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IMMUNOMODULATORYACTIVITY OF ETHANOLIC EXTRACT OF *MELASTOMA MALABATHRICUM* L. IN *BALB / C* MICE

***Patonah Hasimun[1], IKetut Adnyana[2], Nelly Dandan Tibe[1]**

[1]Faculty of Pharmacy, Bhakti kencana University, Sukarno-Hatta no 754, Bandung, Indonesia 40617

[2]School of Pharmacy, Bandung Institute of Technology, Ganesa no 10, Bandung, Indonesia

*Corresponding author E-mail:patonah@bku.ac.id

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ABSTRACT

The non-specific immune system is the body's foremost defense in the face of attacks of various foreign substances on the body that can provide an immediate response. Immunomodulators reactivate the immune system by increasing non-specific immune responses. Traditionally *Melastomamalabathricum* L is used as a reliever of fever, pain relievers, anti-inflammatory, smooth blood flow, and stop the bleeding. The aimed of this study was to determine the immunomodulatory activity of ethanol extract of *Melastomamalabathricum* L leaf using carbon clearance method. A total of 15 mice were grouped randomly into 5 groups consisting of group 1 receiving drug carriers, group 2 receiving Stimuno® Forte 19.5 mg/kg, group 3-5 receiving extract dose 100, 300, and 600 mg / for 7 days. On the 8th day, the mice were injected intravenous with carbon suspension. The measured parameters were phagocytosis index and spleen weights. The results showed an increase in phagocytosis index in the group receiving doses of 100, 300, and 600 mg/kg, with values of 1.04, 1.34, and 1.41 respectively. Groups 3-5 showed an increase in the lymphatic weight index. The result of this research can be concluded that ethanol extract of *Melastomamalabathricum* L. leaf has potential as an immunomodulator.

INTRODUCTION

The immune system is a mechanism by which the body maintains its integrity as a protection against harm that can come from various materials in the environment. The body is equipped with specific and non-specific defense mechanisms to fight potentially damaging substances or to fight microorganisms. The non-specific immune system is the body's foremost defense against microbial attacks and can respond directly to, for example, bacteria through the destruction of non-specific bacteria by phagocytosis where the main cells are mononuclear cells (monocytes and macrophages) and polymorphonuclear cells or granulocytes. [1]The immune system can be decreased with various factors such as unhealthy diet, air pollution, erratic weather changes, lack of physical activity and stress, and various infectious agents such as bacteria, viruses, fungi and parasites that can cause various symptoms of the disease. This disorder, if not well managed, in the absence of efforts to improve the immune system it can lead to more serious illness so that the

necessary immunity boosters. [2]Immunomodulators are substances that have been proven to be used to modify the immune system's response to foreign body threats (as immunostimulants). Immunostimulant is a compound that can increase the body's immune response. Immunostimulators indirectly reactivate the immune system is low by increasing non-specific immune responses. [3]Some herbal remedies are known to have immunomodulatory properties and generally act by stimulating immunity, both specific and non-specific immunities. Various plants that have immunomodulatory activity are used in traditional medicine. Some plants work by stimulating humoral and cellular immunity, while others only activate the cellular components of the immune system as a function of phagocytosis.[4]*Melastomamalabathricum* L is a plant of the Melastomataceae family, which is widely grown in tropical Asia, that grows wild in areas that get enough sunlight. *MelastomaMalabathricum* L plant contains saponin, flavonoid and tannin compounds. Traditionally, the plant is used as an antipyretic, painkiller (analgesic), diuretic, relieve swelling, smooth the flow of blood, and stop the bleeding.[5]*In vitro* study results, it was reported that the *MelastomaMalabathricum* L ethanol extract showed excellent potential as an immunomodulator and was relatively safe to use despite large doses.[6]Other studies have reported that *Melastomamalabathricum* L contains active compounds of asiatic acid, kaempferol, and quercetin.[7]It strengthens its empirical use as anti-inflammatory,[8] antioxidants,[9] anticoagulants,[10] and gastroprotective.[11]Based on the description above, this study aims to investigate the immunomodulatory effect of *Melastomamalabathricum* L leaf in male balb/c mice using carbon-clearance method. The parameters measured were the phagocytosis index and spleen weight index.

METHODS

Materials collection: Leaves *Melastomamalabathricum* L obtained from the area of Mount Seteleng, North PenajamPaser District, East Kalimantan, Indonesia. Botanical determination has been done at the Biology Laboratory at the Faculty of Mathematics and Natural Sciences, Mulawarman University, Samarinda, East Kalimantan, Indonesia (No 006/UN17.8.5.7.16/FMIPA/HA/II/2016).

Melastomamalabathricum L leaf was taken from the center of the stalk to the top of the leaf. Then the leaves are cleaned of impurities using clean running water. Then the leaf that has been washed drained to dry. Furthermore, the leaves are cut small and dried at room temperature (conditions protected from sunlight) for several days to obtain a dry leaf. The dried leaves are powdered and sieved using a 4/18 size sieve. Leaf powder is stored in a sealed glass container and is protected from sunlight at room temperature.

Extractpreparation:
Melastomamalabathricum L. was extracted using 96% ethanol (obtained from Brataco) by maceration method for 3x24 hours, while occasionally stirring. The filtrate was filtered and collected for concentration using a rotary evaporator temperature of 50 ° C to obtain a dry extract. Phytochemical screening was performed on dry extracts to determine the presence of secondary metabolites including flavonoids, alkaloids, saponins, tannins, steroids, triterpenoids.In addition, the examination of total ash content, water content, water-soluble extract, ethanol soluble concentration was also carried out.

Preparation of calibration curve ofcarbon ink: Carbon inks were obtained from the local grocery (Winsor and

Newton Ink brands). The dried ink is then weighed as much as 100 mg. Dispersed in 1% acetic acid 100 mL to obtain concentration 1 mg / ml. Further dilution was performed to produce concentrations of 40, 60, 80, 100, and 120 µg / ml. A total of 4 mL of carbon ink suspension is added to 20 µl of mice blood taken from the tip of the vein of the tail. After the mixture was homogenized, an absorbance measurement was performed using a UV-Vis spectrophotometer at a wavelength of 650 nm. The calibration curve was obtained from absorbance data and carbon concentrations of 40, 60, 80, 100, and 120 µg / ml. The blank solution used is the blood of mice and aquadest.

Animal preparation: This study was conducted on 2-month-old *balb/c* mice with a body weight range of 20-35 g. The procedures in this study have been approved by the local ethics committee (No 511/UN6.C1.3.2/KEPK/PN/2016). Prior to the treatment, a total of 15*balb/c* mice were acclimatized in animal cages for 14 days with free access to food and drinking water.

Immunomodulatory activity: Immunostimulant effect test was performed by the carbon clearance method. Immunomodulatory activity compared to standard drugs that have been clinically tested can improve the immune system and marketed in Indonesia, namely Stimuno forte in the form of capsule preparations. One capsule contains 50 mg active ingredient of Phyllanthus niruri extract.A total of 15 balb / c mice were grouped randomly into 5 groups, consisting of group 1 receiving drug carriers, group 2 receiving Stimuno forte® 19.5 mg/kg (obtained from local pharmacies), group 3-5 receiving extract dose 100, 300, and 600 mg/kg for 7 days. On day 8, all animal groups are injected with carbon ink suspension. The carbon inks are prepared in the form of a 1.6% v / v suspension in 1% gelatine solution (in 0.9% NaCl) and injected intravenously 0.1 ml / 10g dose. Blood sampling was performed at 3, 6, 9, 12, 15 and 20 minutes after injecting carbon suspension. A total of 20 µl of blood is dropped on a drop plate containing sodium citrate as an anticoagulant. Then, the addition of 4 ml of 1% acetic acid solution and measured its absorbance using spectrophotometry at a wavelength of 650 nm. The phagocytic index of each treatment group was calculated to determine the activity of the phagocytosis.[12]Prior to the examination, a calibration curve was created to determine the linearity relationship between the carbon concentration in the blood and the absorbance value. From the calibration curve, it is known that the absorption regression and carbon concentration is $y = 0{,}00459x + 0{,}11158$ with $r = 0{,}99836$. The result showed that there is a linear correlation between the carbon concentration in the blood of the mice and the absorbance value, the increase of carbon concentration in blood, the absorbance value obtained will also increase.

Data analysis: The absorbance data obtained is used to calculate the phagocytosis index. The data obtained were analyzed statistically where the difference value was significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

The yield of *Melastomamalabathricum* L leaf extraction was 15.22%. The results of the quantitative examination of dry matter and dry extract include moisture content, water-soluble content, ethanol soluble content, and total ash content are shown in table 1. The water content check was performed to ensure the water content in the test material. Determination of total ash content provides a description of mineral content derived from the initial process until the formation of the extract. Determination of levels of the extracted compounds to determine the amount of compound content in dry matter or extracts that can be extracted in a particular solvent. In this study, we determined the

levels of water-soluble extracted compounds and the levels of ethanol-soluble extracted compounds.Phytochemical screening is performed to identify secondary metabolites contained in dry matter and extracts. The extraction effectiveness is determined by the presence of active compounds in the dry matter and extracts after the maceration extraction process. The results of the phytochemical screening are shown in Table 2.The method of carbon clearance is done by injecting the ink carbon into the bloodstream intravenously, to measure the phagocytic ability of the reticuloendothelial cells. This method is a non-specific response test to determine the activity of macrophage cell phagocytosis to carbon as a foreign body. The absorbance measurements were performed on Spectrophotometry at 635 nm wavelength because at that wavelength gave the maximum absorbance value of the carbon ink. The rate of carbon clearance is measured at 3, 6, 9, 12, 15 and 20 minutes. The carbon ink suspension used is a stable carbon ink in the bloodstream and does not cause thrombosis. The carbon inks are prepared in the form of a suspension in 1% gelatine in 0.9% physiological NaCl solution so that the carbon ink suspension concentration is 1.6% v/v.The value of absorbance obtained, then used to calculate the value of transmittance by the formula $\tau = 102\text{-}A$. The percent value of transmittance obtained, used to calculate the percent value of absorbance by the formula (% absorbance = 100% -% transmittance). The decrease in the value of the absorbent percent showed less concentration of carbon in the blood (Table 3).The results of absorbance measurements at 3 minutes after intravenous injection of carbon inks increased the amount of carbon in the blood of mice in line with the increase in absorbance values. In the next minute, there is a decrease in absorbance value that indicates the occurrence of phagocytosis by macrophages, which can eliminate carbon contained in the blood. The decrease in absorbance values occurred in different treatment groups. It shows that giving leaf extract of *Melastomamalabathricum* L can increase phagocytic activity against the foreign body (carbon ink). The absorbance value obtained in each group was used to obtain the phagocytic constant's value on the rate curve of carbon ink removal in blood. The value of phagocytic constants is one of the parameters used to determine the rate of phagocytosis where the greater the value of the phagocytic constant, the higher the rate of carbon clearance, which means the faster the phagocytic cells perform the process of phagocytosis. The value of the phagocytic constant is used to obtain the value of the phagocytosis index. The index value of phagocytosis greater than 1 in each group means that each group has the ability to increase the body's defense system against foreign body attack (carbon ink). It has been reported that flavonoid compounds have mechanisms as immunomodulators by activating NK cells to stimulate the production of interferon γ (IFN-γ). [13] IFN-γ produced by various immune system cells is the main cytokine of MAC (Macrophage Activating Cytokine) and plays an important role in providing non-cell-specific immunity. IFN-γ is a cytokine that can activate macrophages, thereby increasing phagocytosis activity quickly and efficiently in removing antigen.[14]There was a significant difference of phagocytosis index between the group receiving Stimuno forte® dose of 19.5 mg/kg compared to the control group ($p<0.05$). The results showed that there was no significant difference ($p> 0.05$) in the phagocytic index between the groups receiving the dose test extract 100 and 300 mg/kg compared to the control group. While the group receiving a dose of 600 mg/kg showed significantly different phagocytic index values than the control group ($p <0.05$).

Table 1. Results of quantitative examination of dry matter and Dry extract from leaf *Melastomamalabathricum* L

No	Inspection	*Melastomamalabathricum* L.	
		Dry matter (%)	Dry extract (%)
1	Water-soluble content	16.0	79.5
2	Ethanol soluble content	17.5	77.7
3	Total ash content	5.5	12.8
4	Water content	5.0	10.5

Table 2. Results of phytochemical screening of dry matter and Ethanol Extract of *Melastomamalabathricum* L

No.	Inspection	Dry leaves	Dry extract
1	Alkaloids	+	+
2	Flavonoids	+	+
3	Saponins	+	+
4	Quinones	+	+
5	Tannin	+	+
6	Steroids/Triterpenoids	+ Steroid	+ Steroid

+ Present, - not present

Table 3. Absorbance values for 20 minutes of measurement for each treatment group

Group	The percent value of absorbance (100 -% transmittance) at minute-						
	0	3	6	9	12	15	20
1	23.7 ±0.6	52.3±3.1	51.6±3.2	52.9±3.6	50.4±3.7	48.5±5.1	46.2±5.3
2	22.9±0.9	50.2±0.9	49.8±1.5	47.7±2.3	45.6±1.8	44.5±2.0	41.7±1.3
3	23.3±1.2	53.5±1.8	53.8±3.2	53.5±3.4	51.2±1.2	50.5±1.1	47.1±3.3
4	23.6±1.2	52.6±1.2	51.6±1.5	49.9±2.5	49.9±1.2	48.7±1.2	43.1±2.1
5	24.1±1.4	48.6±0.6	48.6±3.8	48.0±6.8	47.0±6.6	43.9±7.8	39.7±3.5

Blood turbidity was measured for 20 min, after intravenous administration of carbon ink suspension. Group 1 received 1% CMC, group 2 received Stimuno forte® 19.5 mg / kg, group 3-5 received 100, 300, and 600 mg / kg dose extracts.

Table 4. Phagocytosis index of each treatment group after 7 days of drug administration

Group	Regression equation	Regression coefficient	Index of phagocytosis
1	y = - 1,206x + 54,532	1,2055±0,96	1
2	y = - 1,7346x + 52,635	1,7346±0,14	1,44*
3	y = - 1,2585x + 55,985	1,2585±1,00	1,04
4	y = - 1,6162x + 54,973	1,6162±0,19	1,34
5	y = - 1,704x + 51,908	1,704 ±0,77	1,41*

Group 1 received drug carriers, group 2 received Stimuno forte® 19.5 mg / kg, group 3-5 received extract doses of 100, 300, and 600 mg / kg.

* There was a significant difference ($p<0.05$) compared to the control group.

Table 5. Lymphatic weight index for each treatment group after 7 days of drug administration

Group	Body weight (g)	weight of the spleen (g)	Lymphatic weight index (%)
1	28 ± 6.6	0.15 ± 0.09	0.51 ± 0.18
2	26 ± 1.5	0.17 ± 0.02	0.65 ± 0.07
3	30 ± 4.2	0.15 ± 0.04	0.51 ± 0.22
4	27± 1.2	0.16 ± 0.02	0.58 ± 0.10
5	28 ± 2.1	0.18 ± 0.02	0.62 ± 0.04

Group 1 received drug carriers, group 2 received Stimuno forte® 19.5 mg / kg, group 3-5 received extract doses of 100, 300, and 600 mg / kg.Each group consists of 3 mice

The strength of the phagocytic index in the group receiving a dose of 600 mg/kg was comparable to the group receiving Stimuno forte ($p > 0.05$). It shows that *Melastomamalabathricum* L dose of 600 mg/kg is the best dose because it has phagocytic ability comparable to stimuno forte® (Table 4).The spleen is a secondary lymphoid organ, besides containing many macrophages, its also containing other immune system cells such as dendritic cells, Langerhans, T cells and B cells. The spleen is also an important part of the reticuloendothelial system containing lymphocytes, monocytes, and macrophages. [15] An increase in lymph node weight may indicate an increase in the proliferation of immune cells present within the organ.The weight of the spleen in the 2-5 group increased compared to the control group (Table 5). Based on these results it can be stated that *Melastomamalabathricum* L extract enhances immunomodulatory activity. It can be explained that the spleen contains dendritic cells and macrophages that act as APC (Antigen Presenting Cell) which serves to present antigen to lymphoid cells.

CONCLUSION

MelastomaMalabathricum L leaf has an immunomodulatory effect, which increases its activity in-line with the increase in dose. The extract increases the lymphatic weight index as a marker of the active immune system.

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