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

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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-inflammatory 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 (H0)
and after (H14) 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).

\[
\text{Lymphocytes(\%)} = \frac{L}{L+M+N} \times 100\% \quad (1)
\]

\[
\text{Monocytes (\%)} = \frac{M}{L+M+N} \times 100\% \quad (2)
\]

\[
\text{Neutrophils (\%)} = \frac{N}{L+M+N} \times 100\% \quad (3)
\]

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
prepared 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].

\[
\text{Spleen’s relative weight} = \frac{\text{spleen’s weight}}{\text{body’s weight}} \times 100\% \quad (4)
\]

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 (H0) and after
(H14) the administration of the flamboyant leaf
eExtract 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 (P0)
and other treatment groups (P1, P2 and P3).
Significant leukocyte count difference (p< 0.05)
was also found between P1 and P3, and between
P2 and P3, but not between P1 and P2 (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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(H0)</th>
<th>(H14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0 (Control)</td>
<td>24,80±1,643</td>
<td>27,00±2.23</td>
</tr>
<tr>
<td>P1 (250 mg/kg bb)</td>
<td>24,80±2,588</td>
<td>56,20±3,61</td>
</tr>
<tr>
<td>P2 (500 mg/kg bb)</td>
<td>25,40±1,140</td>
<td>58,40±5,54</td>
</tr>
<tr>
<td>P3 (750 mg/kg bb)</td>
<td>27,00±1,000</td>
<td>74,20±5,73</td>
</tr>
</tbody>
</table>

Note: H0: before flamboyant extract administration. H14: 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. Middelton 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 (NFKB), 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 Campbell [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](image)

The phagocytosis index shows that the flamboyant leaf extract had a moderate immunostimulatory characteristic at P1 (250 mg/kg BW) and P2 (500 mg/kg BW); and strong immunostimulatory potency P3 (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 that 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Spleen relative weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₀ (control)</td>
<td>41.79±1.29</td>
<td>0.57±0.04</td>
</tr>
<tr>
<td>P₁ (250 mg/kg BW)</td>
<td>41.70±1.54</td>
<td>0.61±0.04</td>
</tr>
<tr>
<td>P₂ (500 mg/kg BB)</td>
<td>41.17±2.05</td>
<td>0.64±0.04</td>
</tr>
<tr>
<td>P₃ (750 mg/kg BB)</td>
<td>41.81±2.05</td>
<td>0.65±0.06</td>
</tr>
</tbody>
</table>

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 phosphorylisis the serine or threonine amino acid residues on the target cells. Afterward, the IP3 stimulates the release of Ca²⁺ into the cytoplasm. The increased Ca²⁺ concentration has a critical role in stimulating the action of protein kinase C and 5-lipooxygenase. 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.

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