Pharmacogn J. 2021; 13(6): 1397-1407
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Study of Sungkai (Peronema canescens, Jack) Leaf Extract Activity as an Immunostimulators With *In vivo* and *In vitro* Methods

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History

- Submission Date: 14-09-2021;
- Review completed: 08-10-2021;
- Accepted Date: 29-10-2021.

DOI : 10.5530/pj.2021.13.177

Article Available online

http://www.phcogj.com/v13/i6

ABSTRACT

Introduction: Sungkai (*Peronema canescens*, Jack.) contains polysaccharides, terpenoids, alkaloids, and polyphenols which have pharmacological activity as immunostimulants. **Objective:** This study aimed to see how the effect of Sungkai extract as an immunostimulant agent was carried out *in vitro* and *in vivo*. **Materials and Methods:** This study was conducted using two methods, namely *in vivo* and *in vitro*. *In vivo* research method was conducted to test the activity and phagocytic capacity of macrophage cells, the percentage of leukocytes, and the total number of leukocytes. This study used 30 male white mice as the test animals that were randomly divided into 5 treatment groups. Each group was consisting of 6 mice which were given different treatments. The negative control group was given with the 0.5% NaCMC suspension, the mice test substance group was given with the suspension of Sungkai ethanol extract with various doses of 800, 400, and 200 mg/kgBW, and lastly the comparison group was given with the Stimuno in a dose of 50 mg/kg orally for 7 days. On day 8, blood was taken from the mice's vein to count the number and percentage of its leukocytes, then followed by the intraperitoneal injection of a Staphylococcus aureus bacteria suspension. After 1 hour of administration of the bacterial suspension, the peritoneal fluid was taken to be observed for its activity and phagocytic capacity of macrophage cells. The *in vitro* research method was used to test the viability and immunostimulatory activity of RAW 264.7 cells with the Sungkai extraction at the concentration of 1.10, 100 g/m. This cell viability test using the microtetrazolium (MTT) method aims to see whether the Sungkai sample used is safe and not toxic to RAW 264.7 cells by observing at the cell viability value that should exceed >90%. The concentration of Sungkai extraction at 1.10, 100 g/mL was found to be safe and non-toxic to RAW 264.7 cells with a viability value of >90%. Thus, this concentration of Sungkai extraction can be performed for its immunostimulatory activity test on LPS induced of RAW 264.7 cells by observing their levels of IL-6 and TNF-α. (proinflammatory cytokines) were compared with the LPS alone as a control using the sandwich ELISA (Enzyme-Linked Immunosorbent Assay) method. **Results:** The observations were analyzed by one-way ANOVA and Duncan's follow-up test (significance was taken at $p<0.05$). The results showed that variations in concentration increased significantly ($p<0.05$) on the activity and phagocytic capacity of macrophage cells, along with the total leukocyte cells. The percentage of leukocytes showed that the cells had a significant increase ($p<0.05$). It was found that the Sungkai extraction on 1.10, 100 g/mL could significantly increase the concentration of TNF- and IL-6 ($p<0.05$) which were tested by one-way ANOVA and followed by Duncan's post hoc test. **Conclusion:** Sungkai leaf extract (Peronemacanescsens Jack.) in a dose of800, 400, and 200 mg/kgBW has an immunostimulant effect both *in vivo* and *in vitro*.

Key words: Sungkai (Peronema canescens, Jack), phagocytosis, macrophages, total and percentage of leukocytes, MTT (Microtetrazolium), LPS (lipopolysaccharide), RAW 264.7 cells, cell viability, immunostimulant.

INTRODUCTION

The immune system is the human body's defense mechanism that functions to respond against "attacks" from outside the body (1). When microbes enter the human body, they will pass through 3 layers of immune system defense. The first layer of this defense containing the non-specific, especially physical/mechanical, biochemical, and humoral immune system. This defense will prevent the entry of microbes into the human body. The second layer contains the non-specific immune system, especially cellular defense. This one will prevent microbes that have managed to enter the body by destroying them (2). The third layer contains the specific immune system which will deal with micro-organisms that have not been handled by the non-specific immune system (3).

One of the human body's efforts to defend itself against the invasions of antigens is to destroy the bacteria by the non-specific process of phagocytosis. Phagocytosis is a defense mechanism that works on digesting foreign particles or microorganisms by the phagocytic cells to destroy them into pieces (4).

The immune system can be divided into two types; the non-specific immune system (innate immunity) and the specific immune system (adaptive immunity). The non-specific immune system has a faster activity because it does not involve the memory cells (5). Several components that are involved in the non-specific immune system are macrophage cells (phagocytic white blood cells) and natural killer cells which protect the body from pathogen attacks so that the body eventually builds its defense system. The body's defense system can be activated by providing compounds that can increase the body's immune response. Many of these compounds are found in plants that can stimulate these immune response functions which are called immunomodulators (6).

An immunomodulator is a substance or compound that can modulate the activity and function of the body's immune system by dynamically regulating

Cite this article: Dillasamola D, Aldi Y, Wahyuni FS, Rita RS, Dachriyanus, Umar S, Rivai H. Study of Sungkai (*Peronema canescsens*. Jack.) Leaf Extract Activity as an Immunostimulators With *In vivo* and *In vitro* Methods. Pharmacogn J. 2021;13(6): 1397-1407.

immune cells such as cytokines (6). Immunomodulators are divided into three groups; the immunostimulators that function to increase the activity of the immune system, the immunoregulators that can regulate the immune system, and the immunosuppressors to inhibit or suppress immune system activity (7).

Traditional medicine plays an important role in stimulating and suppressing the immune response. Natural substances derived from plants can be functional as immunomodulators to control certain immune responses (8). One of the plants that can potentially be used as a natural ingredient that might improve the immune system is the Sungkai plant (*Peronema canescens* Jack). Sungkai (*Peronema canescens* Jack) is one of the leading export commodities specifically for Sumatra and Kalimantan. In Fransisca's research (2020), an analysis of the active substances in Sungkai leaves was carried out before it was being made into extracts. The test results showed that the active substances in Sungkai leaves were flavonoids, alkaloids, steroids, phenolics, tannins, and saponins. The Sungkai leaf compounds that were listed in the test results have the main activity as an antibacterial (9).

According to Gresinta (2012), the compounds that might have bioactivity as the immunostimulant agents are the groups of polysaccharide compounds, terpenoids, alkaloids, and polyphenols (10).

In the non-specific immune system, macrophage cells will release some pro- inflammatory cytokines such as interleukins (IL-12, IL-1β, IL-6) and tumor necrosis factor-alpha (TNF-α). IL-6 belongs to a group of pro-inflammatory cytokines. TNF-α has several activities such as increasing the prothrombotic role, stimulating the adhesion molecules from leukocyte cells and inducing endothelial cells, as well as regulating macrophage activation and immune responses in tissues by stimulating its growth factors and other cytokines (11). The presence of macrophage activation is also an early stage of bio-information transfer to produce immunomodulatory activity (12).

RESEARCH METHODS

Place and Time

This research was conducted from January to May 2021 at the Research Laboratory of Immunology and Serology, Faculty of Cell Culture, Pharmacology and the Central Laboratory of the Faculty of Pharmacy, Andalas University.

Tools

The tools used were measuring cup (Pyrex), Erlenmeyer (Pyrex), needle probe (Terumo), rotary evaporator (Ika), filter paper (Whatman), analytical balance (Ohaus), microscope (Olympus), centrifuge (Oregon), pipette leukocytes (Assistant), mouse cage, surgical scissors (OneMed), Evaporator (Buchi R-210 Rotavapor), UV-vis spectrophotometer (Thermo Scientific Genesys 10S UV-Vis), UV-lamp (Camag), Incubator 37°C/5%CO2 (Thermo Scientific®), microbiological safety cabinet air flow class II (Thermo Scientific®), water bath (Memert®), microplate reader (Bio-Rad®), centrifuge (Thermo Scientific®), conickel tube (Falcon®), tube eppendorf, inverted microscope (Zeiss®), plate 96 and 24 wells, plastic bag, tissue, spray bottle, T-25 flask (Iwaki®), micropipette (Ecopipette®), hemacytometer, analytical balance (Mettler Toledo®), cabinet ice (Samsung®), Incubator, 37°C/5%CO2 (Thermo Scientific®), microbiological safety cabinet air flow class II (Thermo Scientific®), water bath (Memert®), micropla te reader (Bio-Rad®), centrifuge (Thermo Scientific®), conickel tube (Falcon®), eppendor tube.

Materials

The materials used were Sungkai extract (Peronema canesens), 0.5% sodium CMC, 70% Ethanol, Ethanol P, NaCl 0.9%, Aquadest, Stimuno (Dexa Medica, Batch No.: SLBZ4289), Quercetin (Sigma-Aldrich , CAS Number: 117-39-5), Aquadest (Andeska Laboratory), Staphylococcusaureus (Microbiology Laboratory, Faculty of Pharmacy, Andalas University), Nutrient Agar (Merck), Nutrient Brooth (Merck), Turk's solution (Sagara Husada Mandiri), RAW 264.7 cells (ATCC®TIB-71TM), Dulbecco's Modified Eagle Medium (DMEM) (Gibco), Fetal Bovine Serum (FBS) (Gibco), Dimethyl Sulfoxide (DMSO), Penicillin-Streptomycin 2% (v/v) (Gibco), Trypsin-EDTA (Gibco), 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl Tetra Zolium Bromide (MTT) (Sigma), Phospate Buffer Saline (PBS), Lipopolysaccharide (LPS) (Sigma), Mouse TNF-α ELISA kit, Mouse IL-6 ELISA kit, and Mouse NO ELISA kit (Bio Technology Laboratory®), F254, Mayer reagent, Dragendorf reagent, HCl, Metal Mg, FeCl3, HgCl2, AlCl3, Sodium Acetate, Geimsa coloring kit (Merck), white male mice, Wright Stain, and EDTA.

Procedure

This study were provided with *in vivo* and *in vitro* test methods. The *in vivo* testing was carried out using mice (Mus musculus), by looking at their phagocytic cells activity, percentage, and total number of the leukocyte cells. Meanwhile, the *in vitro* testing was carried out using the MTT cytotoxic test method and cytokines such as Interleukin 6 and TNF- α.

Sungkai Extract Preparation

The Sungkai plant samples that had been chopped into smaller sizes in a weight of 1 kg was macerated using 70% ethanol solvent in a dark-brown bottle. The ratio number of samples and solvents used were 1:10. After the sample was soaked with the ethanol, it was stirred periodically every 6 hours then let stand for 18 hours, and repeated over for 3 days before the solvent was filtered to obtain the macerate. The filtration process was being repeated at least 2 times with the same amount and type of solvent (13). The macerations that were collected was going through an evaporation process with a rotary evaporator until a desired thick extract was able to be obtained (14). The viscous extract obtained was used for organoleptic examination, phytochemical screening, and TLC profile.

In vivo Research Method Test Animal Setup

The animals used were 30 white male mice of 2-3 months aged with body weights of 20-30 gram and had never been treated with drugs. These test animals were grouped into 5 groups which were consist of 6 mice each. The first group was given with 5% NaCMC solution, meanwhile the 2nd, 3rd, and 4th group were given with Sungkai extract in a dose of 800, 400, and 200 mg/kgBW. The last group was given with Stimuno supplement in a dose of 50 mg/kg. The test preparation was given orally for 7 days.

Bacterial Culture

Staphylococcusaureus (SA) was cultured on an inclined Nutrient Agar (NA). A single oseof SA culture was inoculated into new NA medium, then incubated at 37°C for 24 hours in an incubator. The Staphylococcusaureus that growing on Nutrient Agar media was transferred to Nutrient Broth, continued to be incubated for 24 hours at 37°C, then centrifuged at 2500 rpm for 25 minutes until pellets were formed and suspended with NaCl 0.9% (15).

Total Leukocyte Calculation

Fresh blood was taken into a leukocyte pipette to the exact number of 0.5 and the following truck solution was taken up to the exact number

of 11. It was shaken for three minutes before one until two drops were discarded from the leukocyte pipette and another drop was added to the hemacytometer counting chamber. The liquid was let sit for 2 minutes for the leukocytes to settle. The number of leukocytes then was counted on the four count rooms (16).

$$\text{Total Leukocytes Count} = \text{Total Leucocytes} \times \frac{20}{0.4}$$

On the eighth day, the tails of mice were cut and blood smears were made and then dried. After drying, it was dripped with methanol, so that it coats the entire blood smear and was left for 5 minutes. The blood smear then was stained with Giemsa and was left for 20 minutes. It was then washed with distilled water, dried, and added with immersion oil before it was observed under an ocular microscope. The number of eosinophils, rod neutrophils, segment neutrophils, lymphocytes, and monocytes was counted at 1000x magnification (16).

Determination of Macrophage Cell Phagocytic Activity and Capacity

After acclimatization for 7 days, the mice were given with a suspension of Sungkai plant ethanol extract for 7 days. On the eighth day, after the blood of the mice was taken, the mice in each group were injected with 0.5 mL of Staphylococcusaureusbacteria suspension intraperitoneally, then left for 1 hour. After that, the mice were killed and dissected. Peritoneal fluid was taken using a micropipette and smears were made on slides and fixed with absolute methanol for 5 minutes, then stained with Giemsa which had been diluted with distilled water for 20 times, allowed to sit for 20 minutes, then rinsed with running water before getting dried. The preparations were viewed under an ocular microscope using immersion oil at a magnification of 1000 times. The phagocytic activity of macrophage cells was determined based on the percentage of phagocytes that carried out phagocytosis from 100 phagocytic cells. The value of phagocytic capacity was determined based on the number of SA bacteria phagocytized by 50 active phagocytic cells (17).

$$\% \text{ Phagocytic Activity} = \frac{\text{Total Active Macrophages X } 100\%}{\text{Total Macrophages}}$$

The data on the activity and phagocytic capacity of macrophage cells, the total number of leukocytes, and the percentage of leukocytes obtained from the research results were statistically analyzed using the one-way Analysis of Variance (ANOVA) method and continued with Duncan's analysis using SPSS statistical software.

In vitro Research Method

Cell Viability Test (MTT) Cell Laying

Add as much as 180 L of the preparation and calculated cell suspension (amount and volume measured) into each plate 96-well plate except for blank wells which only contains the medium. It was incubated for 24 hours at 37°C, 5% CO2 (18).

Test Solution Laying

20 L of the prepared test solution was transferred into each test well on a 96-well plate containing the previous cell suspension. The control well was only filled with 180 L of cell suspension, and the blank one was only filled with 200 L of medium. The plate was again incubated for 24 hours in an incubator for 24 hours at 37°C, 5% CO2. Observe the changes that occur in the cells during the incubation period.

MTT Solution Laying

20 L of 2 mg/mL MTT solution was pipetted into each well then was incubated for 3-4 hours at 37°C, 5% CO2. After 3-4 hours, a purple precipitate of formazan crystals will be seen. The supernatant was discarded, then the remaining supernatant was drained with a tissue. After leaving only the formazan crystal violet precipitate, the precipitate was dissolved with 100 L of DMSO in each well. The absorption was measured with a microplate spectrophotometer with a wavelength of 550 nm (18).

By using absorbance data that was obtained from measurements, the percentage of cell viability can be determined using the following formula: (18).

Cells/mL = Average Cells per Room x Dilution Factor x 104

Immunomostimulant Activity Test (TNF-α and IL-6)

Cell Laying

Enter as much as 900μL of the cell suspension that has been made and counted (amount and volume measured) into each 24-well plate except for blank wells which only contain medium. Then incubate in an incubator for 24 hours at 37°C, 5% CO2.

Test Solution Laying

100 L of the prepared test solution was transferred into each of the test wells on a 24-well plate containing the previous cell suspension. The normal control well was only filled with 900 L of cell suspension, and the blank was only filled with 900 L of medium. The plates were again incubated for 2 hours. After 2 hours, add the LPS solution into each well until a concentration of 1 g/mL was obtained and incubated again for 24 hours. After that, the medium was taken and centrifuged at 2000 RPM for 20 minutes at 2-8oC. The supernatant portion was taken to measure the levels of TNF-α, and IL-6 by ELISA method.

Data analysis

All data were expressed as mean ± standard deviation (SD) of the results obtained from 3 repetitions of one-way Analysis of Variance (ANOVA) with Duncan's test using SPSS statistical software. The p value < 0.05 indicates a significant difference.

RESULTS AND DISCUSSION

The identification was carried out at the Herbarium of Biology Department, Faculty of Mathematics and Natural Sciences, Andalas University. The plant used was Sungkai herbs (*Peronema canescsens* Jack.) from Lamiaceae family (Figure 1).

In the extraction process, 402.32 g of extract was obtained from 1 kg of simplicia powder with a yield value of 21.35%. The results of the extraction process have met the requirements set by the 2008 Herbal Pharmacopoeia. Organoleptically, Sungkai extract was obtained in the form of a thick extract, greenish brown in color, characteristic odor, and bitter taste. The results of the phytochemical screening examination revealed that the ethanol extract of Sungkai contained alkaloids, flavonoids, phenolics, and saponins and terpenoids.

Figure 1: *Peronema canescsens* Jack

Thin layer chromatography profile examination is a qualitative test to identify the identity compound of quarcetin in Sungkai herbal extract. The stationary phase used is silica gel F254 TLC plate. The mobile phase used was n-hexane: ethyl acetate (6:4). TLC profile of extract viewed under UV light with a wavelength of 254 nm, the Rf value of Sungkai extract was 0.51 while the Rf of pure quarcetin was 0.51. This indicates that the extract used contains quarcetin compounds because it has the same Rf value (Figure 2).

The results of the phagocytic test activity of groups 1, groups 2, 3 and 4 which are test groups with doses of 800, 400, and 200 mg/kgBW and group 5 (Stimuno) at a dose of 50 mg/kg can be seen in Table 1. Animals given sungkai extract showed greater phagocytic activity of macrophage cells than animals given 0.5% Na CMC. After ANOVA was performed, it turned out that the sungkai extract significantly affected the phagocytic activity of macrophage cells ($p<0.05$). Next, Duncan's further test was carried out, it turned out that the larger the dose of Sungkai extract given the macrophage activity also increased ($p<0.05$). The phagocytic activity of animal macrophage cells given a dose of 800 mg/kgBW Sungkai herb extract was the same as that of animals given stimulation ($p>0.05$).

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The results of the calculation of the phagocytic capacity of macrophage cells can be seen in Table 2. The test results of the test group at doses of 800, 400, and 200 mg/kgBW of ethanolic extract of Sungkai herb significantly affected the phagocytic capacity of macrophage cells in test animals ($p<0.05$). Animals given ethanol extract of Sungkai herb showed greater phagocytic capacity of macrophage cells than animals given 0.5% Na CMC ($p<0.05$). The dose of 50 mg/kgbw showed the phagocytic capacity of macrophage cells which was not significantly different from the dose of 200 mg/kgbw ($p>0.05$). According to research that had been done previously, flavonoid compounds have been shown to increase IL-2 and lymphocyte proliferation. Lymphocyte proliferation will affect CD4+ cells, which will activate Th1 cells.

Activated Th1 cells will affect SMAF (Specific Macrophage Activating Factor). SMAF (Specific Macrophage Activating Factor) is a multiple molecule, one of which is IFN-γ. IFN-γ will activate macrophages, so that macrophages will experience increased phagocytic activity and capacity. This will cause macrophages to kill bacteria more quickly (20).

In the calculation of the total number of leukocytes, the results of the increase in the total number of leukocytes from the five experimental animal groups can be seen in Table 3. The test results of the test group at doses of 200, 400 and 800 mg/kgbw of the ethanol extract of the Sungkai herb significantly affected the total leukocyte cell count of the test animals. ($p<0.05$). Animals given ethanol extract of Sungkai herb showed a higher total leukocyte cell count than animals given 0.5% Na CMC ($p<0.05\%$). At a dose of ethanol extract of the Sungkai herb 800 mg/kgbw, the total leukocyte count was greater than the doses of 200 and 400 mg/kgbw ($p<0.05$). At a dose of 50 mg/kgbw, the total leukocyte count was not significantly different from the dose of 800 mg/kgbw ($p>0.05$). The total leukocyte cell count of animals given a dose of 800 mg/kgbw of ethanol extract of Sungkai herb was not significantly different from that of animals given stimulation ($p>0.05$). According to previous research, an increase in the total number of leukocytes indicates an increase in the immune response.

The results of the calculation of the percentage of leukocytes can be seen in Table 4. The results of the Anova test group at doses of 200, 400, and 800 mg/kgbw of sungkai extract significantly affected the percentage of neutrophil cell segments and the percentage of lymphocytes ($p<0,)5$)

Table 1: Average percentage of phagocytic activity of peritoneal macrophage cells of male white mice after administration of Sungkai extract (Peronema canescsens Jack.).

Percentage of the phagocytic activity of	macrophage cells (%)
Dosage	Average ± SD
Na CMC 0,5 %	55,83 ± 7,11
Stapylococcusaureus	60,17 ± 1,95
200 mg/kgbW	72,00 ± 3,52[c]
400 mg/kgbw	85,33 ± 4,41[d]
800 mg/kgBW	88,00 ± 3,41[d]
Stimuno 50 mg/kgbw	90,67 ± 6,62

Table 2: Average phagocytic capacity of peritoneal macrophage cells of male white mice after administration of Sungkai extract (PeronemacanescsensJack.)..

Macrophage Cells Phagocytic Capacity	
Dosage	Average ± SD
Na CMC 0,5 %	67,17 ± 7,91
Stapylococcusaureus	134,83 ± 18,35
200 mg/kgbw	188,33 ± 8,52
400 mg/kgBW	197,33 ± 8,41
800 mg/kgBW	309,17 ± 7,88
Stimuno	363,83 ± 16,14

Table 3: Average total number of leukocytes in male white mice after administration of Sungkai extract (PeronemacanescsensJack.).

Total Leukocytes (/µL of blood)	
Dosage	Average ± SD
Na CMC 0,5 %	5533± 163,29
Stapylococcusaureus	8676 ± 190,44
200 mg/kgBW	11368± 321,58
400 mg/kgBW	11983 ± 385,57
800 mg/kgBW	12516± 952,72
Stimuno	13141 ± 252

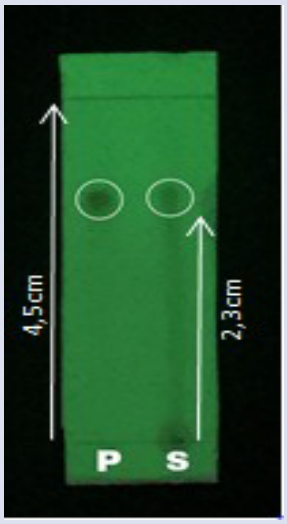

Figure 2: Thin layer chromatography results of ethanol extract of Sungkai herb. Description: S = sungkai, P = quercetin.

Table 4: Average number of leukocyte cell percentages in male white mice after administration of Sungkai extract (*Peronema canenscens*. Jack)

Leukocyte Cells Percentage (%)					
Dosage	Average ± SD				
	Segment Neutrophils	Stem Neutrophils	Eosinophils	Lymphocytes	Monocytes
Na CMC 0.5%	50.50 ± 4.50	10.50 ± 3.27	3.83 ± 1.34	27 ± 2.08[a]	6 ± 1.63
Stapylococcus aureus	61.66 ± 3.66	14.83 ± 2.78	3.83 ± 1.34	41.83 ± 0.76	5.83 ± 5.33
200 mg/kgbw	61.33 ± 3.50	24.66 ± 7.11	6.5 ± 0.74	50.50 ± 4.85[ab]	7.66 ± 3.67
400 mg/kgbw	66.83 ± 3.18	25.66 ± 3.44	5.83 ± 2.30	53.83 ± 8.89	7.83 ± 3.85
800 mg/kgbw	72.33 ± 3.93	30.33 ± 2.42	7 ± 0.95	61.50 ± 8.21	8.83 ± 3.19
Stimuno	81.50 ± 5.35[ab]	34.16 ± 3.18	8 ± 0.94	63 ± 1.63[c]	9.83 ± 1.10

Table 5: Absorbance value of sungkai treatment on cell viability test of RAW 264.7.

Sungkai (g/mL)	Absorbent			Cell Viability (%)			average ± SD
	1	2	3	1	2	3	
1	2,304	2,350	2,891	130,43	124,32	128,89	128,10 ± 2,85
10	2,896	2,721	2,661	135,32	127,01	124,16	128,83 ± 5,79
100	2,793	2,677	2,762	135,69	136,69	135,08	135,82 ± 0,81

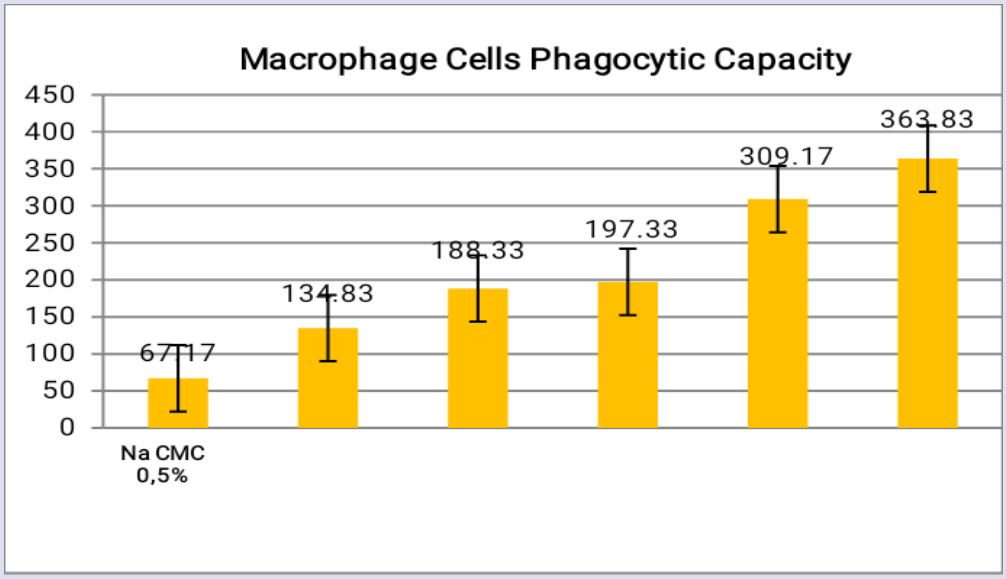

Figure 3: Bar graph of the dose of Sungkai extract on the phagocytic activity of macrophage cells of male white mice.

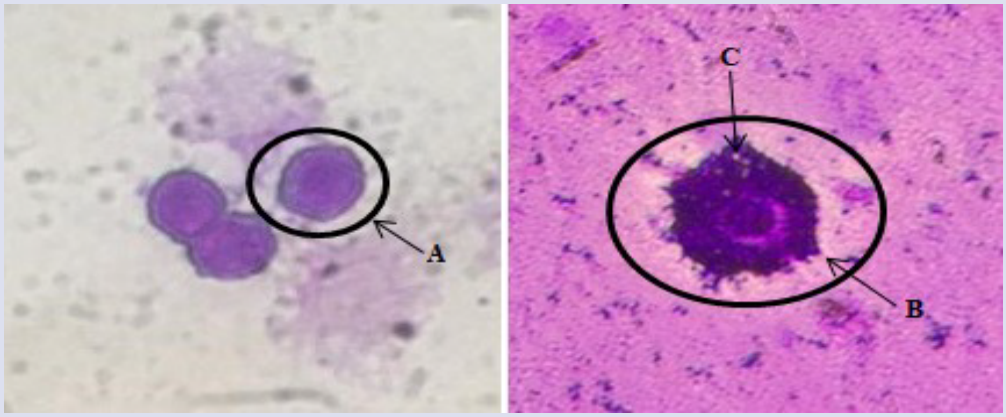

Figure 4: Bar graph of the dose of Sungkai (*Peronema canescens* Jack.) extract. on the phagocytic capacity of peritoneal macrophage cells in male white mice.

Table 6: Absorbance data and TNF- levels from control, LPS, and Sungkai in 264.7. RAW cells.

Groups	Absorbent			TNF-α Concentration (ng/mL)			Average ± SD
	1	2	3	1	2	3	
Untreated	0,069	0,070	0,073	144,515	148,577	160,048	151,05 ± 8,06
Control	0,230	0,235	0,227	436,201	441,837	432,779	436,94 ± 4,57
Sungkai (1 g/mL)	0.214	0,297	0,301	417,568	505,752	509,557	477,63 ± 52,05
Sungkai (10µ g/mL)	0,466	0,516	0,555	644,608	679,480	705,293	676,46 ± 30,46
Sungkai (100µ g/mL	0,372	0,422	0,462	572,257	611,986	641,722	608,66 ± 34,85

Table 7: Absorbance data and IL-6 levels from control, LPS, and sungkai in 264.7 RAW cells.

Groups	Absorbent			IL-6 Concentration (ng/mL)			Average ± SD
	1	2	3	1	2	3	
Untreated	0,108	0,114	0,116	1,280	1,403	1,444	1,38 ± 0,09
Control	0,211	0,226	0,245	3,613	4,016	4,560	4,06 ± 0,48
Sungkai (1 g/mL)	0,238	0,274	0,262	4,346	5,470	5,075	4,97 ± 0,57
Sungkai (10µg/mL)	0,251	0,248	0,258	4,731	4,641	4,948	4,77 ± 0,3
Sungkai (100µ g/mL	0,256	0,263	0,255	4,886	5,107	4,855	4,95 ± 0,15

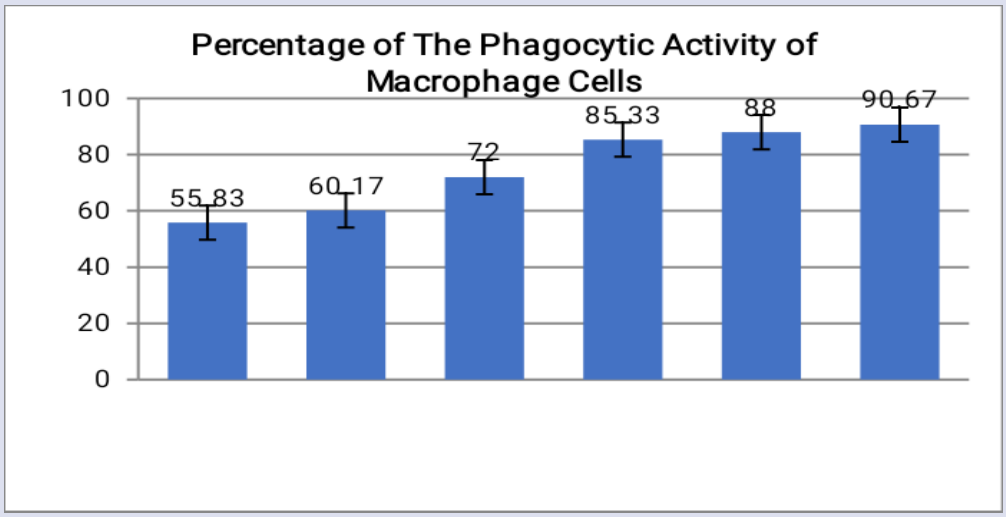

Figure 5: Peritoneal macrophage cells of male white mice Description: (A) macrophages (B) active macrophages (C) Staphylococcus aureus bacteria.

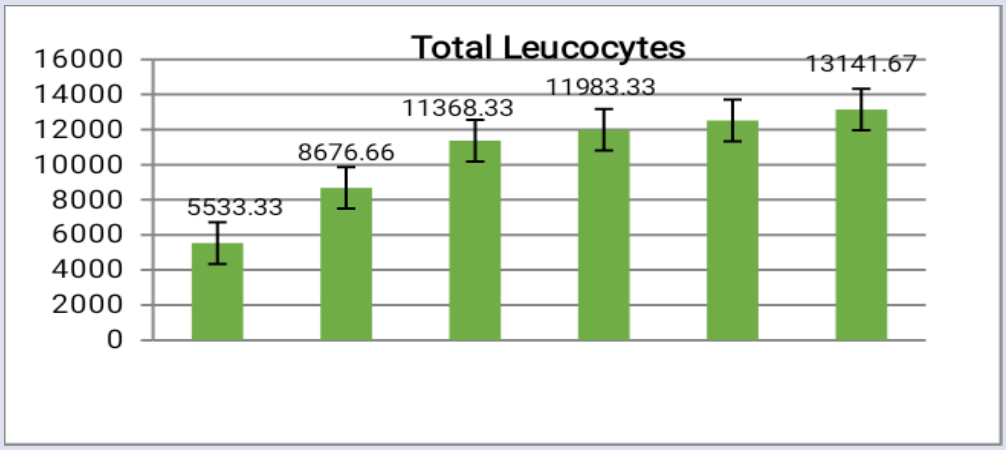

Figure 6: Bar graph of the dose of Sungkai (*Peronemacanescsens*.) extract. on the total number of leukocytes in male white mice.

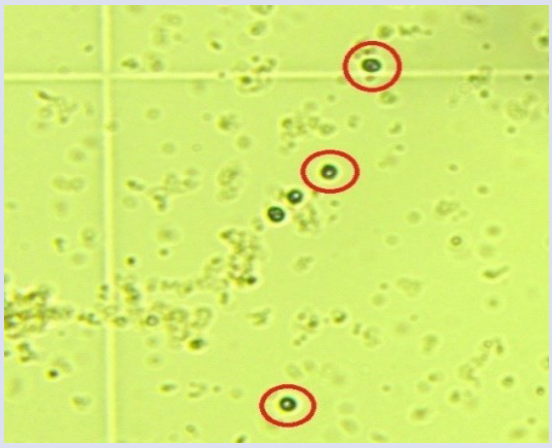

Figure 7: Leukocyte cells of male white mice observed under a microscope at 400 times magnification.

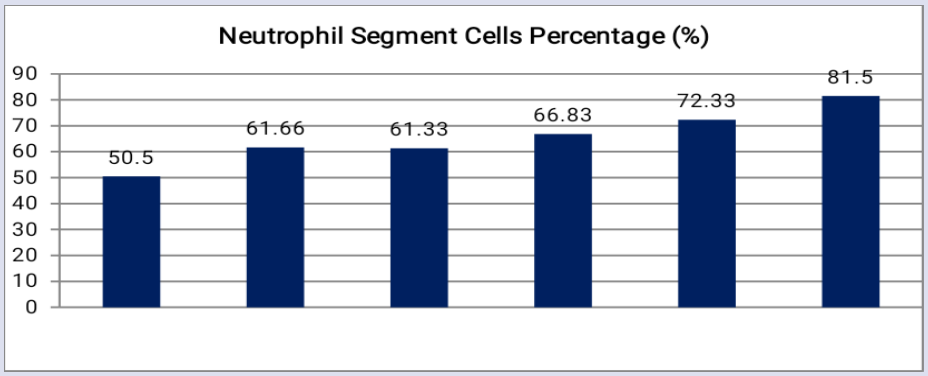

Figure 8: Bar graph of the dose of Sungkai extract on the percentage of leukocytes in male white mice.

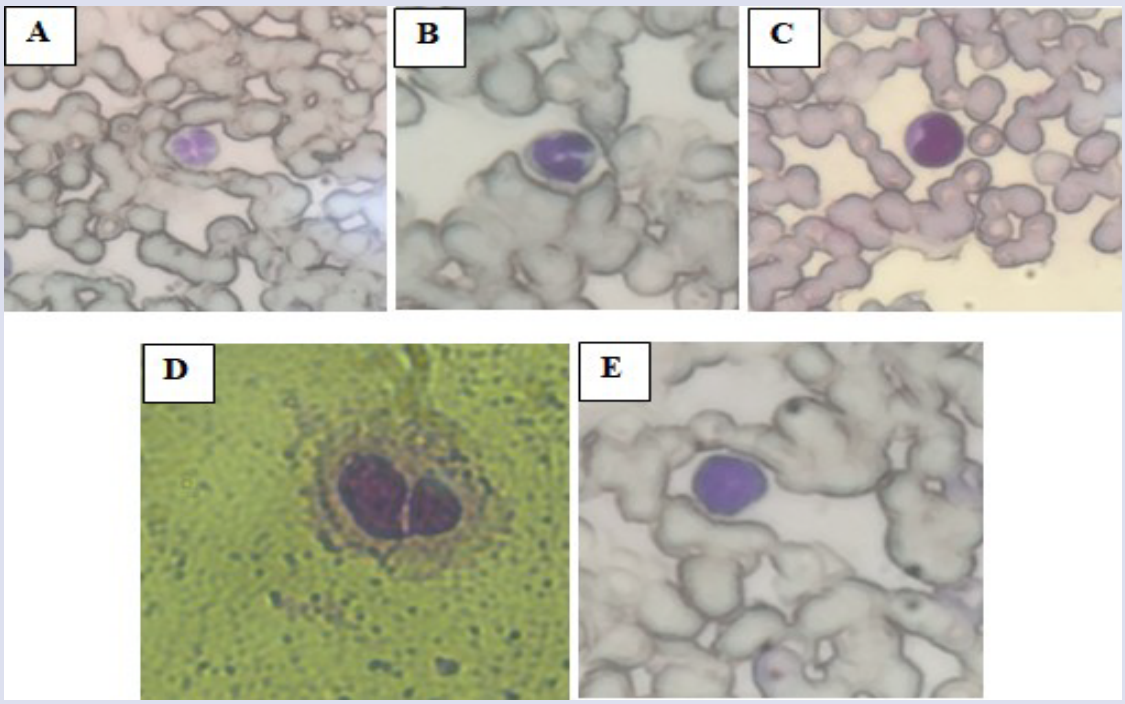

Figure 9: White mle mouse leukocyte cells. Description: (A) Segment neutrophils, (B) Rod neutrophils, (C) Monocytes, (D) Eosinophils, (E) Lymphocytes.

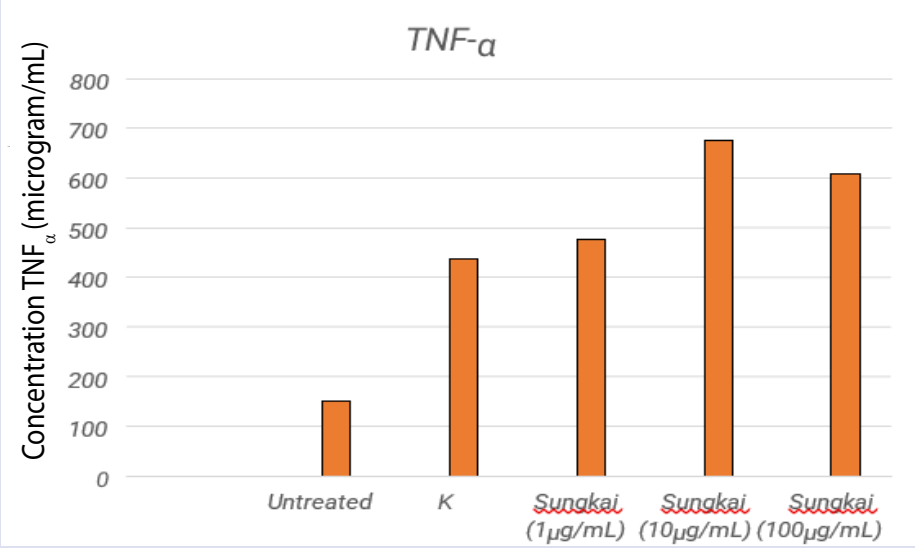

Figure 10: Effect of sungkai on TNF- production in LPS induced RAW 264.7 cells.

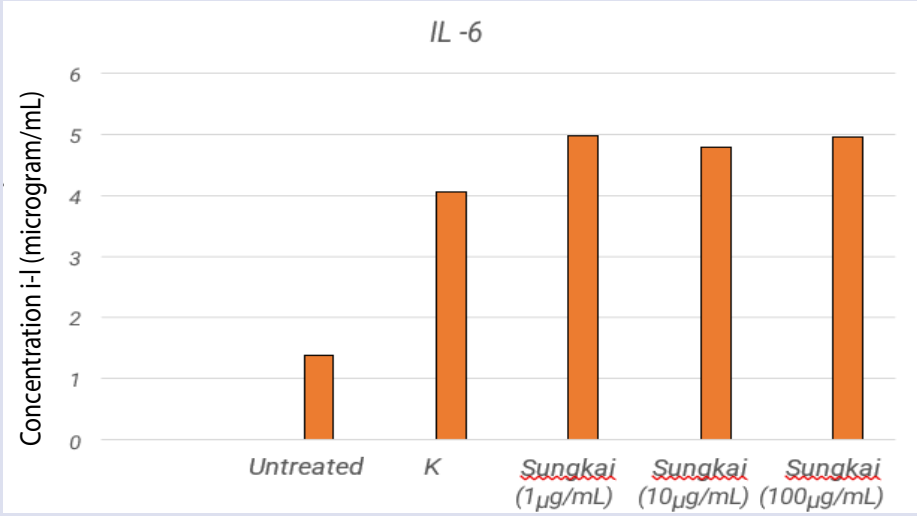

Figure 11: Effect of Sungkai on IL-6 production in RAW 264.7 cells induced with LPS.

and did not affect the percentage of leukocyte cells. the percentage of stem neutrophils, eosinophils, and monocytes was significantly ($p>0.05$). At a dose of 200 mg/kg body weight of Sungkai extract, the percentage of neutrophil cell segments was not significantly different at a dose of 400 mg/kg body weight ($p>0.05$) and a dose of 800 mg/kg body weight of Sungkai extract the effect was the same as that given by stimuno at a dose of 50 mg/kg. kgbw ($p>0.05$).

The effect of Sungkai extract on the percentage of leukocyte cells increased significantly ($p<0.05$) as shown in Figure 6. Animals given sungkai extract doses of 200 mg/kgbw and 400 mg/kgbw showed the same percentage of lymphocyte cells as the control group ($p> 0.05$). Meanwhile, the dose of Sungkai extract 800 mg/kgbw showed a higher percentage of lymphocyte cells than the doses of 200 and 400 mg/kg and the same effect as the stimuno at a dose of 50 mg/kgbw ($p<0.05$). This increase in the percentage of lymphocyte cells indicates that sungkai ethanol extract can stimulate an increase in specific immune responses.

The sungkai test on cell viability aims to determine whether sungkai is safe and not toxic to RAW 264.7 cell viability using the MTT (microtetrazolium) method, which is counting the number of living cells (viability) by looking at the absorbance value measured using a microplate reader with a wavelength of 550 nm. The cell viability obtained with 3 repetitions for concentrations of 1, 10, and 100µg/mL was 128.08; 128.83; 135.82%. The sample can be said to be safe and non-toxic to cells if the cell viability value is above 90% (21). Based on the results obtained, sungkai is safe and non-toxic to RAW 264.7 cells. The results of the Sungkai 1, 10, and 100µg/mL tests had a significant effect on the viability of RAW 264.7 cells ($p<0.05\%$) which was followed by Duncan's test, namely there was no difference at each concentration of 1, 10, 100µg/mL.

After knowing that sungkai with concentrations of 1, 10, and 100 g/mL are safe and non-toxic, further testing can be done, namely the immunostimulatory activity of sungkai by measuring the levels of cytokines TNF- and IL -6 which is a pro- inflammatory cytokine with lipopolysaccharide (LPS) as an inducer and uses the sandwich ELISA (Enzym-Linked Immunosorbent Assay) method, which is a specific reaction of complex bonds between antigens and antibodies using enzymes as reagents. ELISA results can be observed qualitatively and

quantitatively. Qualitative results can be seen from the change in color to yellow which is the result of the reaction of antibodies with antigens. The higher the intensity of the yellow color produced, the higher the absorbance value and the more antigen-antibody bonding reactions that occur, which means the higher the number of tested antigens. Quantitative results can be assessed from the optical density measured using a microplate reader with a wavelength of 450 nm (22). From the TNF- test, the untreated level was 151.047 ng/mL and the control level was 436.939 ng/mL. The 1, 10, and 100µg/mL streams were 477,626; 509.557; 705.293 ng/Ml. This indicated that the 1, 10, and 100µg/mL ribs significantly increased TNF-α levels in RAW 264.7 cells ($p<0.05\%$) compared to controls. Then, continued with Duncan's test, it was found that there were differences in of 1, 10, 100µg/mL compared to the control.

In the IL-6 test, the untreated level was 1.367 ng/mL and the control level was 4.063 ng/mL. the 1, 10, and 100µg/mL streams were 4.964; 4,773; 4,949 ng/mL. This indicated that the 1, 10, and 100µg/mL ribs could significantly increase IL-6 levels in RAW 264.7 cells ($p<0.05\%$) compared to controls. Then, continued with Duncan's test, it was found that there were differences in each concentration of 1, 10, 100µg/mL compared to the control.

The presence of immunostimulant activity in the sungkai is due to the flavonoid content contained in it. According to Devagaran & Diantini (2012) that flavonoids, curcumin, limonoids, and catechins can increase the activity of the immune system (23).

CONCLUSION

From the research conducted, it was proven that the extract of Sungkai (Peronema canescens Jack.) at doses of 800,400 and 200 mg/kgbw was immunostimulant by increasing the activity and phagocytic capacity of macrophage cells, total number of leukocytes, percentage of lymphocyte cells, decreasing segmental neutrophil cells and increasing cytokine levels. proinflammatory (TNF-α. and IL-6) as well as safe and non-toxic to RAW 264.7 cells. A dose of 200 mg/kg body weight of Sungkai extract (Peronema canescens Jack.). gave the same immunostimulant effect as stimuno at a dose of 50 mg/kg body weight.

ACKNOWLEDGMENT

Profound Thanks towards Research Institution and Community Service (LPPM) of Andalas University, and this research was supported by PNBP ANDALAS UNIVERSITY, Padang, Indonesia, in the scheme of "Riset Dasar (RD) Batch 1 Contract Number. T/5/UN.16.17/PT.01.03/KO-RD/2021" signed on march, 30th 2021.

CONFLICTS OF INTEREST

There is no conflicts of interest in the research.

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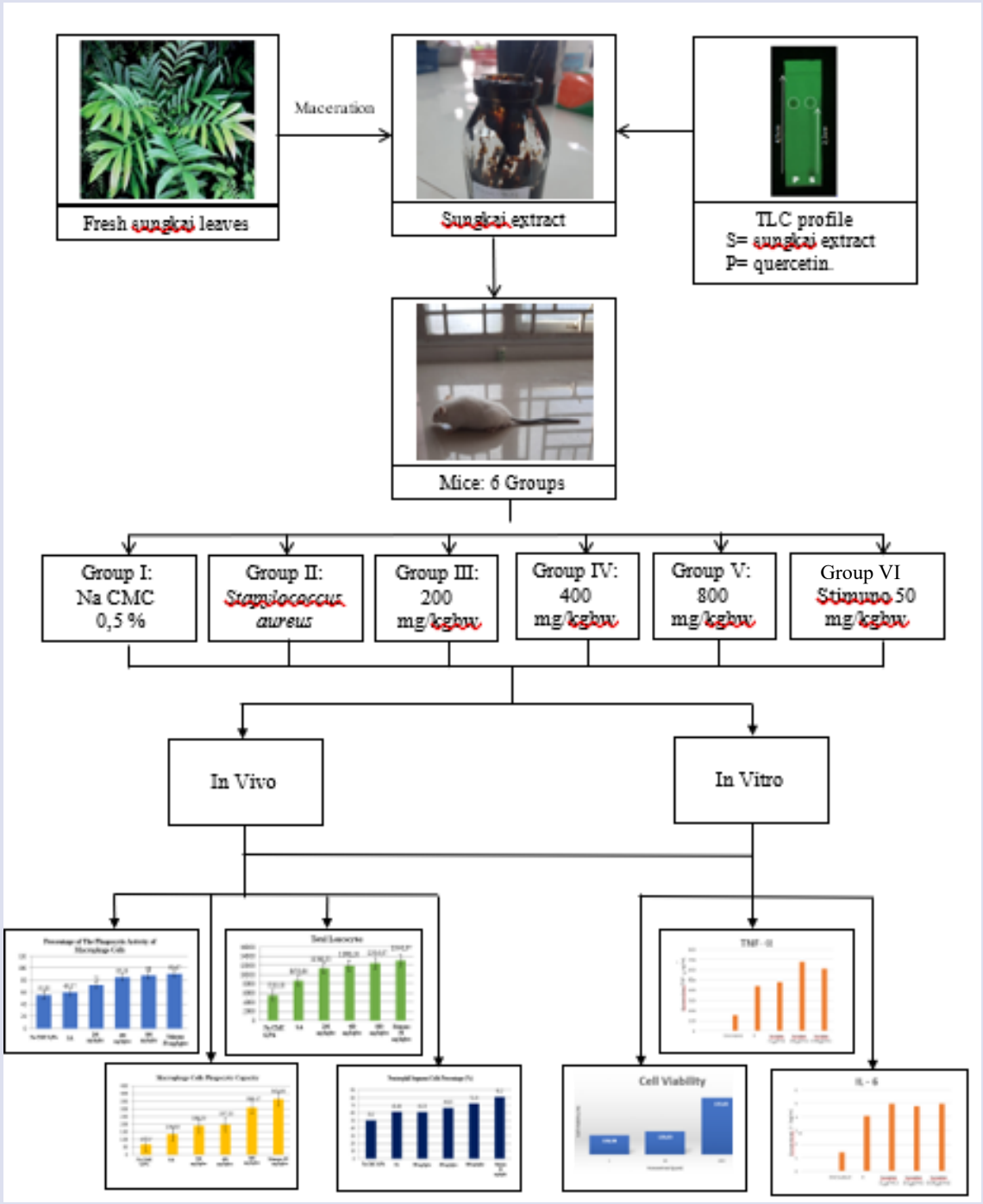
GRAPHICAL ABSTRACT



SUMMARY

Sungkai (*Peronema canescsens* Jack.) contains polysaccharides, terpenoids, alkaloids, and polyphenols which have pharmacological activity as immunostimulants. The aim of this research is to see how the effect of Sungkai extract as an immunostimulant agent was carried out in vitro and in vivo. 30 male mice were divided into 6 groups. From the research conducted, it was proven that the extract of sungkai (*Peronema canescens* Jack.) at doses of 800, 400 and 200 mg/kgbw was immunostimulant by increasing the activity and phagocytic capacity of macrophage cells, total number of leukocytes, percentage of lymphocyte cells, segmental neutrophil cells and increasing cytokine levels. proinflammatory (TNF-α. and IL-6) as well as safe and non-toxic to RAW 264.7 cells. A dose of 800 mg/kg body weight of sungkai extract (*Peronema canescens.Jack*) gave the same immunostimulant effect as stimuno at a dose of 50 mg/kg bodys weight.

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Cite this article: Dillasamola D, Aldi Y, Wahyuni FS, Rita RS, Dachriyanus, Umar S, Rivai H. Study of Sungkai (*Peronema canescsens* Jack.) Leaf Extract Activity as an Immunostimulators With *In vivo* and *In vitro* Methods. Pharmacogn J. 2021;13(6): 1397-1407.