Research Article

Immunomodulatory activity of *Plectranthus scutellarioides* (L.) R.Br. leaves ethanolic extract and its fraction on rat using carbon clearance method

Moelyono Moektiwardoyo*, Siti Rositah, Sri Agung Fitri Kusuma

ABSTRACT

Aim: The aim of this study was to investigate the immunomodulatory activity of the ethanolic extract and fractions (n-hexane, ethyl acetate, and water fraction) obtained from *Plectranthus scutellarioides* (L.) R.Br. leaves which employed in the immunity experimental model. **Materials and Methods:** The cellular immunity was conducted by a carbon clearance method in comparison to commercial immunostimulant containing other standardized plant extract, *Phyllanthus niruri* (50 mg/kg, orally) in male rats (*Rattus norvegicus*) of Wistar strain. The dose of the *P. scutellarioides* ethanolic extract and fractions was administered at 102 mg/kg and 510 mg/kg orally once a day for 6 days, respectively. Each experimental group consists of four animals. **Results and Discussion:** The result of this study revealed that the *Plectranthus* leaves extract produced a significant increase in the phagocytic index (>1.5) compared to its fraction. According its phagocytosis index, the extract was classified as a power strong immunomodulator candidate. The strength of immunomodulatory effect of the extract was almost having similar protection as the *P. niruri* extract as the standard agent. **Conclusion:** It can be concluded that the ethanolic extract of *Plectranthus* leaves possesses potential immunostimulant properties.

KEY WORDS: Carbon clearance, Extract, Immunomodulator, Leaves, *Plectranthus scutellarioides*

INTRODUCTION

Recently, immunodeficiency diseases are increasing every year in worldwide. The major cause of immunodeficiency is reported from infectious diseases such as HIV/AIDS, diarrheal diseases, acute respiratory tract infections, yellow fever, hepatitis A and E, and tuberculosis.[1] Thus, our body must have defense mechanisms to control it and it can be done through the immunomodulation process. However, the defense capabilities of our immune system can be strengthened by taking proper nutrition.[1-4] Immunomodulator or biological response modifiers are substances that affect the body's biological reactions to foreign substances. Immunomodulators consist of immunostimulators that function to enhance immune system function and activity and immunosuppressors that can inhibit or suppress immune system activity.[5] The immunomodulators have biphasic effects, some play a role to stimulate the immune system while others mechanism inhibit normal host parameters or already activated parameters.[6] Both immunosuppression and immunostimulation need to be handled to regulate the normal immunological function.[7] Immunomodulatory agents are supplemented as alternatives to treat various immunologic diseases.[8,9] Several groups of drugs such as anticancer-chemotherapeutic agents, anti-allergic, nonsteroidal anti-infla mmatory drugs, corticosteroids have been administered to control pathogens and immunological emergencies. In addition, they are difficult to access and these drugs have been reported to have many adverse effects and tend to be expensive for poor people.[10] Besides those drugs, interferon-γ (IFN-γ) as recombinant cytokines in combination with vaccines was produced as immunomodulator agent using recombinant technology.[11,12] However, the weakness of this agent is easily degraded and has undesired effects such as lymphopenia, neutrophilia, and monocytopenia.[13] Therefore, recently, several studies have been employed to obtain immunomodulatory agents from plant material as an alternative of conventional chemotherapy for a variety of diseases, especially when the body's defense mechanism has to be induced under the low immune response.

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Many traditional plants have been reported to possess immunomodulatory activities. The bioactive compounds in plants can stimulate both specific and nonspecific immunity.[14] As a result, the people, especially in the rural areas of the developing country, back to utilize the alternative medicinal plants that are widely accessible, accepted, cheaper, and admitted to having minimal side effects. The plant derivatives contain active metabolites such as flavonoids, polysaccharides, peptides, lectins, and tannins, have been reported to induce the immune system in different experimental methods.[15] Thus, the leaves of *Plectranthus scutellarioides* that contain the similar compounds such as flavonoid, polyphenolic, monoterpenoids, and sesquiterpenoids, exhibited that this plant can be a prospective source of immunomodulatory agents.[16] In addition, the *P. scutellarioides* leaves belonging to the *Lamiaceae* family also have been reported have an immunomodulatory role in the prevention of tuberculosis performed against Wistar strain mice. Leaf extract of this plant proved to increase the level of T lymphocytes, CD4 T cells, IFN-γ, and TNF-α and potentially capable of reducing of *Mycobacterium tuberculosis* colonies that infected the lungs of tested animals.[17] The utilization of the *P. scutellarioides* as a raw material on the immunomodulatory natural drug has not been done previously, indicated the novelty of this finding research. Therefore, this research was carried out to obtain the immunomodulatory activity of ethanolic extract of *P. scutellarioides* and its fraction *in vivo* in white rats *Rattus norvegicus* Wistar strain using the carbon clearance method.

MATERIALS AND METHODS

Materials

Solvents and all of the chemical reagents are analytical grade and obtained from Sigma Aldrich. The standardized immunomodulatory agent containing *Phyllanthus niruri* extract was purchased from Dexa Medica Laboratories.

Extract and Fractions Preparation

P. scutellarioides leaves were collected from Manoko Herbs Plantation, Lembang, West Java, Indonesia, and identified at the Department of Biology, Faculty of Mathematical and Natural Sciences, Universitas Padjadjaran. The extraction of *Plectranthus* leaves was performed by a maceration method using 70% ethanol as solvent within 3 × 24 h.[18] The solvent was evaporated using a rotary evaporator at 60°C to obtain a liquid extract. Then, the liquid extract was reevaporated on the water bath at 60°C to obtain viscous extract. The ethanol crude extracts of *Plectranthus* leaves then dissolved in water, then fractioned with n-hexane in separating funnel, and repeated for 3 times. Furthermore, the water fraction was retrieved and reconstituted with ethyl acetate using a separating funnel with three replications to achieve a clear ethyl acetate fraction. The fraction of ethyl acetate, n-hexane, and the water fraction obtained was separately collected, and then concentrated using a rotary evaporator until to attain a constant weight of each fraction.

Phytochemical Screening

The systematic screening of *Plectranthus* leaves extracts and fractions was analyzed to discover bioactive compounds for immunomodulator agents. The alkaloids, flavonoid, quinones, saponin, tannin, polyphenol, steroid, triterpenoid, monoterpenoid, and sesquiterpenoid were detected using the standard methods.[19]

Preparation of Test Animals

The research proposal has been submitted to the Health Research Ethics Commission of the Faculty of Medicine, Padjadjaran University and has obtained ethical approval with number 432/UN6. KEP/EC/2018. The test animals used in this study were male rats (*R. norvegicus*) Wistar strain on the weight of 150–250 g and age ranged from 2 to 3 months and obtained from educational hospital of Padjadjaran University, Eyckman Bandung, Indonesia. The experiment had seven common groups consisting of four animals each. The rats were housed under the standard laboratory for a week before treatment. The groups were as follows: Group I was served as normal control that was not treated at all, Group II was negative control group and received Na-CMC once daily (0.5%, oral), Group III was positive control and administered with standard product (50 mg/Kg, oral), Group IV received the ethanolic extract of *Plectranthus* leaves at a dose of (102 mg/kg, oral), whereas Groups V, VI, and VII were administered by the fraction of n-hexane, ethyl acetate, and water (510 mg/kg, oral), respectively. The experimental procedure was approved by the Institutional Ethical Committee (432/UN6.KEP/EC/2018).

Carbon Standard Curve

A weight of 100 mg dried carbon was grounded, and then dispersed in 100 mL of acetic acid to attain a concentration of 1 mg/mL carbon solution. Each solution was pipetted for 2, 3, 4, 5, and 6 mL then treated with 1% of acetic acid to a volume of 50 mL to get carbon levels of 40, 60, 80, 100, and 120 µg mL. Then, the solution was taken for 4 mL and mixed with 75 µL of rat blood taken from the tip of the tail vein and homogenized. The absorbance was measured by an ultraviolet (UV)-visible spectrophotometer at a wavelength of 639 nm. The obtained absorbance plot was used to create a carbon calibration curve.

Preparation of Colloidal Carbon Suspensions

A weight of 1.6 g of dried black ink was suspended in 0.5% of Na-CMC (diluted in 25 mL of 0.9% of physiological NaCl solution).[20]

Sample Preparation

A total of 50 mg of 0.5% Na-CMC was developed with hot water in a volume of 20-fold times. After expanding, it was crushed and mixed with ethanol extract or fraction according to the test concentration that has been designed. Finally, the mixture was crushed to a homogeneous level and added with distilled water up to a volume of 10 mL.[20]

Immunomodulatory Activity Test

For 6 days, the rats were given the suspension of the leave extract and fractions orally once a day; meanwhile the negative control was only given Na-CMC 0.5%, positive control was given a Stimuno suspension and the normal group did not receive any treatment at all. On the past day of testing with a 24 h break, the rats were relaxed using ether and the rat's blood was drawn through retro-orbital bleeding using capillary tubes (microhematocrite). The blood collected in Vacutainer that contained ethylenediaminetetraacetic acid and then homogenized. After being homogeneous, a volume of 75 µL blood was taken then lysed with 4 mL of 1% acetic acid. This first blood sample was used as a blank (0 min). Then, 0.1 mL/10 g of carbon suspension was injected intravenously into the tail, followed by the rat blood taking for 75 µL on the 3rd, 6th, 9th, 12th, and 15th min after injection. Each blood was lysed with 4 mL 0.1% acetic acid and its absorption was measured at 639 nm wavelength using a UV-visible spectrophotometer.[20] The amount of carbon will be reduced in proportion to the time increasing due to phagocytic processes by cells, especially neutrophils, monocytes, macrophages, and eosinophils.[21] The obtained absorbance was then plotted into the equation to calculate the phagocytic constant value of each extract and fraction dose. Phagocytic constant a can be found using the following formula:

$$K = \frac{\text{Log OD1} - \text{Log OD2}}{t2 - t1}$$

Where, K (phagocytic constant); OD1 (absorbance at the 1st time); OD2 (absorbance at the 2nd time); t (times).

Phagocytic constant values that have been obtained were used as a reference to get the magnitude of the phagocytic index. The average value of the phagocytic index can indicate the phagocytic activity of phagocytic cells against carbon particles as antigens due to the effect of giving extracts and fractions of the leaves. The equation to determine the phagocytic index value was as follows:

Table 1: The values of phagocytosis constant of *Plectranthus* leaves extract and the fractions

Time (min)	Phagocytosis constant						
	I	II	III	IV	V	VI	VII
3	0.00103±0.0001	0.00605±0.0000	0.01366±0.0001	0.00640±0.0001	0.00256±0.0001	0.00605±0.0001	−0.00484±0.0000
6	0.00089±0.0001	0.00420±0.0000	0.01218±0.0020	0.00744±0.0001	0.00306±0.0001	0.00689±0.0001	−0.00400±0.0001
9	0.00309±0.0020	0.00376±0.0001	0.00912±0.0000	0.00943±0.0001	0.00036±0.0001	0.00568±0.0001	−0.00253±0.0000
12	0.00382±0.0001	0.00495±0.0001	0.01038±0.0001	0.01074±0.0001	0.00502±0.0001	0.00834±0.0001	−0.00104±0.0000
15	0.00466±0.0002	0.00605±0.0001	0.01334±0.0000	0.01250±0.0001	0.00732±0.0001	0.00637±0.0001	0.00086±0.0000

I: Normal control group; II: Negative control group; III: Positive control group; IV: Group of extract treatment; V: Group of n-hexane fractions treatment; VI: Group of ethyl acetate fraction treatment; VII: Group of water fraction treatment

$$IF = \frac{\text{Konstanta fagositosis tikus X}}{\text{Konstanta fagositosi rata-rata kontrol}}$$

Data Analysis

The value of the phagocytosis rate of the antigen or foreign body by the body is calculated as the index of phagocytosis. The data were then analyzed statistically using ANOVA followed by Duncan's test.

RESULTS AND DISCUSSION

Phytochemical Screening Result

Chemical compounds group of flavonoids, polyphenols, monoterpenoids, sesquiterpenoids, and steroids was obtained in the *Plectranthus* leaves extract. The same secondary metabolites were detected in *Aegle marmelos* as fruit extract that produced a significant increase in the phagocytic index using carbon clearance assay.[22] Therefore, the chemical profile of *Plectranthus* leaves extracts indicated as potential sources of immunomodulatory agents.

Immunomodulatory Activity Testing

The effect of modulation on nonspecific immune responses was seen using the carbon clearance method. The test was conducted to evaluate the drug's effect on the system of reticuloendothelial. The method performs the ability of immune cells to phagocytose antigens and the carbon act as the antigen. The system of reticuloendothelial is a diffuse system consisting of phagocytic cells, which plays an important role in the particle clearance from the bloodstream.[23] Thus, when ink containing colloidal carbon particles is directly injected into the bloodstream, the clearance rate of carbon in the blood by macrophage is built by an exponential equation.[24] The amount of carbon will be reduced in proportion to the increase in time due to phagocytic processes by cells, especially neutrophils, monocytes, macrophages, and eosinophils.[22]

The extract and fractions showed different phagocytes constant values when compared to the positive control in the clearance of colloidal carbon from the blood after administration of these drugs. Phagocytosis constant is a parameter that shows the rates of phagocytosis, the greater the value of phagocytosis constant, and the higher the speed of carbon clearance. The clearance capability of the *Plectranthus* leaf extract was more significant than its fractions, presented in Table 1 and Figure 1. The values of phagocytosis constants were used as a reference for obtaining the phagocytosis index size. The increased carbon clearness index illustrated the increased function of phagocytes capability of mononuclear macrophages and nonspecific immune systems.[24] The mean value of phagocytosis index showed the phagocytosis activity of phagocytic cells against carbon particles as antigen, performed in Table 2 and Figure 2.

The immunomodulatory activity of the test material was classified according to its phagocytosis index, if its phagocytosis index value (k) <1.2, it means that the drug has a weak immunostimulatory activity, meanwhile if the value of k is between 1.3 and 1.5 means having moderate immunostimulatory activity, and if the value of k higher than 1.5 means having immunostimulatory power strong.[24] The *Plectranthus* leaves extract was found to be most effective at low dose (102 mg/kg, p.o) was classified as a power strong immunomodulator candidate. The strength of immunomodulatory effect of the extract was almost having similar protection as the *P. niruri* extract as the standard agent. Since the dose of the extract showed remarkable augmentation in the phagocytic

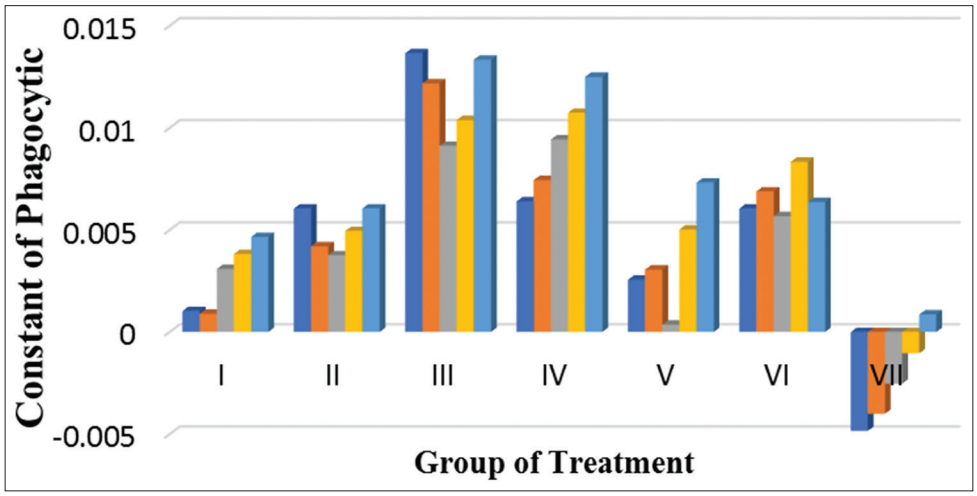

Figure 1: The comparison of phagocytes constant among the groups. I: Normal control group; II: Negative control group; III: Positive control group; IV: Group of extract treatment; V: Group of n-hexane fractions treatment; VI: Group of ethyl acetate fraction treatment; and VII: Group of water fraction treatment; treatment duration: 3 min (dark blue); 6 min (orange); 9 min (gray); 12 min (yellow); and 15 min (light blue)

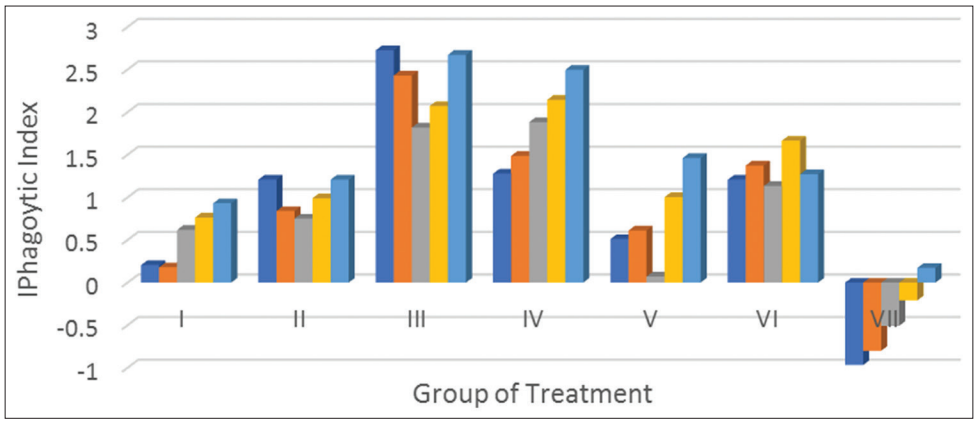

Figure 2: The phagocytosis index values at various time of observation. I: Normal control group; II: Negative control group; III: Positive control group; IV: Group of extract treatment; V: Group of n-hexane fractions treatment; VI: Group of ethyl acetate fraction treatment; and VII: Group of water fraction treatment

Table 2: Phagocytosis index values at various time of observation

Time (min)	Phagocytosis index						
	I	II	III	IV	V	VI	VII
3	0.2062	1.2088	2.7270	1.2791	0.5124	1.2080	−0.9668
6	0.1793	0.8399	2.4305	1.4877	0.6114	1.3755	−0.7994
9	0.6192	0.7519	1.8201	1.8838	0.0723	1.1341	−0.5063
12	0.7650	0.9904	2.0720	2.1449	1.0039	1.6659	−0.2079
15	0.9322	1.2088	2.6737	2.4963	1.4624	1.2731	0.1723

I: Normal control group; II: Negative control group; III: Positive control group; IV: Group of extract treatment; V: Group of n-hexane fractions treatment; VI: Group of ethyl acetate fraction treatment; VII: Group of water fraction treatment

Table 3: Variance analysis of phagocytosis

Group of treatment	*n*	Subset			
		1	2	3	4
VII	5	−0.002312851			
I	5		0.002704899		
V	5		0.003669995		
II	5		0.005005111	0.005005111	
VI	5			0.006670120	
IV	5				0.09308060
III	5				0.11746969
Sig.		1.000	0.90	0.192	0.060

index, it is suspected that the *Plectranthus* leaf extract might be due to the increasing activity of the reticuloendothelial system, whereas the fraction with higher dose (510 mg/kg, p.o) than the extract resulted in the lower phagocytic index than the extract. The fractions of n-hexane and ethyl acetate exhibited moderate immunomodulatory activities. The water fraction showed exhibited the opposite graph with the other group. From the graph, it assumed that the water fraction has not immunostimulant activity but immunosuppressant. However, in the body its activity can increase to be immunostimulant as the constant value and phagocytosis index increases. Thus, among the groups, the water fraction of *Plectranthus* leaves showed the weakest immunomodulatory activity. This finding data may contribute to immune compromised patients using the extract of *Plectranthus* leaves to modulate immune reaction either by stimulation or suppression and may assist as a supportive therapy along with conventional drugs.[14]

Statistical Analysis

The calculated data on phagocytosis constants and phagocytosis index that have been obtained were then analyzed statistically by using variant analysis (ANOVA) with $\alpha = 0.05$ to determine if there were significant differences in immunomodulatory activity between test groups. The results on Tables 3-4 showed that there were significant differences in immunomodulatory activity among the four test groups. There were significant differences in the results of the analysis so that further analysis was performed. Further analysis is done by the Duncan's method to see which groups have the best immunomodulatory activity. Further test results using Duncan's method of phagocytosis constants and phagocytosis index.

Table 4: Statistical analysis of phagocytosis index by Duncan method

Group of treatment	*n*	Subset			
		1	2	3	4
VII	5	−0.461646804			
I	5		0.540427497		
V	5		0.732533997		
II	5		1.000000000	1.000000000	
VI	5			1.331361276	
IV	5				1.858436224
III	5				2.344704329
Sig.		1.000	0.090	0.193	0.060

Based on the results of the analysis, it can be seen that the four test groups are in a different subset, indicating that these groups have significant differences in immunomodulatory activity. Group IV was in the same subset as Group III, supported the important finding that the ethanolic *Plectranthus* leaf extract was not significantly different from the positive group response. This statistical data supported that the immunostimulant activity of *Plectranthus* leaf extracts can be as potent as *P. niruri* extract as the immunomodulatory standard agent. In the phagocytosis index calculation results, the n-hexane and ethyl acetate fractions were seen to have immunostimulant activity based on the index value of phagocytosis (>1), but the statistical analysis results showed that both fractions did not exhibit significantly different from the response of the normal and negative groups. While the water fraction group was at a different subset with others, has shown no immunomodulatory activity.

CONCLUSION

This finding research showed that *P. scutellarioides* (L.) R.Br. leaf extract with a dose of 102 mg/kg BW has scientifically been studied and evaluated has immunomodulatory activity.

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