Jurnal Natural
Vol.18 (1) 2018
DOI 10.24815/jn.v18i1.9830
Published February 2018
pISSN 1411-8513
eISSN 2541-4062

IMMUNOSTIMULATORY EFFECT OF METHANOL EXTRACT OF FLAMBOYANT LEAF [*Delonix regia* (Boj. ex Hook.) Raf.] IN MICE

Kartini Eriani[1*], Ainsyah[1], Rosnizar[1], Yunita[1], Ichsan[2] and Al Azhar[3]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Syiah Kuala University, Jl.Syech Abdurrauf No. 3, Banda Aceh, 23111, Indonesia
[2]Microbiology division, Faculty of Medicine, Syiah Kuala University, Jln.Hasan Krueng Kalee Darussalam-Banda Aceh, 23111, Indonesia
[3]Faculty of Veterinary Medicine, Universitas Syiah Kuala, Indonesia
*corresponding Author E-mail : kartini_eriani@unsyiah.ac.id

Abstract. Flamboyant [Delonix regia (Boj. ex Hook) Raf.] leaf contains flavonoid compounds that are expected to have immunostimulatory effect. This research was done to determine the effect of flamboyant leaf extract on immune response by accessing the activity of immune cells and capability test the extract as immunostimulant in mice. Leaf extraction was done by maceration using methanol in the Laboratory of Biology of Chemistry Department, Faculty of Mathematics and Natural Sciences of Syiah Kuala University whereas animal treatment and testing were carried out Micro-technique Laboratory of Biology Department of the same faculty. This research used 20 male mice strain Swiss-Webster aged 7-8 weeks were randomly assigned to 4 treatment groups with five replications each. Group 1 (P0) was untreated control; group 1-3 were mice administration flamboyant leaf extract 250 mg/kg BW (P1), 500 mg/kg BW (P2), and 750 mg/kg BW (P3) per oral. The treatments were given for 14 days after one week of adaptation period. Blood samples were collected before and after extract treatment and used for leukocyte count analysis. Phagocytosis activity was accessed by carbon clearance assay on day 15. At the end of the study, all mice were sacrificed for spleen weight analysis. Data obtained was analyzed by Analysis of Variance followed by Tukey test (Leukocyte count and spleen weight) or regression analysis (carbon clearance). The results showed a flamboyant leaf extract administration resulted in increased leukocyte counts that were significantly different ($p<0.05$) between treatment groups. Phagocytosis test indicated the extract had moderate to strong immunostimulatory effect whereas spleen weight analysis did not show any difference among treatment groups. In conclusion, flamboyant leaf methanol extract was able to increase immune cells and had potential immunostimulatory activity in mice.

Keywords: *Delonix regia*, immunostimulant, leukocytes, lymphocyte proliferation.

I INTRODUCTION

Medicinal herbs have been and widely used by Indonesian people for various health purposes. According to fundamental health research, Riset Kesehatan Dasar (RISKESDAS), in 2010, approximately 59.12% of Indonesian people consumed medicinal herbs, and 95.6% of them felt healthier from consuming these herbs [1]. One of the benefits of medicinal herbs is their function as immunostimulators; compounds improve immune response. The use of these herbs as immunostimulators can detain or decrease the infection of viruses and intracellular bacteria, surmount the mechanisms of immunodeficiency and stimulate the growth of immune cells in the immune system [2]. Immunostimulants can activate the immune system through various ways such as by increasing the number and activity of T lymphocyte, the natural killer (NK) cells, increasing the activity of macrophage and by releasing interferon and interleukins [3]. One of the medicinal herbs that have benefited as an immunostimulant is flamboyant [Delonix regia (Boj. ex Hook.) Raf.]. The previous study shows that flamboyant can be used as anti-malaria, anti-bacteria, analgesic, anti-sore, anti-inflammation and anti-microbial [4]. Flamboyant contains alkaloids, flavonoids, terpenoids, and tannins [5]. Based on its chemicals contents, flamboyant is expected to

have a function as an immunostimulant. Therefore, it is necessary to conduct a test toward the effect of extracts prepared from different parts of the herbs as an immunostimulant. This study was done to investigate the effect of methanol extract of flamboyant leaves given orally on the activity of immune cells and the relative weight of spleen as well as to test immunostimulant quality of the extract using mice as experimental animals.

II METHODOLOGY

This study was done in the Microtechnique Laboratory of Department of Biology, Faculty of Mathematics and Natural Sciences, Syiah Kuala University, in Banda Aceh. This study used the complete randomized design consisting of four treatments and five repetitions each. Here, 20 male mice aged 7-8 weeks were randomly assigned into four treatment groups. Group 1 (P0) was untreated control, and group 2 (P1), 3 (P2) and 4 (P3) were mice given flamboyant leaf extract of 250, 500 and 750 mg/kg BW per oral, respectively. The treatments were given for 14 days after a week of adaptation period.

Blood preparation

Blood collection was done twice, before (H_0) and after (H_{14}) the extracts were given. The blood was collected from coccygeal vein and dropped onto the surface of an object glass. The thin blood smear was then prepared by streaking the blood out with another object glass with 30° angle. The smear was dried, fixed in methanol solution and let to dry at room temperature for a minute [6]. Blood smear was rinsed in Giemsa staining solution (1:9 dilutions in phosphate buffered saline pH 6.8) for 20 to 30 minutes. The smear was gently washed with running water, dried at room temperature and observed at five fields/viewpoints using a light microscope at magnification of 10 x 100 with immersion oil [7]. The percentages of white blood cells (lymphocytes, monocytes, and neutrophils) were quantified according to the formula of Field and Shute [7], as Eqs (1) to (3).

$$\text{Limphocytes}(\%) = \frac{\text{L}}{\text{L+M+N}} \text{ x } 100\% \quad (1)$$

$$\text{Monocytes } (\%) = \frac{\text{M}}{\text{L+M+N}} \text{ x } 100\% \quad (2)$$

$$\text{Neutrophils } (\%) = \frac{\text{N}}{\text{L+M+N}} \text{ x } 100\% \quad (3)$$

where L is the number of lymphocytes, M is the number of monocytes and N is the number of neutrophils.

Phagocytosis test

On day 15th, the phagocytosis potency in every mouse was measured by using carbon clearance method. Here, the tint of carbon suspension was prepraed by mixing 1.6 ml of pelican carbon tint and 8.4 ml of gelatin 1% (m/v) in physiological NaCl solution. Carbon suspension, 0.1 ml/10 g BW, was injected intravenously via coccygeal vein. Blood was withdrawn at minute of 0, 30, 60, and 90 post injection. The blood was dropped on the drop plate pre-coated with sodium citrate and dropped by using pipette as much as 50 µl of dialysis in 4 ml of 1% acetic acid solution (minute 0). Carbon content in the blood was measured using UV-Vis spectrophotometer at wavelength 627 nm [8].

Spleen weighing

At the end of experiment all mice were sacrificed by neck dislocation and dissected for spleen collection. The spleens were washed in physiological NaCl solution and being weighed using the electrical balance. Spleen's relative weight was counted according to Eqs (4) [9].

$$\text{Spleen's relative weight} = \frac{\text{spleen's weight}}{\text{body's weight}} \text{ x } 100\% \quad (4)$$

Data Analysis

The number of leukocytes and the relative weight of the spleen were analyzed Analysis of Variance (ANOVA) followed by the Turkey test. Phagocytosis data were analyzed using the regression analysis.

III RESULT AND DISCUSSION

Effect of Flamboyant Leaf Extract on Leukocytes

Leukocyte count of mice before (H_o) and after (H_{14}) the administration of the flamboyant leaf extract 0, 250, 500, and 750 mg/kg BW for 14 days are presented in Table 1. This study found that there were significant differences ($p< 0.05$) in leukocyte counts between the control (P_0) and other treatment groups (P_1, P_2, and P_3). Significant leukocyte count difference ($p< 0.05$) was also found between P_1 and P_3, and between P_2 and P_3, but not between P_1 and P_2 (Table 1). Increased leukocyte counts are found after flamboyant extract administration. The increase

is expected due to the flavonoid bioactive compounds contained in the flamboyant leaf extract. These secondary metabolite compounds have character as immunostimulants to improving immune system activity against viruses, bacteria and other microbes [10]. Immunostimulatory effect of flavonoids in roselle's petal (*Hibiscus sabdariffa* L.) on lymphocyte proliferation has been reported earlier [11].

Table 1 Average (mean±SD) leukocyte count before and after the administration of flamboyant leaf extract for 14 days

Treatment	(H_0)	(H_{14})
P_0 (Control)	24,80±1,643	$27,00^{a}$±2,23
P_1 (250 mg/kg bb)	24,80±2,588	$56,20^{b}$±3,61
P_2 (500 mg/kg bb)	25,40±1,140	$58,40^{b}$±5,54
P_3 (750 mg/kg bb)	27,00±1,000	$74,20^{c}$±5,73

Note: H_0: before flamboyant extract administration. H_{14}: 14 days after flamboyant extract administration.

The flavonoids in flamboyant are expected to have a role as mitogen-activated protein kinase (MAPK) such as the stimulation of the immune cells as well. Middleton *et al*. [12] suggested that flavonoid compounds could stimulate the activity of MAPK. MAPK can initiate phosphorylation of various proteins including protein transcription factor which is needed in protein synthesis required for cell cycle process. Craxton *et al*. [13] added that MAPK could induce activity nuclear factor kappa *B* (NFK*B*), the transcription factor stimulate proliferation and differentiation of leukocytes through cytokine regulation mechanism. The immunostimulatory role of flavonoid might also induce production of IL-2 that takes part in improving the proliferation of T helper cell. According to Cambpell [14], interaction between antigen presenting cells (APCs) and T helper cells improves in the presence of T cell surface protein called as CD4 that has affinity to some class II major histocompatibility complex (MHC) protein. Interaction between CD4 and class II MHC helps keep the T helper cells and APC integrated although the specific antigen activity is under process. When T helper cells interact with specific class II MHC complex and antigen in an APC, they reproduce themselves and differentiate into activated T helper cell clones and memory T helper cells.

Phagocytosis test

The results of the regression analysis showed that there was a linear correlation between blood carbon content and absorption values (Figure 1). The higher carbon concentration in blood was, the higher absorption value could be. The comparison of the regression values showed the immunostimulant potency of the tested materials (Table 2).

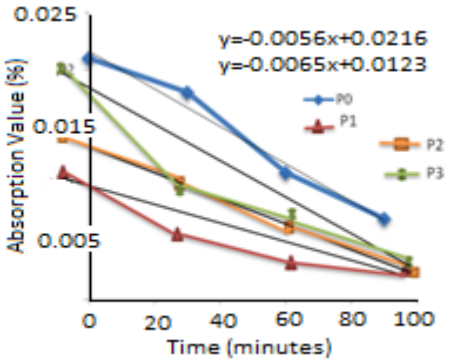

Figure 1 The rate of carbon clearance in all treatment groups. P_0: the control (mice given distilled water). P_1: mice give flamboyant leaf ethanol extract 250 mg/kg BW. P_2: mice given flamboyant leaf extract 500 mg/kg BW. P_3: mice given flamboyant leaf extract 750 mg/kg BW.

Table 2 Phagocytosis index and immuno-stimulatory effect criteria of flamboyant leaf extract.

Treat-ment	Regression coefficient	Phagocytosis index (ku/kk)	Imunostimu-latory criteria
P0	-0.006	1	-
P1	-0.005	1.3	Moderate
P2	-0.008	1.4	Moderate
P3	-0.009	1.5	Strong

Note : P_0: control (mice given distilled water). P_1: mice given flamboyant leaf extract 250 mg/kg BW. P_2: mice given flamboyant leaf extract 500 mg/kg BW. P3: mice given flamboyant leaf extract 750 mg/kg BW. KU: regression coefficient of treatment group. KK: regression coefficient of control group.

The phagocytosis index shows that the flamboyant leaf extract had a moderate immunostimulatory characteristic at P_1 (250 mg/kg BW) and P_2 (500 mg/kg BW); and strong immunostimulatory potency P_3 (750 mg/kg BW). These results implied that the higher the dosage given, the stronger the immunostimulatory potency is. However, at dosages range from 2000 to 6000 mg/kg BW, the extract could be toxic to livers and kidneys [15].

The existence of the immunostimulatory effect of flamboyant leaf extract might be caused by the presence of bioactive compounds could act as immunostimulants such as flavonoids. This is supported by data from previous research conducted by Susilo [16], flavonoid contents in Jamblang leaf extract (Syzygium cumini L.) could improve the immune activity in mice. Similar result was also reported by Yuswantina [17] that the flavonoid content in bread fruit leaf extract (Artocarpus altilis) could affect

phagocytosis activity of macrophage. According to Baratawidjaya [18], flavonoid compounds in plants stimulate the production of interferon γ (IFN-γ) by activating the natural killer (NK) cells. The IFN-γ produced by the cells of immune system is the main cytokines of macrophage activating cytokine (MAC) and takes in part in cellular non-specific immunity. Samuel [19] adds that IFN-γ is cytokines can activate macrophage so that phagocytosis proceeds rapidly and efficiently in discarding antigens. IFN inducing substances stimulate cells to activate IFN genes expression that in turn, result increased IFN protein synthesis. The binding of IFN protein produced to its receptors on cell membrane stimulates effector producing genes to obstruct the antigen replication [19].

Effect of flamboyant leaf extract on spleen proliferation

Spleen's relative weight of mice in treatment groups might reflect the effect of flamboyant leaf extract on spleens. As shown in Table 3, this study found no significant effect of the extract (p>0.05) on spleen. This result can be seen in Table 3.

Table 3 Average spleen's relative weight of mice after the administration of flamboyant leaf extract

Treatment	Body weight (g)	Spleen relative weight (%)
P_0 (control)	41.79±1.29	0. 57^a±0.04
P_1 (250 mg/kg BW)	41. 70±1.54	0.61^a±0.04
P_2 (500 mg/kg BB)	41.17±2.05	0.64^a±0.04
P_3 (750 mg/kg BB)	41.81±2.05	0.65^a±0.06

The results showed that spleen's relative weight tended to increase in treatment groups compared to the control. The increase might be related to increase lymphocyte proliferation in the organ. This is in agreement with those stated by Aldi [20] that the enhancement of the spleen's weight was followed by the increased rate of lymphocyte cells production in the organ

Based on the phagocytosis test in this research, the flamboyant leaf extract might have an immunostimulatory potency that also affects spleen proliferation. Wijisekera [21] stated that the main effect of immunostimulant in the immune system is by increasing phagocytosis process via increased macrophage activity. This output complies with a study conducted by Nurkhasanah [22] indicating that Nigella sativa extract containing flavonoids could increase the number of lymphocytes and cause the spleen proliferation. Roitt [23] suggested that the general mechanism of lymphocyte cell proliferation occurred when antigens tied to the surface of T and B cell together with IL-1 from the APCs. This, in turn, could activate the G-protein to activate phospholipase C to initiate hydrolysis phosphatidylinositol bisphosphate (PIP2) to diacylglycerol (DAG) and inositol triphosphate (IP3) in the plasma membrane.

DAG directly activate the protein kinase C by phosphorolysis the serine or threonine amino acid residues on the target cells. Afterward, the IP3 stimulates the release of Ca^{2+} into the cytoplasm. The increased Ca^{2+} concentration has a critical role in stimulating the action of protein kinase C and 5-lipoxygenase. These enzymes might stimulate the production of IL-2 that in turn activates the proliferation of lymphocyte cells.

CONCLUSION

The results show that methanol extracts of flamboyant leaf had potential immunostimulatory effect toward the immune system by increasing immune cells and spleen proliferation in mice. Based on these results, it is recommended to carry out further research to investigate the effect of the extract on cytokine expression and to measure the immune activity using the antibody titer method.

ACKNOWLEDGMENT

The authors thank to Research Institute of Syiah Kuala University that funded this research under a competitive grant of the financial year 2016 and all who have contributed to this study.

REFERENCES

1. Departemen Kesehatan. Indonesia Cinta Sehat, Saatnya Jamu Berkontribusi. http://www.depkes.go.id/index.php?vw=2&id=1723 : access date: 30 May 2014
2. Block, K. J and Mead, M. N. 2003 Immune System Effects of Echinacea Ginseng and Astragalus. *Integr. Cancer Ther.* **2**(3): 247-267.
3. Tan H T and Rahadja K 2007 Obat-Obat penting, Khasiat, Penggunaan dan Efek-Efek Sampingnya. Elex Media Komputindo, Jakarta.
4. Roy, P., S. Kamlesh, P. Ramji, G. Dipanwita, B. Nikunj, P. Archana, B. Gautam, S. Neerav, G., and Kamadasan T (2013) Evaluation of anti-ulcer effects of ethanolic extract of Delonix regia flower.

Indian J. Res. Pharm. Biotechnol. **1**(3):440-459

5. Kumar A R Rijwana S and Yeshwanth D 2013 Phytochemical evaluation of Delonix regia Samanea saman, Bauhinia variegatga. *Intern. J. Res. Pharm. Chem.* **3**(4): 2231-278.
6. Aldi Y Nisya O dan Dian H (2013) Uji Imunomodulator beberapa subraksi ekstraksi Asetat Meniran (*Phyllanthus niruri* L.) pada Mencit Putih Jantan dengan Metoda Carbon Clearance. *Seminar Nasional Perkembangan Terkini Sains Farmasi dan Klinik III* 2339-2592.
7. Fild J W and Shute P G 1965 The Microscopy Diagnosis of Human Malaria: II-A Morphological Study of Erythrocytic Parasites. (Kuala Lumpur: The Government Press)
8. Susilo J 2012 Efek Imunomodulator Fraksi Etil Asetat Daun Jombang (*Taraxacum officinale* Weber et Wiggers) Terhadap Respon Imun Nonspesifik pada Mencit Jantan Galur Balb/C. *Bachelor Thesis*. STIKES Ngudi Waluyo, Ungaran.
9. Farizal, J. 2012. Pengaruh pemberian ekstrak etanol umbi bidara upas (Meremia mammosa) terhadap proliferasi limfosit dan produksi Roi Makrofag Study Eksprimental Infeksi Salmonella Typhimurium Pada Mencit Balb/C. *Thesis* Universitas Diponogoro, Semarang.
10. Suhirman, S., Christina. W. 2013. Prospek Dan Fungsi Tanaman Obat Sebagai Imunomodulator. Jurnal Penelitian, 121-122.
11. Ulfah, M. 2014. Uji Aktivitas Imunostimulan Fraksi Asetat Ekstrak Etanol Kelopak Rosella (Hibiscus sabdariffa L.) terhadap Proliferasi Sel Limfosit Mencit Galus Swiss Secara In Vitro Beserta Identifikasi Kandungan Senyawa Kimianya. *Bachelor Thesis*. Universitas Wahid Hasyim, Semarang.
12. Middleton E Kandaswami C and Theoharides T C 2000 The Effect of Plant Flavonoid on Mammalian cell: Implication for Inflamation, Heart Disease and Cancer. *J. Pharmacol*. **52**(4): 673-751.
13. Craxton A Shu G Satlatvala J Kreb E G and Clark E A 1998 P38 MPAK Is Required for CD40- Induced Gene Expression and Proliferation in B Lymphocytes. *J. Immunol.* **161**: 3225-3236.
14. Campbell N A Jane B R dan Lawrence G M 2004. *Biologi.* Translation from: Biology, by Wasmen, W. (Jakarta: Erlangga).
15. Fitri F 2015 Uji Toksisitas Akut Ekstrak Methanol Daun Flamboyan (*Delonix regia* (Boj. Ex.Hook) Raf.) Terhadap Mencit Strain Swiss-Webster. *Bachelor Thesis*. Universitas Syiah Kuala, Banda Aceh.
16. Susilo J 2012 Efek Imunomodulator Fraksi Etil Asetat Daun Jombang (Taraxacum officinale Weber et Wiggers) terhadap Respon Imun Nonspesifik pada Mencit Jantan Galur Balb/C. *Bachelor Thesis* STIKES Ngudi Waluyo, Unara
17. Yuswantina R Efek Imunomodulator Ekstrak Etanol Daun Sukun (*Artocarpus Altilis* (Park) Fosberg) Terhadap Respon Imun Non Spesifik pada Mencit Jantan Galur Balb/C. *Bachelor Thesis*. Stikes Ngudi Waluyo. Unggaran.
18. Bratawidjaja G K and Rengganis I 2012 Imunologi Dasar (Jakarta: FK UI)
19. Samuel C E 2001 Antiviral Actions of Interferon. *Clinical microbial* **14**(4): 778-809.
20. Aldi Y dan Suharti 2011 Aktivitas Ekstrak Etanol Biji Jintan Hitam (*Nigella sativa* L.) Terhadap Titer Antibodi dan Jumlah Sel Leukosit Pada Mencit Putih Jantan. *Scienti* **1**(1): 2087-5045.
21. Wijisekera R O B 1991 The Medicinal Plant Industry. (Florida: CRC Press).
22. Nurkhasanah 2009 Pengaruh Pemberian Ekstrak Jintan Hitam (*Nigella sativa*) Terhadap Respon Proliferasi Limfosit Limpa Mencit Balc/C yang Diinfeksi *Salmonella typhimurium*., *Bachelor Thesis* Universitas Dipenogoro. Semarang.
23. Roitt I Jonathan B and David M 1997 Essential Immunology (London: Mosby International)
24. Strite S and Morkoc H 1992 *J. Vac. Sci. Technol.* B **10** 1237
25. Jain S C, Willander M, Narayan J and van Overstraeten R 2000 *J. Appl. Phys.* **87** 965
26. Nakamura S, Senoh M, Nagahama S, Iwase N, Yamada T, Matsushita T, Kiyoku H and Sugimoto Y 1996 *Japan. J. Appl. Phys.* **35** L74