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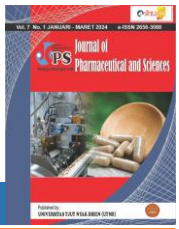

Evaluation of effectiveness of ethanol extract of green amaranth leaves (*Amaranthus hybridus L.)* as an immunostimulant in male white mice (Mus musculus)

Uji efektivitas ekstrak etanol daun bayam hijau (*Amaranthus hybridus L.)* sebagai imunostimulan pada mencit putih jantan (*Mus musculus*)

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Abstract

The immune system plays a critical role in defending the body against pathogens, and enhancing its activity through immunostimulants is essential for improving health. This study aims to evaluate the immunostimulatory potential of ethanol extract from green amaranth leaves (*Amaranthus hybridus* L.) in male white mice (*Mus musculus*). The ethanol extract green amaranth leaves was prepared from dried green amaranth leaves and tested for its effect on phagocytic activity using the carbon clearance method. Phytochemical screening revealed the presence of flavonoids, saponins, tannins, and steroids. The animals were divided into five groups: negative control (0.5% Na CMC), positive control (Stimuno Forte®), and three experimental groups receiving varying doses of the ethanol extract (125 mg/kg, 250 mg/kg, and 500 mg/kg body weight). The results indicated that the ethanol extract significantly enhanced phagocytic activity, with the most optimal effect observed at 125 mg/kg body weight. The stimulation index increased with higher doses, demonstrating the dose-dependent immunostimulatory effect. The study concludes that the ethanol extract of green amaranth leaves can serve as an effective natural immunostimulant, with the 125 mg/kg body weight dose being the most effective in enhancing immune function in male white mice. These findings suggest the potential of green amaranth as a therapeutic agent for immune-related disorders.

Keywords*: *Green Amaranth Leaves, Ethanol Extract, Immunostimulant, Phagocytosis, Carbon Clearance Method

Abstrak

Sistem kekebalan tubuh memainkan peran penting dalam melindungi tubuh dari patogen, dan peningkatan aktivitasnya melalui imunostimulan sangat penting untuk meningkatkan kesehatan. Penelitian ini bertujuan untuk mengevaluasi potensi imunostimulasi dari ekstrak etanol daun bayam hijau (*Amaranthus hybridus* L.) pada mencit putih jantan (*Mus musculus*). Ekstrak etanol daun bayam hijau disiapkan dari daun amaranth hijau yang dikeringkan dan diuji untuk pengaruhnya terhadap aktivitas fagositosis menggunakan metode penghilangan karbon. Penyaringan fitokimia menunjukkan adanya flavonoid, saponin, tanin, dan steroid. Hewan percobaan dibagi menjadi lima kelompok: kontrol negatif (0,5% Na CMC), kontrol positif (Stimuno Forte®), dan tiga kelompok eksperimental yang menerima dosis ekstrak etanol yang berbeda (125 mg/kg, 250 mg/kg, dan 500 mg/kg berat badan). Hasil penelitian menunjukkan bahwa ekstrak etanol secara signifikan meningkatkan aktivitas fagositosis, dengan efek yang paling optimal diamati pada dosis 125 mg/kg berat badan. Indeks stimulasi meningkat dengan dosis yang lebih tinggi, menunjukkan efek imunostimulasi yang bergantung pada dosis. Penelitian ini menyimpulkan bahwa ekstrak etanol daun amaranth hijau dapat menjadi imunostimulan alami yang efektif, dengan dosis 125 mg/kg berat badan menjadi yang paling efektif dalam meningkatkan fungsi kekebalan tubuh pada tikus putih jantan. Temuan

ini menunjukkan potensi amaranth hijau sebagai agen terapeutik untuk gangguan yang berkaitan dengan sistem kekebalan.

Kata Kunci: Daun Bayam Hijau, Ekstrak Etanol Daun Bayam Hijau, Imunostimulan, Fagositosis, Metode Penghilangan Karbon.

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Introduction

The immune system plays a crucial role in maintaining health and defending the body against pathogens such as viruses, bacteria, and other microorganisms. It consists of various components working in unison, including physical barriers like the skin, as well as specific immune responses involving immune cells such as lymphocytes and macrophages. One way to enhance immune function is by using immunostimulants—substances that can increase the activity or responsiveness of the immune system [1].

Immunostimulants can be derived from synthetic or natural sources. Natural immunostimulants, which are commonly found in medicinal plants, have gained increasing attention due to their perceived safety and lower side effects compared to synthetic drugs. The use of medicinal plants as immunostimulants is not only prevalent in traditional medicine but has also gained support in modern pharmacology [2,3].

Amaranthus hybridus L., commonly known as green amaranth, is a plant belonging to the Amaranthaceae family. It is widely distributed in tropical and subtropical regions, including Indonesia. Apart from being a nutritious food source, green amaranth also has potential medicinal properties. Various studies have demonstrated that green amaranth contains bioactive compounds such as flavonoids, alkaloids, tannins, and saponins, which are associated with pharmacological effects [4-6].

Flavonoids and saponins, for example, are known to possess antioxidant, anti-inflammatory, and immunomodulatory effects. As a result, extracts from green amaranth leaves are frequently studied for their potential therapeutic benefits in a variety of diseases, including infections and immune-related disorders. However, while research on green amaranth's medicinal benefits is relatively abundant, further investigations are needed to explore its potential as an immunostimulant [7,8].

Ethanolic extracts are commonly used to extract bioactive compounds from plants because ethanol can dissolve both polar and non-polar compounds, allowing for a more efficient extraction of bioactive molecules. In the case of the ethanolic extract of green amaranth leaves, compounds such as flavonoids, saponins, and alkaloids can be extracted, potentially offering immunostimulatory effects [9-11].

Previous studies have indicated that these compounds may enhance immune cell activity, such as macrophages and lymphocytes, and stimulate the production of cytokines involved in immune responses. For instance, flavonoids are known to boost humoral immune responses by stimulating antibody production, while saponins can enhance phagocytosis by immune cells. Therefore, the ethanolic extract of green amaranth leaves holds significant potential as a natural immunostimulant [12,13].

White male mice (*Mus musculus*) are one of the most commonly used animal models in pharmacological and immunological research due to their similarities with the human immune system and their ease of

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handling. Mice can be induced to experience either a weakened or enhanced immune response, making them ideal subjects for testing immunostimulants [14,15].

This study aims to evaluate the effectiveness of ethanolic extract from green amaranth leaves as an immunostimulant in white male mice.

Experimental Section

Experimental Section should be described with sufficient details to allow others to replicate and develop the published results.

Materials and Apparatus

The materials used in this study include green amaranth leaves (*Amaranthus hybridus* L.), ethanol, Pelikan brand Chinese ink, 0.9% NaCl, CMC-Na, aquadest, Stimuno Forte® capsules containing 50 mg of *Phyllanthus niruri* extract per capsule (Dexa Medica, Palembang), K2EDTA, Turk's reagent, Na_2CO_3, and the test animals used are male white mice (*Mus musculus*).

The apparatus used in this study includes a distillation apparatus, rotary evaporator, analytical balance, Eppendorf tubes, dropper pipette, vials, capillary tubes, evaporating dish, scalpel, micropipette, UV-Vis spectrophotometer 1800 (Shimadzu, Japan), hemocytometer, microscope, aluminum foil, animal cages, syringe, tubes, oven, furnace, desiccator, blender, water bath, porcelain crucible, tongs, and round-bottom flask.

Preparation of Green Amaranth Leaf Simplisia (*Amaranthus hybridus* L.)

Fresh green amaranth leaves weighing 10 kg are used as samples. The process begins with wet sorting, followed by weighing, washing, chopping, and then drying in a drying cabinet at a temperature of approximately 40°C. Afterward, dry sorting is conducted, and the dried leaves are ground using a blender and sieved with a 40-mesh sieve to obtain a fine powder. The dry powder is then weighed and stored in a well-sealed container.

Phytochemical Screening

Alkaloid Test

0.5 g of simplisia powder is added to 1 ml of 2 N HCl and 9 ml of distilled water, then heated in a water bath for a few minutes. The mixture is cooled and filtered. The obtained filtrate is used for the alkaloid test. Three test tubes are prepared, each containing 0.5 ml of filtrate. To each test tube, add 2 drops of Mayer's reagent, Bouchardat's reagent, and Dragendorff's reagent. Alkaloids are considered positive if a precipitate forms. If at least two of the three reagents produce a positive result, the sample is declared to contain alkaloids, indicated by the formation of a white or yellow precipitate [16,17].

Flavonoid Test

One gram of simplicia powder is added to 10 mL of hot water, boiled for 5 minutes, and filtered while hot. A 5 mL aliquot of the filtrate is taken, then a small amount of magnesium powder and 1 mL of concentrated hydrochloric acid are added, followed by 2 mL of amyl alcohol. The mixture is shaken and allowed to separate. The formation of a yellow, orange, or red color in the amyl alcohol layer indicates the presence of flavonoids [16].

Saponin Test

0.5 g of simplicia powder is placed into a test tube, then 10 mL of hot water is added, cooled, and shaken for 10 seconds. If foam forms for no less than 10 minutes, with a height of 1-10 cm, and does not disappear when 1 drop of 2 N HCl is added, it indicates the presence of saponins [16,17].

Tannin Test

One gram of simplicia powder is boiled with 10 mL of distilled water for 3 minutes, then cooled and filtered. The filtrate is diluted until it is colorless, and 1-2 drops of 1% $FeCl_3$ reagent are added. If a greenish-black color appears, it indicates the presence of tannins [16].

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Steroid Test

One gram of simplicia is macerated with 20 mL of n-hexane for 2 hours, then filtered, and the filtrate is evaporated. Anhydrous acetic acid and concentrated sulfuric acid are then added. If a purple, red, or a color that changes to blue-purple or blue-green forms, it indicates the presence of terpenoids/steroids [16,17].

Preparation of Ethanol Extract of Green Amaranth Leaves (*Amaranthus hybridus* L.)

Weigh 500 grams of simplicia powder and macerate it using ethanol as the solvent, with a solvent-to-material ratio of 1:10, using 5 liters of solvent. The 500 grams of simplicia powder is placed in a glass jar and soaked with 75 parts of ethanol solvent (3750 mL) for 5 days, occasionally stirring. After 5 days, the filtrate is filtered using filter paper, and the residue is separated, obtaining filtrate I. The residue is then dissolved again with 25 parts of ethanol solvent (1250 mL) for 2 days, kept away from light, and occasionally stirred. The filtrate is then filtered again, and the residue is discarded, yielding filtrate II. Filtrate I and II are combined, concentrated, and evaporated using a vacuum rotary evaporator until a thick extract is obtained.

Determination of Carbon Clearance

At the 5th and 15th minutes after the injection of carbon ink, 50 µL of blood is collected from the retro-orbital vein of the mice and added to 4 mL of 1% Na_2CO_3 (w/v). The absorbance is then measured using a UV-Visible spectrophotometer at a wavelength of 675 nm [18].

Preparation of Stimuno Forte® Suspension

The Stimuno Forte® suspension is prepared by weighing 0.5 g of Na CMC and expanding it with 20 times its weight in hot water. After it expands, it is triturated, and then 0.065 g of Stimuno Forte® powder is added. The mixture is triturated until homogeneous and diluted with distilled water to the planned volume of 100 mL.

Preparation of Green Amaranth Leaf Extract Suspension

The ethanol extract of green amaranth leaves is weighed at 125 mg, 250 mg, and 500 mg, and then placed into a mortar. A 0.5% Na-CMC suspension is gradually added while triturating until a homogeneous mixture is formed, reaching a final volume of 10 mL.

Preparation of Experimental Animals

The test animals used were 25 male mice weighing 20–30 grams. The mice were divided into 5 treatment groups, with each group consisting of 5 male mice. Group I served as the negative control (Na CMC 0.5%), Group II as the positive control (Stimuno Forte®), and Groups III, IV, and V as the test groups (varying doses of the extract). Before treatment, the animals were acclimatized for 2 weeks in suitable cages to adjust to their new environment.

Testing of Immunostimulant Effects

The immunostimulant effect was tested using the carbon clearance method. In this test, the mice were divided into 5 groups, with each group consisting of 5 mice. Group I (negative control) was given a 0.5% Na CMC suspension, Group II (positive control) was given Stimuno Forte® suspension, Group III was given the extract suspension at a dose of 125 mg/kg body weight, Group IV was given the extract suspension at a dose of 250 mg/kg body weight, and Group V was given the extract suspension at a dose of 500 mg/kg body weight.

Each test animal was given the test preparation orally once a day for 6 days. On the 7th day, the mice were injected with a carbon suspension via the tail vein at a dose of 0.2 mL/20 g body weight, and blood samples were collected at 5 and 15 minutes through the retro-orbital vein using capillary tubes, each containing 50 µL of blood. The samples were then lysed with 4 mL of 1% sodium carbonate and their absorbance was measured using a UV-Visible spectrophotometer at a wavelength of 675 nm. After 12 hours, the mice were euthanized, and the liver and lymph nodes were collected, weighed, and recorded.

Carbon Elimination Rate Constant : $K = \frac{\text{InOD1} - \text{InOD2}}{\text{t2} - \text{t1}}$

Description:
K : Phagocytosis constant

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In ODI : Absorbance at minute 5
In OD2 : Absorbance at minute 15
t1 : Initial time (minute 5)
t2 : Final time (minute 15)

$$\text{Stimulation index} = \frac{\text{Phagocytosis constant of the extract}}{\text{Phagocytosis Constant of the Negative Control}}$$

$$\text{Phagocytosis Index} = \frac{K^{1/3}x \text{ Animal weight}}{\text{Liver weight} + Lymph\ node\ weight}$$

Data Analysis

The data from the study were analyzed using the Statistical Product and Service Solutions (SPSS) program to determine the homogeneity and normality through a one-way ANOVA. This was done to assess the differences in means among the treatments. If significant differences were found, post-hoc analysis was conducted using the Homogeneous Subsets test to identify which variables differed. A significance level of $p < 0.05$ was considered statistically significant.

Results and Discussion

Phytochemical screening results

The phytochemical screening results are summarized in Table 1 below.

Table 1. Phytochemical Screening Results of Green Amaranth Leaves (*Amaranthus hybridus* L.)

Phytichemical Compound	Result
Alkaloid	Negative
Flavonoid	Positive
Saponin	Positive
Tannin	Positive
Steroid	Positive

Based on table 1, the test for alkaloids was negative, as no precipitate formed after treatment with Mayer's, Bouchardat's, and Dragendorff's reagents. Alkaloids are known for their pharmacological activities, including analgesic, anti-inflammatory, and antimicrobial effects. However, the absence of alkaloids in *Amaranthus hybridus* suggests that this plant does not possess these specific bioactive compounds, or they may be present in very low concentrations that cannot be detected through standard testing methods [19].

Flavonoids were found to be present in the leaves of *Amaranthus hybridus*, as evidenced by the formation of a yellow, orange, or red color in the amyl alcohol layer. Flavonoids are a group of polyphenolic compounds with potent antioxidant, anti-inflammatory, and antimicrobial properties. Their presence in this plant suggests potential health benefits, particularly in combating oxidative stress and inflammation, which are involved in various chronic diseases [8,20].

Saponins were also detected, as stable foam formed when the leaf extract was heated. Saponins are glycosides that possess various bioactive properties, including antimicrobial, immunomodulatory, and cholesterol-lowering effects. The presence of saponins in *Amaranthus hybridus* highlights its potential for use in treating infections, improving immune function, and managing cholesterol levels [21].

Tannins were found to be present, as indicated by the formation of a greenish color when ferric chloride solution was added to the extract. Tannins are polyphenolic compounds that have astringent properties and are known for their antimicrobial and antioxidant activities. Their presence in the leaves of *Amaranthus hybridus* further supports the potential therapeutic value of this plant, particularly for wound healing, gastrointestinal issues, and infections [22].

Steroids were detected through Liebermann's reagent, as evidenced by the observed color change. Steroids have significant anti-inflammatory, immunosuppressive, and antiallergic properties. The presence of steroids in *Amaranthus hybridus* suggests that this plant may possess potential therapeutic effects in managing inflammatory conditions, autoimmune disorders, and other steroid-responsive diseases [23].

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Carbon Elimination Rate

The carbon elimination rate was measured to assess phagocytic activity in mice, using UV-Vis spectrophotometry at a wavelength of 675 nm. A decrease in carbon absorbance in the blood reflects the phagocytosis process, where the lower the absorbance value, the less carbon remains in the blood. The measurement results show a decrease in absorbance values across all treatment groups, indicating an increase in phagocytic activity (Figure 1).

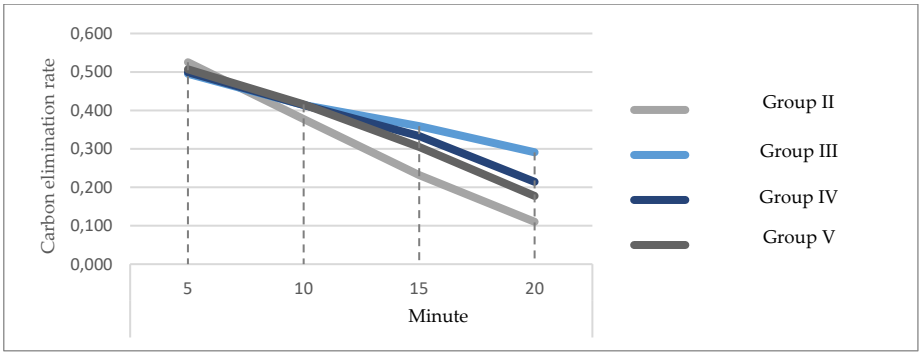

Figure 1. Carbon Elimination Rate in Mice Blood

Based on Figure 1, the results showed a decrease in absorbance over time due to carbon elimination. The faster the decrease in absorbance, the higher the increase in the carbon elimination rate in the blood.

The phagocytosis constant can be used as an indicator to determine the carbon elimination rate. The results of the phagocytosis constant can be seen in Table 2.

Table 2. The average results of the phagocytosis constant

Group	Phagocytosis Constant
I	0,0032±0,0005
II	0,0261±0,0008
III	0,4118±0,0004
IV	0,0076±0,0003
V	0,1312±0,0007

The phagocytosis constant is used to determine the rate of carbon elimination. The larger the value of the phagocytosis constant, the higher the carbon clearance rate, meaning that the phagocytic cells perform phagocytosis more quickly. Once the value of the phagocytosis constant is obtained, the phagocytosis index is then calculated. The phagocytosis index is directly proportional to the value of the phagocytosis constant, meaning that the larger the values of both, the faster the phagocytosis process carried out by phagocytic cells in eliminating carbon in the blood [24,25].

From the carbon elimination rate, the carbon elimination rate constant is calculated. The carbon elimination rate constant is one of the parameters used to determine the speed of phagocytosis. The higher the value of the carbon elimination constant, the higher the rate of carbon elimination, meaning that phagocytic cells perform phagocytosis more quickly [26].

Phagocytosis Index

The phagocytosis activity test using the carbon clearance method reflects the nonspecific immune system's response to phagocytosis of foreign particles in the blood. Carbon ink is used as an antigen because it is stable in the bloodstream and does not cause thrombosis. Upon intravenous injection, the carbon is phagocytosed by macrophages, and its amount decreases over time due to phagocytosis by leukocytes, especially neutrophils, monocytes, and macrophages [27]. Phagocytosis activity is expressed as the phagocytosis index (Figure 2).

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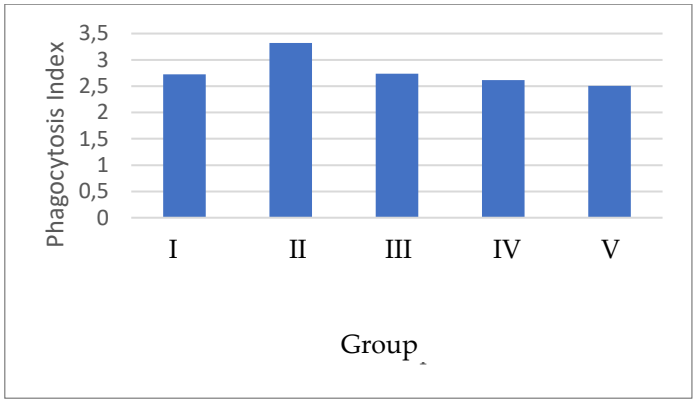

Figure 2 Phagocytosis Index

Figure 2 shows that Stimuno Forte® has the highest phagocytosis index, followed by ethanol extract of green amaranth leaves (*Amaranthus hybridus L.)* at doses of 125 mg/kg body weight and 250 mg/kg body weight. The 500 mg/kg body weight dose of the extract had a lower index than Stimuno Forte® but was still significantly different from the negative control group (CMC Na 0.5%) and other doses. The ANOVA test showed significant differences ($p < 0.05$) between the treatment groups.

The results indicate that the ethanol extract of green amaranth leaves at doses of 125 mg/kg body weight, 250 mg/kg body weight, and 500 mg/kg body weight enhances phagocytosis activity, with the most optimal effect at 125 mg/kg body weight. The lack of significant difference between 125 mg/kg body weight and 250 mg/kg body weight suggests that 125 mg/kg body weight is the most effective dose for enhancing phagocytosis. Higher doses do not always lead to higher efficacy due to potential opposing effects from other compounds in the extract, which may reduce its overall effectiveness [28,29].

Stimulation Index

The stimulation index is the ratio between the test group and the control group. A compound with a stimulation index greater than 1 is classified as an immunostimulant, meaning it can enhance the body's defense system, while a value less than 1 indicates immunosuppressive properties (Figure 3).

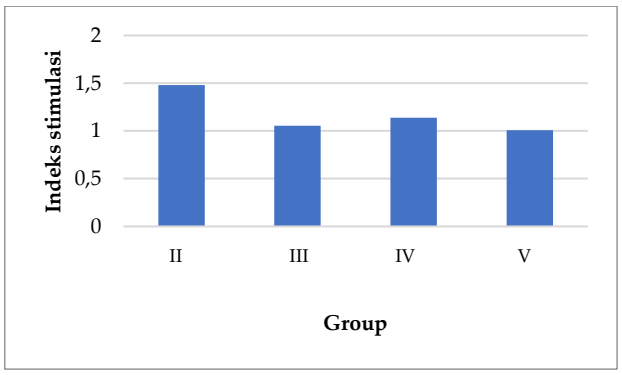

Figure 3. Stimulation Index.

The stimulation index of the ethanol extract of green amaranth leaves (*Amaranthus hybridus L.)* at doses of 125 mg/kg, 250 mg/kg, and 500 mg/kg shows a clear correlation between dose and stimulation index. As the dose of the ethanol extract increases, the stimulation index also increases, indicating a dose-dependent effect.

The data in Figure 3 show that the stimulation index for extract ethanol green amaranth leaves at 125 mg/kg body weight is 1.053, at 250 mg/kg body weight is 1.139, and at 500 mg/kg body weight is 1.007. These values demonstrate that extract ethanol green amaranth leaves acts as an immunostimulant. The stimulation index increases with the dose, suggesting that higher doses enhance the immune stimulation effect. The value of the stimulation index at the 250 mg/kg body weight dose closely approximates that of the positive control group, Stimuno Forte®, which contains 50 mg of meniran herb per capsule and is known to improve the immune system.

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The phytochemical screening of green amaranth leaves (*Amaranthus hybridus L.)* revealed the presence of secondary metabolites such as alkaloids, tannins, saponins, glycosides, steroids/triterpenoids, and flavonoids. Flavonoid compounds can enhance the immune system by increasing neutrophil oxidative activity, promoting B and T cell proliferation, and enhancing phagocytosis and macrophage activation, thus acting as immunostimulants [30].

The effectiveness of a substance is closely related to the chemical compounds it contains. The presence of secondary metabolites, particularly flavonoids, in green amaranth leaves likely contributes to its immunostimulant activity by boosting metabolic activity within cells. Based on these findings, it can be concluded that the ethanol extract of green amaranth leaves stimulates macrophages, thereby increasing phagocytosis and enhancing immune function [31].

Conclusions

The administration of ethanol extract of green amaranth leaves (*Amaranthus hybridus L.)* has an effect on increasing phagocytosis activity in male white mice. Based on the variation of doses of ethanol extract of green amaranth leaves, the dose of 125 mg/kg body weight is the most optimal dose in providing immunostimulant effects in male white mice.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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