried leaf samples were powdered for extraction.

2.3. Sample extraction

Extraction of sungkai leaves was carried out by maceration method. The dry sample was put into a maceration bottle as much as 402.32 grams, then it was added the ethanol solvent until all submerged and tightly closed. It was left for 24 hours, and then stirring occasionally.

It was filtered by a filter, next it was separated from the dregs and the filtrate. Extract was evaporated and concentrated with a low pressure rotary evaporator at a temperature of 40°C. The results of the rotary evaporator were dried in an evaporation cup over a water bath until a thick extract is obtained.

2.4. Immunostimulant test

2.4.1. Acclimatization of experimental animals

All mice were adapted to the environment for ± 7 days. Experimental animals were only given food and water every day. Test animals are declared suitable for study if their body weight is not more than 10% after the acclimatization period.

2.4.2. Determination of Carbon Content

Five grams of Chinese ink was weighed and dried in an oven at 105^{o}C for 30 minutes. Drying was continued in a desiccator until a constant weight was obtained.

2.4.3. Manufacturing of Colloidal Carbon Suspension

The dried Chinese ink was suspended 1.6 grams with 25 mL of 0.5% (w/v) Na CMC into 0.9% (w/v) physiological NaCl solution. So, the concentration was obtained 64 mg/mL (6.4 %).

2.4.4. Carbon Raw Curve

The dried Chinese ink was weighed 100 mg, then 1% acetic acid was added until 100 mL. So the concentration of 1000 ppm was obtained. Each solution was 6, 5, 4, 3, 2 mL and it was supplemented by 1% of acetic acid to a volume of 50 mL. So the concentration of carbon content was 120, 100, 80, 60, 40 ppm. Four mililiters of each concentration were pipetted and 50 L of mouse blood was added. After homogenizing, UV-Vis spectrophotometer was used to measure the absorption at a wavelength of 636.5 nm which is the absorption area for carbon. The absorbance plot was used to make a calibration curve as a blank using male white mouse blood and aquadest [7].

2.4.5. Carbon clearance method

2.4.5.1. Calculation of phagocytic constants;

$$K = \frac{\text{Log A(n)} - \text{Log A(n} - 1)}{\text{t(n} - 1) - \text{t(n)}}$$

Definition:

K	= Phagocytosis constant
A(n)	= Absorbance at time-n
A(n-1)	= Absorbance at time n-1
t	= Time (3, 6, 9, 12, 15)
n	= Pick up period (1, 2, 3, 4, 5)

2.4.5.2. Calculation of the phagocytic index;

$$PI = \frac{\text{Phagocytosis constant of micex}}{\text{average phagocytic constant in control mice}}$$

Definition:
PI = Phagocytic index
$Mice_x$ = The mice that have been treated and the phagocytosis constant value was determined

2.5. Sample Preparation

Mice were divided into 6 groups that consist of group I as a negative control (only given 0.5% Na CMC), group II was given propolis, and the other groups were given sungkai leaf extract preparations of 10, 50, 100, and 200 mg/kg body weight, it can be seen in Table 1. The test preparation was administered orally once a day for six days respectively. On the 7^{th} day after administration of the suspension preparations, the tails of mice were smeared methanol by a cotton swab, then the ends of the mice's tails were cut and the blood was collected on a drip plate that was dripped with heparin until homogeneous. Fifty liters of blood was taken and four mililiters of 1% acetic acid was added. This first blood is called a blank sample (zero minute). Then 0.1 mL/10 gram BW of carbon suspension was injected intravenously. Mice blood was taken 50 L at 3, 6, 9, 12, and 15 minutes after carbon injection and then the absorbance was measured at a wavelength of 636.5 nm.

Table 1. Treatment of experimental animals

Experimental Animal Groups	**Treatment**
Group I	0.5% Na CMC suspension
Group II	195 mg/kgbw propolis
Group III	sungkai leaf extract suspension dose 10 mg/kgbw
Group IV	sungkai leaf extract suspension dose 50 mg/kgbw
Group V	sungkai leaf extract suspension dose of 100 mg/kgbw
Group VI	sungkai leaf extract suspension dose of 200 mg/kgbw

2.6. Leukocyte cell test

2.6.1. Calculation of the percentage of leukocyte cell types

Mice blood in day-0 is the blood that are used in the carbon clearance test. One drop of blood was dripped on the slide. Then the droplets were flattened by another slide, a blood smear was obtained and then it was dried. Next, the blood smear was dripped by methanol and then it was left for five minutes. One drop of Giemsa solution has been diluted by distilled water (1:20) that was added and left for 20 minutes, rinsed with distilled water and air-dried. Eosinophils, rod neutrophils, segment neutrophils, lymphocytes and monocytes were counted by a microscope at a magnification of 40 times [7]. The percentage of leukocyte type can be calculated by the following formula:

$$\text{Leukocyte count} = \frac{t}{n} x\ 100\%$$

Definition:
t = increasing/normal/decreasing of value
n = Number of samples

2.6.2. Leukocyte count

Fresh blood was sucked by a leukocyte pipette up to 0.5 scale, then turk's solution was added up to 11 scale. The pipette was shaken between 15 and 30 seconds. Then, the first three drops were discarded, while the next drops were put in the counting chamber of the hemacytometer. It was left around three minutes for the leukocytes to settle and then it was count the total leukocyte cells under a microscope with a magnification of 10 times at the four corners of the counting chamber [7]. Calculation of the number of leukocytes can be calculated by the following formula:

$$WBC = \frac{NI \times P}{0.4} (8)$$

Definition :
WBC = White blood cell
Nl = Number of leukocytes in 64 counting boxes
P = Dilution value (20 times)
0.4 = The total of blood in five boxes

2.7. Data analysis

The research data was processed by the one way ANOVA method statistically and it was continued by Duncan's Post Hoc method.

3. RESULTS AND DISCUSSION

The extract that obtained 402.32 g of the sungkai plant was 85.9 g with the yield percentage was 21.35%. In this study, standardization was carried out to determine the identity of the sample. Organoleptic examination showed that the extract was thick, greenish brown, characteristic odor, and bitter taste. Phytochemical examination in this study showed that samples of ethanol extract of sungkai leaves were known to contain flavonoids, alkaloids, saponins, and phenolics. These results are in line compare to previous studies that ethanol extract of sungkai leaves have bioactivity as immunostimulant agents consist of polysaccharides, terpenoids, alkaloids and polyphenols [5].

This study used ethanol extract of sungkai leaf and liquid propolis as a comparison. Sungkai leaf extract is insoluble in water. Therefore, the preparation was made in the form of a suspension using 0.5% Na CMC as a suspending agent. Na CMC 0.5% does not affect the efficacy of the active substance and it can dissolve the extract. Examination of drying shrinkage and total ash content of sungkai leaf extract obtained levels of less than 10%. Examination of the chromatographic profile of leaf samples showed an Rf value of 0.64.

Chinese ink suspension was used as an antigen to identify the ability of cells to phagocytize the foreign substances. A compound is declared to be foreign substances if it has a molecular size of 14.000 Da to 6.000 kDa. The use of Chinese ink as an antigen because of it is stable in the blood and it does not blockage of blood vessels and lungs. Standard curve determination of cell phagocytosis ability aims to identify the linearity between the carbon content in the blood of mice and the measured absorbance. The results of the determination of the carbon have a linearity value. It can be seen in Figure 2.

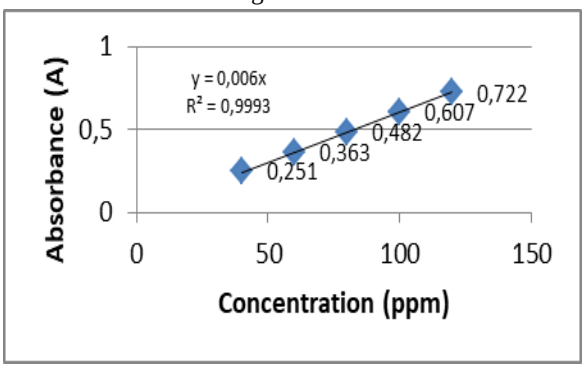

Figure 2. Graph of the carbon calibration curve at a wavelength of 636.5 nm

Based on the results of the total leukocyte cell test, the group of animals that were given sungkai extract at a dose of 200 mg/kgbw showed the highest total number of leukocytes. It can be seen in Figure 3.

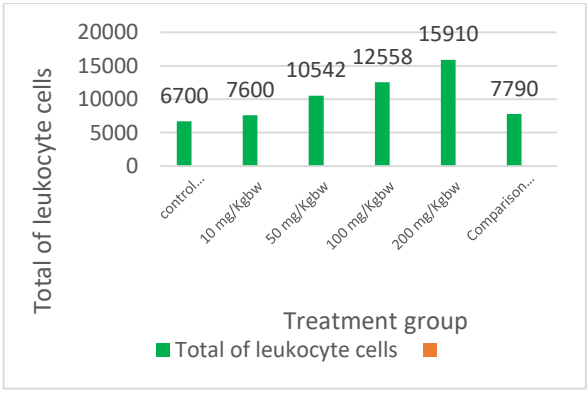

Figure 3. Graph of leukocyte cell count in male white mice after giving the test preparation for sixs days

Examination of mice blood samples on the percentage of leukocyte cell types after administration of sungkai leaf ethanol extract can be seen in Figure 4. In this study, An increasing of the number of segmented neutrophils was carried out in the blood of mice that was given the test preparations of sungkai leaf extract and propolis. Segment neutrophils are non-specific body defense systems that play a role in attacking foreign substances when it enter the body for the first time.

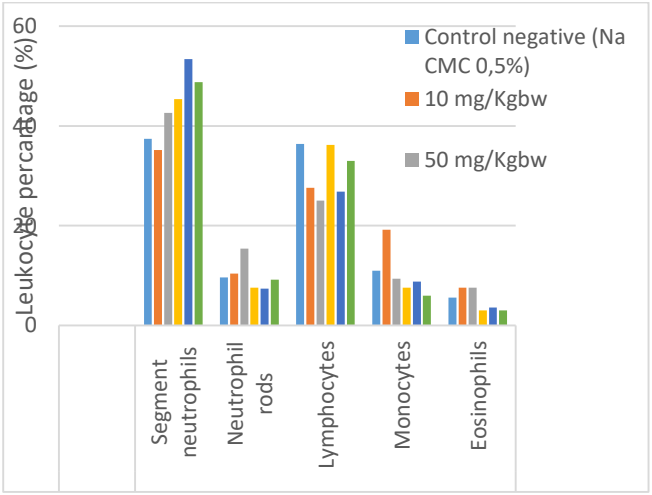

Figure 4. Graph of the percentage of leukocyte cell types

Meanwhile, the observation of basophil cells was not seen on the smears because basophil cells are soluble in Giemsa dye [10]. The smears can be seen in Figure 5 clearly.

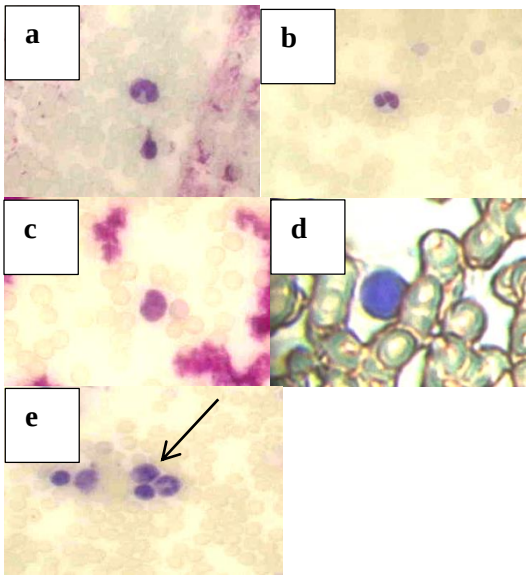

Figure 5. Types of leukocytes: a) Stem neutrophils; b) Eosinophils; c) Monocytes; d) Lymphocytes; e) Segment neutrophils

Determination of the value of the Phagocytic Index (PI) aims to eliminate microorganisms and foreign substances in the body. An increasing of the PI value indicates an increase of the phagocytic function of mononuclear macrophages and the non-specific immune system [9]. Examination of mice blood samples that use the carbon clearance method showed the PI value in Figure 6.

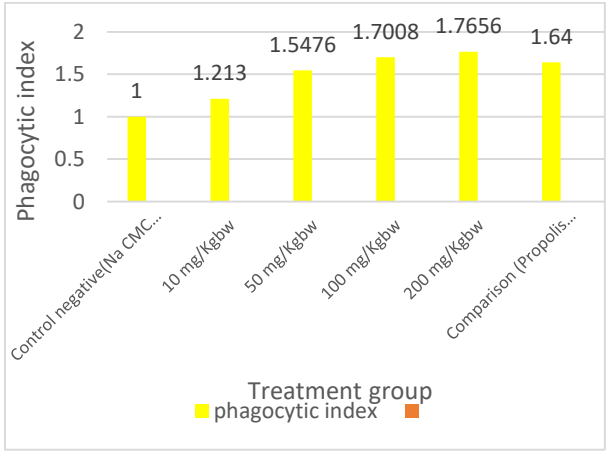

Figure 6. Graph of the phagocytic index of male white mice after giving the test preparation for six days

Based on the Figure 6., it can be seen that there has been an increase of the PI value. It indicates an increase of the blood ability phagocytize cells. The rate of carbon clearance will increase in the mice blood so carbon levels will decrease automatically. Based on these results, it can be concluded that the PI value on the test preparation sample and the comparison group is more than 1 (PI>1). These results showed that sungkai leaf extract has an immunostimulant effect.

4. CONCLUSION

The results showed that the ethanol extract of sungkai leaves had immunostimulant effect based on phagocytosis index (PI) value. It means that the ethanol extract of sungkai leaf can affect the percentage of neutrophil cell types in male white mice and affect the increase in the total number of leukocytes in male white mice significantly.

ACKNOWLEDGMENT

This work was supported by Universitas Andalas 2021, with number of contract: SP DIPA 023.17.2.677513/2021, signed on November 23rd 2020

REFERENCES

[1] Baratawidjaja K, Imunologi Dasar Edisi ke-7, Fakultas Kedokteran Universitas Indonesia Press, 2006.

[2] Tao Y, Wang D, Hu Y, Huang Y, Yu Y, Wang D, The immunological enhancement activity of propolis flavonoids liposome in vitro and in vivo. Evidence-based Complement Altern Med, 2014.

[3] Saravanan S, Prakash Babu N, Pandikumar P, Karunai Raj M, Gabriel Paulraj M, Ignacimuthu S, Immunomodulatory potential of Enicostema axillare (Lam.) A. Raynal, a traditional medicinal plant. J Ethnopharmacol. 140(2) (2012) 239–46. DOI: http://dx.doi.org/10.1016/j.jep.2012.01.010

[4] Fransisca D, Kahanjak DN, Frethernety A. Uji aktivitas antibakteri ekstrak etanol daun sungkai (Peronema canescens Jack) terhadap pertumbuhan Escherichia coli dengan metode difusi cakram Kirby-Bauer. J Pengelolaan Lingkung

Berkelanjutan. Journal Environ Sustain Manag. 4(1) (2020) 460–70.

[5] Gresinta E. Uji Potensi Ekstrak Daun Etlingera Hemisphaerica Terhadap Jumlah Leukosit Mus Musculus, 2019, pp.476–83.

[6] Das Manika, SC Dasgupta AG. Immunomodulatory and anti-neoplastic activity of common Indian. Indian J Physiol Pharmacol. 53(1) (2009)

[7] Aldi Y, Dewi ON, Uthia R. Uji Imunomodulator Dan Jumlah Sel Leukosit Dari Ekstrak Daun Kemangi (Ocimum Basilicum L.) Pada Mencit Putih Jantan. Sci J Farm dan Kesehat. 6(2) (2016) pp.139

[8] Simmons A. Technical Hematology 3rd ed, J.B. Lippicot Company, Toronto, 1980.

[9] Aldi, Yufri, Oktavia S YSB. Uji Efek Immunomodulator Dari Ekstrak Daun Manggis (*Garcinia mangostana* L) Dengan Metode Carbon Clearence Dan Menghitung Jumlah Sel Leukosit Pada Mencit Putih Jantan. 8(1) (2016)

[10] Aldi Y, Dillasamola D, Effect of *Elephantopus scaber* Linn . leaf extract on mouse immune system, 18(10) (2019) pp.2045–50.