Trop J Nat Prod Res, March 2024; 8(3):6503-6507 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

The Immunostimulatory Effects of *Peronema canescens*. Jack Leaves Extract in *Mus musculus* L. Using the Carbon Clearance Method

Syofyan Syofyan[1], Almahdy Almahdy[2], Fitri Rachmaini[2], Dwisari Dillasamola[2]*

[1]*Department of Pharmaceutics, Faculty of Pharmacy, Universitas Andalas, Padang 25163, Indonesia*
[2]*Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Andalas, Padang 25163, Indonesia.*

ARTICLE INFO

Article history:
Received 09 September 2023
Revised 29 February 2024
Accepted 05 March 2024
Published online 01 April 2024

ABSTRACT

Sungkai leaf extract *(Peronema canescens* Jack.) has been used traditionally to cure various diseases. This research aimed to determine the immunostimulant effect of sungkai leaf extract using the carbon clearance method, the total leukocyte cell count, and the percentage of leukocyte cell types. Twenty-five male mice divided into 5 groups were used for this study. The extract was administered orally for 6 days at 25, 50, and 100 mg/kgbw, Na CMC 0.5% as negative control, and Stimuno 50 mg/kgbw as a comparison (positive control) group. The phagocytic index, total leukocytes, and the percentage of leukocyte cell types were determined on the 7th day. The phagocytotic index value was analysed by one-way ANOVA followed by Duncan's post-hoc test. The increase in phagocytosis index showed that the effect of each dose was significantly different ($p< 0.05$) compared to the control group. The highest phagocytosis index was obtained from a 100 mg/kg bw dose. There was a significant difference ($p<0.05$) in the total leukocyte cell count 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

Introduction

Infectious diseases 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 have been a significant health problem that continues to develop. The critical role of the immune system in recognising and destroying pathogenic microorganisms is needed to protect the body.[1] The immune system is divided into non-specific and specific immune systems. The non-specific immune system is the innate body defence that responds quickly to destroying all foreign pathogenic microorganisms. It works through a phagocytosis mechanism by white blood cells, predominantly neutrophils, monocytes, and tissue macrophages. In contrast, specific or adaptive immune systems give the definitive response towards individual 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 and infections and as a preventive measure to increase the body's resistance to disease. One herbal plant that has the potential to be an immunostimulant is Sungkai *(Peronema canescens* Jack.). Traditional people use sungkai leaf as a medicine for fever and malaria. Sungkai contains alkaloids, flavonoids, terpenoid-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]

*Corresponding author. E mail: dwisaridillasamola@phar.unand.ac.id
Tel: +62-811-6608-808

Citation: Syofyan S, Almahdy A, Rachmaini F, Dillasamola D. The Immunostimulatory Effects of *Peronema canescens*. Jack Leaves Extract in *Mus musculus* L. Using the Carbon Clearance Method. Trop J Nat Prod Res. 2024; 8(3):6503-6507. https://doi.org/10.26538/tjnpr/v8i3.5

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

This study aimed to determine the immunostimulant activity of sungkai leaf extract using the carbon clearance method, total leukocyte cells, and the percentage of leukocyte cell types.

Materials and Methods

Plant Materials and Collection

Sungkai leaves (*Peronema canescens* Jack) were collected on January 2022 in the Aia Pacah region, Padang City, West Sumatera, with GPS location -0.8654234, 100.3869164. The plant was identified and validated at the Andalas University Herbarium, and a voucher specimen number 259/K-ID/ANDA/V/2022 was assigned.

Equipment and Chemicals

These include a Rotary evaporator (Buchi®), maceration container (brown bottle), analytical balance (Ohaus®), measuring cup (Pyrex®), oven (Memmert®), Stimuno (Dexa Medica), quercetin (Sigma-Aldrich), Chinese ink (Yamura), methanol (Brataco), and Giemsa dye (Merck). All reagents and chemicals were of analytical grade.

Preparation of the Extract

Fresh sungkai leaves (4 kg) were dried under shade and ground to a fine powder. The powdered sample (650 g) was subsequently macerated with 70% ethanol at 1:10 (2.5L x 3), sample-to-solvent, in a dark-coloured glass container for 18 hours.[9] The filtrate was dried using a rotary evaporator (Buchi®, Switzerland) at reduced pressure.[10]

Characterisation of the Extract

The plant extract was characterised using the following parameters: organoleptic examination, drying shrinkage, determination of total ash content, TLC profile, and phytochemical screening to test the content of alkaloids, flavonoids, phenolics, saponins, terpenoids, and steroids using standard methods.[11]

Determination of total ash content.
The powdered plant sample was ashed in a furnace at 500 - 600°C for 2 - 8 hours, and then the residue left was weighed and recorded. The total ash content was calculated from the weight of the powdered material before ashing.
The TLC profile of the extract was determined by spotting two test samples (T1 and T2) and a standard (S) quercetin on an F_{254} (Merck) TLC plate and developed in a chromatographic tank using a solvent system of n-hexane: ethyl acetate (2:3). The presence of phytochemical was determined by a colourimetric method.

Animal Study
Twenty-five male mice (*Mus musculus* L) weighing 35-40 grams were used for this study. The animals were acclimatised for one week, and standard animal food and water were provided *ad libitum.* Ethical approval was obtained from The Ethics Committee of The Faculty of Medicine of Universitas Andalas with an ethical letter contract number 405/UN.16.2/KEP-FK/2021. All animals were randomly divided into 5 groups, each consisting of 5 mice. The normal control group was given NaCMC 0.5%, the treatment group was given sungkai leaf extract at 25, 50, and 100 mg/kg bw, and the positive control group was given Stimuno® 50 mg/kg bw. All extracts and control were given orally a day for 6 days.

Immunomodulator Activity Test with Carbon Clearance Method
On the seventh day, 75 µL of mice blood was taken from the tail capillary and lysed with 4 mL of acetic acid 1%. This first blood was collected as a blank sample at zero (0) minutes. Then, 0.1 mL/10 g bw of carbon suspension was injected into the animals intravenously. At the 3, 6, 9, 12, and 15 minutes after carbon injection, 75 µL of mice blood was taken. Each blood was lysed with 4 mL of 1% acetic acid, and then the absorption was measured using the double beam UV-Vis spectrophotometer UV-1900i (Schimadzu®, Germany) at a wavelength of 650 nm.[10]

Total Leukocyte Cells Count with Hemocytometer
Fresh blood collected in EDTA was aspirated into a leukocyte pipette until the number 0.5, and then Turk's solution was taken until the number 11 and then shaken for 3 minutes. One drop of the mixed blood and Turk's solution was placed into a Neubauer Counting Chamber (Marienfeld Superior®, Germany). It was left to stand for 3 minutes, and the number of leukocytes was counted at the four corners of the counting chamber using the formula below.[11]

$$Total\ leucocyte\ count = Total\ leucocytes\ x \frac{20}{0.4}$$

Percentage of Leukocyte Types with Blood Smear
Fresh blood obtained from the carbon clearance method procedure was used for this determination. One drop of fresh blood was dropped on the slide, mixed with another slide, and dried. After drying, it was fixed with methanol for 5 minutes. The smeared blood on the microscopic slide was stained with a drop of Giemsa solution diluted with distilled water (1:20) for 20 minutes. The slide was washed with distilled water and then dried. The number of segmented neutrophils, banded neutrophils, lymphocytes, monocytes, and eosinophils was counted under a microscope with 1000x magnification.[1] The phagocytic activity (phagocytosis index) was determined based on the percentage of phagocytes involved in phagocytosis out of 100 phagocytes. Phagocytised beads were counted for each cell as 0-6 or more. The phagocytotic index (PI) was computed from the equation below.
PI = (% phagocytic cells containing ≥ 1 bead) × (mean number of beads/phagocytic cell containing beads).[11]

Data analysis
Phagocytosis index, total leukocytes, and percentage of leukocyte types were statistically analysed by SPSS®29.0 statistical software, one-way ANOVA, followed by Duncan's post-hoc test.

Results and Discussion

The percentage extraction yield of the powdered sungkai leaf was 21.35% crude extract. The organoleptic evaluation shows that the crude extract of sungkai leaf *(Peronema canescens* Jack.) is blackish-green and has a characteristic odour with a bitter taste. The phytochemical screening test showed the presence of different bioactive secondary plant metabolites: flavonoids, alkaloids, saponins, phenolic, and terpenoids. This result agrees with that reported by Andespal.[13] Drying shrinkage obtained in the proximate parameter determination of Sungkai leaf *(Peronema canescens* Jack.) was 9.54% and 2.77% for the ash content. The TLC profile test is a qualitative test to determine the presence or absence of compounds in sungkai leaf extract. The reference 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 F_{254} (Merck®) at the wavelength of 254 nm in the ultraviolet (UV) region.[9] From the test, the R_f point for quercetin was 0.72, equal to the R_f obtained in sungkai leaf extract. The existence of the R_f point equation from quercetin and sungkai leaf extract indicates that sungkai leaf extract contains quercetin, as seen in Figure 1.
The carbon clearance method is an immunomodulatory activity test that uses carbon particles as an antigen, given intravenously. The speed of carbon clearance was checked at intervals of 3, 6, 9, 12, and 15 minutes. Carbon is used as a marker because of its advantages, such as small and stable particle size, which does not cause blockage of blood vessels and lungs.[14] Carbon also has the characteristics of an antigen when injected into the body since it is not generally found in the body[14]. The effect of sungkai leaf extract phagocytosis was determined from the standard curve of 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 the standard carbon curve, the regression equation was y = 0.0059 x + 0.0048 with R^2 = 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]

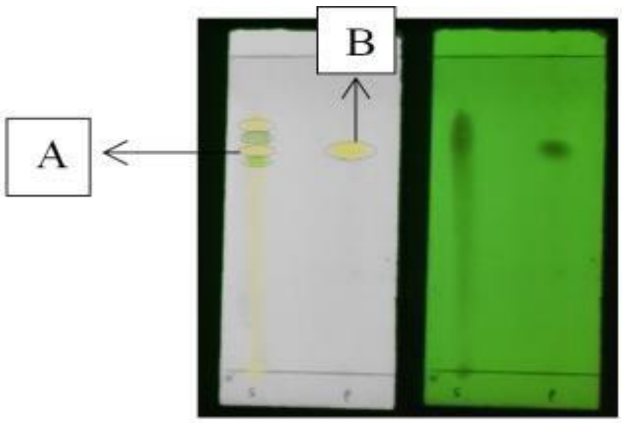

Figure 1: Thin layer chromatography profile of sungkai (*Peronema canescens* Jack.) extract. (A) Sungkai extract, (B) Quercetin.

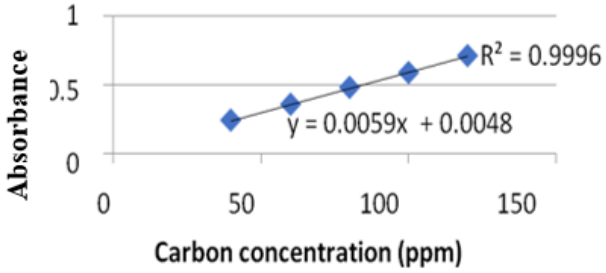

Figure 2: Calibration curve of carbon content in the blood of mice

The phagocytic constant is one of the phagocytosis parameters. The greater the value of the phagocytosis constant, the higher the carbon clearance and the ease of the phagocytosis process.[16] 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 immunostimulant activity.[16] Based on the phagocytic index values, 25, 50, and 100 mg/kgbw doses possess immunostimulant effects. The extract dose of 100 mg/kg bw showed the highest phagocytic index value, as shown in Figure 3. The phagocytic index values of the different groups, the negative control group (Na CMC 0.5%), treatment groups 25, 50, 100 mg/kg bw, and the comparison (positive control group) were 1.00, 1.269, 1.548, 1.737, and 1.555, respectively (Figure 3). The results showed that the group given 100 mg/kgbw of plant extract had the best phagocytic ability compared to the negative and positive control groups. A previous study reported that sungkai extract contains flavonoids that have immunostimulatory effects by increasing the effectiveness of lymphokine proliferation and the activity of IL-2 (interleukin 2). Activated Th1 cells (T helper 1) will produce IFN-γ (interferon-gamma), which can activate macrophages to produce nitric oxide, which can destroy pathogenic microorganisms.[17]
Total leukocyte count is determined with a Hemacytometer, in which Turk solution is used as a leucocyte staining reagent and helps to dilute and lyse erythrocytes.[18] The administration of sungkai leaf extract can increase the total number of leukocytes, as shown in Table 1 and Figure 4. The groups given leaf extract at 25, 50, and 100 mg/kgbw showed a significant difference ($p< 0.05$) compared to the control Na CMC 0.5 %. Sungkai leaf extract at 100 mg/kgbw showed the highest total leukocytes compared to doses of 25 and 50 mg/kgbw and the comparison group.
The leukocyte cell types percentage was calculated from equation 2 above after Giemsa staining. Giemsa stain only showed eosinophils, Banded neutrophils, segment neutrophils, lymphocytes, and monocytes. Basophil cells cannot be seen because the cells are soluble in Giemsa stain.[19] Leukocyte calculation is done by cross-sectioning or counting the leukocytes starting from the object's edge- 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, as shown in Table 2 and Figure 5. All the groups showed a significant difference ($p< 0.05$) compared with Na CMC 0.5% as control. The result shows an increased 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. Neutrophils are highly mobile phagocytic specialists that eat and destroy pathogenic materials in an organism.[17]

Conclusion

This study showed that sungkai leaf extract at doses of 25, 50, and 100 mg/kg bw exhibited an immunostimulating effect on male white mice (*Mus musculus* L.) through phagocytosis demonstrated by phagocytotic index value greater than 1(PI>1). The extract showed a definite increase in the total leukocytes and the percentage of segmented neutrophil cells in the treated animals. Sungkai leaf extract holds potential for use as an immunostimulatory agent, especially in patients with compromised immune systems.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors express their profound thanks to the Research Institution and Community Service (LPPM) of Universitas Andalas. This research was supported by DIPA Universitas Andalas 2021 with number SP DIPA 023.17.2.677513/2021 23rd November 2020 Padang Indonesia in the scheme of "Riset Dasar (RD) Batch 1 Contract PNBP fund number T/5/UN.16.17/PT.01.03/KO- RD/2021" signed on march, 30th 2021. In addition, the author would like to thank Dr Nurainas of Andalas University Herbarium (ANDA) from the Department of Biology, Faculty of Mathematics and Natural Science, Universitas Andalas, who supported the research.

Table 1: The 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/kgBW	8170 ± 2762.607
50 mg/kgBW	10542 ± 1910.385
100 mg/kgBW	12558 ± 2536.990
Stimuno 50 mg/kgBW	11750 ± 1560.849

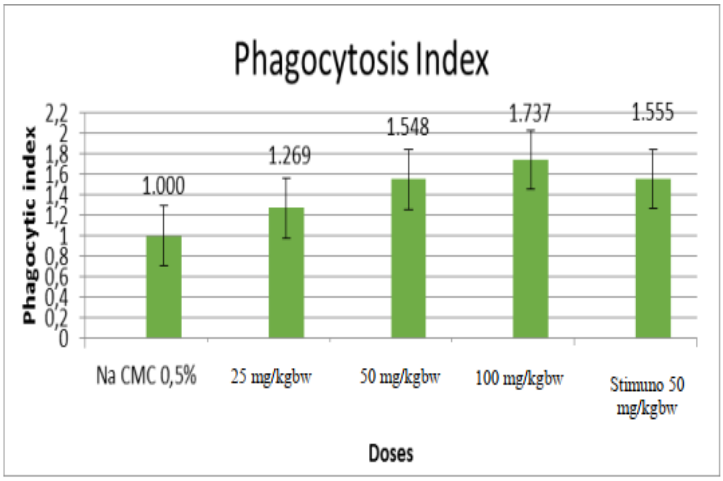

Figure 3: Graphic phagocytic index values from male white mouse blood after administration of sungkai extract (*Peronema canescens* Jack.)

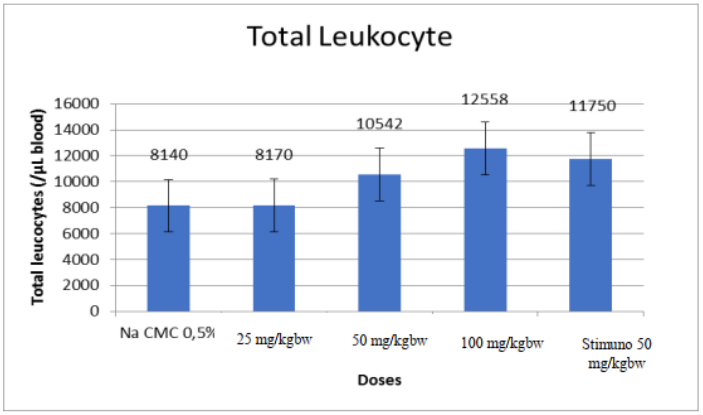

Figure 4: Bar graph of total leukocytes after administration of sungkai extract (*Peronema canescens* Jack.)

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

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

	NaCMC 0.5%	25 mg/kgBW	50 mg/kgBW	100 mg/kgBW	Stimuno 50 mg/kgBW
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

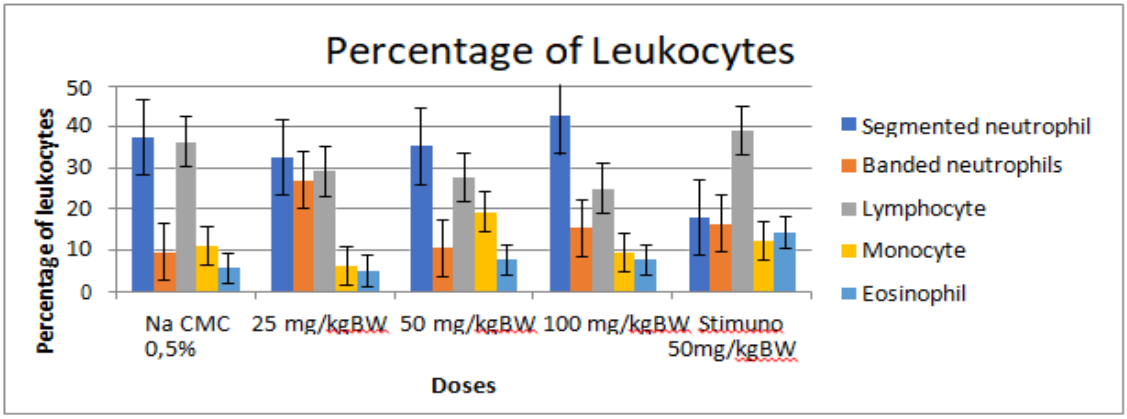

Figure 5: Bar graph of the percentage of leukocytes after administration of sungkai extract (*Peronema canescens Jack)*

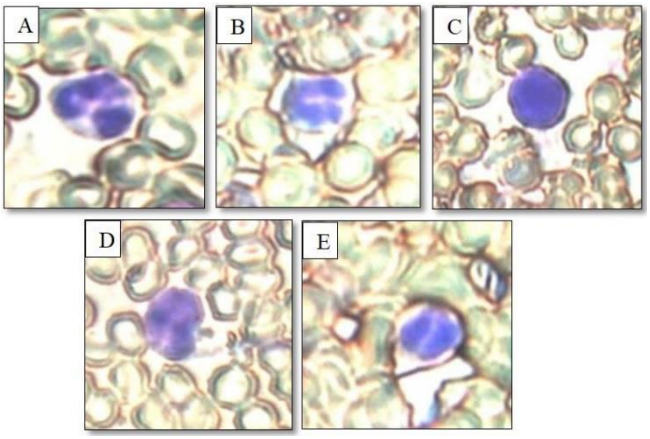

Figure 6: White male mice leukocytes. (A) Segmented neutrophil, (B) Banded neutrophils, (C) Lymphocyte, (D) Monocyte, (E) Eosinophil

References

1. Baratawidjaja KG RI. Basic Immunology Edition 11. Jakarta: Balai Penerbit Fakultas Kedokteran Indonesia; 2018.
2. Abbas K, Lichtman A PS. Basic Immunology : Function and Disorders of The Immune System. 5th Editio. Canada: Elsevier Inc; 2016.
3. Aldi Y, Rasyadi Y, Handayani D. Immunomodulatory Activity of Meniran Extracts (*Phyllanthus niruri* Linn.) on Broiler Chickens. J Sains Farm Klin. 2014;1(1):20–6.
4. Ibrahim A, Kuncoro H. Identification of Secondary Metabolites and Antibacterial Activity of Sungkai Leaf Extract (*Peronema canescens* Jack.) Against Several Pathogenic Bacteria. J Trop Pharm Chem. 2012;2(1):8–18.
5. Ningsih, Arna dan Ibrahim A. Antimicrobial Activity of Sungkai Leaf (*Peronema canescens* Jack) n-Hexane Fraction Extract Against Some Bacteria Using TLC-Bioautography Method. J Trop Pharm Chem. 2013;2(2):76–82.
6. Andriani Fenny, Agus Sundaryono, Nurhamidah. Antiplasmodium Activity Test of the N-Hexane Fraction of *Peronema Canescens* Leaves Against *Mus Musculus*. Alotrop. 2017;1(1):33–8.
7. Fransisca D, Kahanjak DN, Frethernety A. Test antibacterial activity of ethanol extract of sungkai leaves (*Peronema canescens* Jack.) on the growth of *Escherichia coli* using the Kirby-Bauer disc diffusion method. J Environ Sustain. 2020;4(1):460–70.
8. Yani AP. Potential Test of Sungkai Young Leaves (*Peronema canescens*) for Health (Immunity) in Mice (*Mus Muculus*). Pros Semin Biol. 2014;11(1):245–50.
9. RI Ministry of Health. Indonesian Herbal Pharmacopoeia. Edisi 2. Jakarta: Ministry of Health of The Republic of Indonesia; 2017.
10. Dillasamola D, Aldi Y, Kolobinti M. The effect of coriander ethanol extract (*Coriandrum sativum* L.) against phagocytosis activity and capacity of the macrophage cells and the percentage of leukocyte cells in white male mice. Pharmacogn J. 2019;11(6):1290–8.
11. Aldi Y, Dillasamola D, Florina T, Friardi D. Test immunomodulatory effects of ethanol extract skin of purple sweet potato (*Ipomoea batatas* (L.) Lam) with carbon clearance method and the number of leukocytes. Res J Pharm Biol Chem Sci. 2016;7(5):178–86.
12. Afriwardi, Aldi Y, Dillasamola D, Larakhansa YA, Badriyya E. Immunostimulatory activities of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) in white male mice. Pharmacogn J. 2021;13(2):368–75.
13. Andespal. Phytochemical Profiles of Sungkai Leaves (*Peronema canescens*) and Antioxidant and Cytotoxic Activity Tests of Sungkai Leaf Ethanol Extract. Universitas Bengkulu; 2020.
14. Aldi Y, Oktavia S, Yenni B. Immunomodulatory Effect Test of Mangosteen Leaf Extract (*Garcinia mangostana* L.) Using Carbon Clearance Method and Counting Leukocyte Cell Counts in Male White Mice. J Farm Higea. 2016;8(1):20–31.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

15. Alfitasari, D. A., Kusuma, A. M. and Hakim ZR. Immunomodulatory Activity of Shallot Ethanol Extract (*Allium cepa* L.) on Non-Specific Immune Response in BALB/C Strain Male Mice with Carbon Clearance Method. Biosfera. 2016;34(2):75.
16. Aldi Y, Ogiana N, Handayani D. Immunomodulator Test of Several Subfractions of Meniran Ethyl Acetate Extract (*Phyllanthus niruri* L.) in Male White Mice Using Carbon Clearance Method. B-Dent, J Kedokt Gigi Univ Baiturrahmah. 2018;1(1):70–82.
17. Aldi Y, Husni E, Yesika R. Activity of kincung flowers (*Etlingera Elatior* Jack) on total leukocytes and percentage of leukocytes in allergic male white mice. Pharmacogn J. 2020;12(1):44–51.
18. Moyes, C. D. & PS. Principles of animal physiology. Vol. 754. San Francisco: Pearson/Benjamin Cummings.; 2008.
19. Aldi Y, Megaraswita, Dillasamola D. Effect of *Elephantopus scaber* linn. Leaf extract on mouse immune system. Trop J Pharm Res. 2019;18(10):2045–50.
20. Nugroho RA. Get to know mice as laboratory animals. Samarinda: Universitas Mulawarman; 2018.