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Research Article

Immunomodulatory Activity of Enau (*Arenga pinnata*) Leaf Extract on Macrophage Phagocytosis in Mice

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Abstract

The immune system is vital in protecting the body from pathogens and harmful foreign substances. This study aims to evaluate the immunomodulatory activity of *Arenga pinnata* leaf extract on macrophage phagocytosis activity in mice (*Mus musculus*) and determine its optimal dosage. Extraction was conducted using the maceration method with 96% ethanol as the solvent. The immunomodulatory test was performed by measuring macrophage phagocytosis activity. Male mice were divided into six groups: a negative control (0.5% Na-CMC), a positive control (Stimuno® 0.013 mg/kgBW), and four treatment groups receiving *A. pinnata* leaf extract at doses of 100 mg/kgBW, 200 mg/kgBW, 300 mg/kgBW, and 400 mg/kgBW. After seven days of treatment, the mice were injected intraperitoneally with *Staphylococcus aureus* bacteria, and peritoneal fluid was collected for macrophage phagocytosis activity analysis. The results showed that *A. pinnata* leaf extract significantly increased macrophage phagocytosis activity at 300 mg/kgBW and 400 mg/kgBW compared to the positive control. Phytochemical analysis revealed the presence of flavonoids, saponins, tannins, and triterpenoids, which contribute to its immunomodulatory effects. In conclusion, *A. pinnata* ethanol leaf extract exhibits potential as an immunomodulatory agent, particularly at 300 mg/kgBW and 400 mg/kgBW doses Further studies are necessary to explore its pharmaceutical applications and elucidate its mechanisms of action.

Keywords: Enau Leaf (*Arenga pinnata*), Immunostimulant, Macrophage, Phagocytosis

Aktivitas Imunomodulasi Ekstrak Daun Enau (*Arenga pinnata*) terhadap Fagositosis Makrofag pada Mencit

Abstrak

Sistem imun memiliki peran penting dalam melindungi tubuh dari patogen dan zat asing berbahaya. Penelitian ini bertujuan untuk mengevaluasi aktivitas imunomodulator dari ekstrak daun enau (*Arenga pinnata*) pada aktivitas fagositosis makrofag mencit (*Mus musculus*) dan menentukan dosis optimalnya. Ekstraksi dilakukan menggunakan metode maserasi dengan pelarut etanol 96%. Uji imunomodulator dilakukan dengan pengukuran aktivitas fagositosis makrofag. Mencit jantan dibagi menjadi enam kelompok: kontrol negatif (0,5% Na-CMC), kontrol positif (Stimuno® 0,013 mg/kgBB), dan empat kelompok perlakuan dengan ekstrak daun enau 100 mg/kgBB, 200 mg/kgBB, 300 mg/kgBB, dan 400 mg/kgBB. Setelah tujuh hari perlakuan, mencit diinfeksi bakteri *Staphylococcus aureus* melalui injeksi intraperitoneal, kemudian cairan peritoneum diambil untuk penghitungan aktivitas fagositosis makrofag. Hasilnya, ekstrak daun enau meningkatkan aktivitas fagositosis makrofag secara signifikan pada dosis 300 mg/kgBB dan 400 mg/kgBB dibandingkan kontrol positif. Analisis fitokimia menunjukkan adanya flavonoid, saponin, tannin, dan triterpenoid yang berkontribusi terhadap efek imunomodulator. Simpulannya, ekstrak etanol daun enau berpotensi sebagai agen imunomodulator, terutama pada dosis 300 mg/kgBB dan 400 mg/kgBB. Penelitian lanjutan diperlukan untuk pengembangan sediaan farmasi.

Kata Kunci: Daun enau (*Arenga pinnata*), Fagositosis, Imunostimulan, Makrofag

1. Introduction

The environment surrounding us contains various pathogens such as bacteria, viruses, protozoa, parasites, and irritants. These substances can enter the body and cause different diseases or tissue damage. These pathogens are unwanted materials that need to be eliminated.[1] The body consistently defends itself when foreign substances attempt to enter, a process known as immunity. Various tissues and cells of the body unite to form an immune system that generates an immune response. The immune system serves multiple functions, including defense against foreign substances, clearing dead cells, and repairing damaged tissues.[2]

Immunomodulation refers to substances or drugs used to restore the balance of a disturbed immune system by stimulating and improving immune function.[3] The healing of infections is faster when the body's immune system function is enhanced. Various plant-based materials can enhance the function of several components of the immune systems of nonspecific (macrophages, NK cells) and specific (T cell proliferation, antibody-producing B cells).[4]

Phagocytosis is the process of engulfing particles by cells. Macrophages and polymorphonuclear leukocytes are the most critical phagocytic cells. The majority of foreign substances entering tissues are eliminated through phagocytosis mechanisms.[5] Macrophage phagocytosis is widely used as an immunological parameter to evaluate the health/function of the immune system. The phagocytosis capacity/activity assessment can be measured by phagocytosis capacity and index. Phagocytosis is one of the most commonly used methods for screening active substances affecting immune responses.[6]

The role of herbs as immunomodulators is to stimulate, suppress, or modulate various components of the innate and adaptive immune systems. Multiple studies have found that herbs can affect cells in the immune system, antibodies cytokines produced, play a role in cell apoptosis, autoimmune diseases, and more.[7] One herbal plant suspected to be an immunostimulant is the enau leaf (*Arenga pinnata*), as it positively contains alkaloids, flavonoids, saponins, phenols, tannins, and steroids.

The enau tree belongs to the palm family and is naturally found in forested areas. It falls under the subfamily Arecoidae and the tribe Caryoteae.[8] Enau or aren are extensively utilized by communities. These trees are native to Southeast Asia, thriving in tropical and dry forest habitats.[9] In Southeast Sulawesi, enau is empirically used as a traditional medicine, including immunomodulatory.[10] Based on that, research is conducted to test the immunomodulatory activity of ethanol extract from enau leaves (*Arenga pinnata*) on macrophage phagocytosis in male mice (*Mus musculus*).

2. Methods

2.1. Tools

The tools used in the study included rat scales, analytical balances, glassware (pipettes, funnels, beakers, test tubes, stirring rods, spatulas, porcelain crucibles, Petri dishes), autoclave, jars, blender, cotton, a set of mouse surgery tools (scalpel, sterilized forceps), microscope, injection syringe, oral syringe, oven, rotary evaporator (Buchi®), hot plate (Ika®), and UV-vis spectrophotometer (Shimadzu®).

2.2. Materials

The materials used in this study were distilled water, enau leaf extract (*Arenga pinnata*), sodium-CMC, ether, Stimuno® (Dexamedika, Indonesia), Staphylococcus aureus bacteria, 0.9% NaCl, 4% Giemsa dye, 96% ethanol, 1% $BaCl_2$, 1% H_2SO_4, methanol, Mayer's reagent, HCl, FeCl3, chloroform, sulfuric acid, cotton, filter paper, and adhesive tape.

2.3. Procedures

2.3.1. Sample Preparation and Extraction

Enau leaves (*Arenga pinnata*) used in this research were collected from West Muna District, Southeast Sulawesi, and were determined at the Pharmacognosy-Phytochemistry Laboratory, Universitas

Mandala Waluya, under the reference number 197/09.03.01/VIII/2023. Enau Leaves were thoroughly washed with running water, chopped, and air-dried without direct sunlight until completely dry. 500 grams of dried enau leaves were weighed and placed into a maceration container, where 96% alcohol was added to submerge the sample. The container was sealed with black tape lined with aluminum foil and stored at room temperature for 3 days, protected from light, with daily agitation for 3×24 hours. After maceration, the extract was filtered, collected, and concentrated using a rotary evaporator (40-65˚C, 60 rpm) to produce a thick extract.

2.3.2. Phytochemical Screening

a. Alkaloid test: A sample of 0.05 grams of Enau leaf extract was added to a test tube, followed by adding H_2SO_4 until thoroughly mixed. Then, it was filtered, and Meyer's reagent was added. If a white precipitate formed, the sample was considered positive.
b. Flavonoid test: A sample of 0.05 grams of the extract was mixed with $FeCl_3$ and HCl in a test tube until thoroughly combined. A deep red, yellow, or orange precipitate indicated a positive result.
c. Saponin test: A sample of 0.05 grams of extract was placed in a test tube, and hot water was added to dissolve it completely. After 30 minutes, 1 drop of 2N HCl was added. The formation of stable foam indicated a positive result.
d. Tannin test: A sample of 0.05 grams of extract was steeped in boiling water for 3 minutes. After filtration, it was treated with $FeCl_3$ solution. The formation of a dark blue or dark green solution indicated a positive result.
e. Triterpenoid test: A 0.5-gram extract sample was mixed with 2 ml chloroform. Then, 3 ml of sulfuric acid was slowly added until a colored layer formed. A reddish-brown color indicated a positive result.

2.3.3. Preparation of Research Materials

a. Preparation of Stimuno Suspension

0.13 mg of Stimuno® was weighed, ground into powder, and added to a mortar. It was mixed with an appropriate amount of 0.5% sodium-CMC suspension. After homogenization, it was transferred to a 25 mL volumetric flask and topped up with 0.5% sodium-CMC suspension to the mark.

b. Preparation of McFarland Solution

0.05 mL of 1% $BaCl_2$ was mixed with 9.95 mL of 1% H_2SO_4 to obtain a suspension equivalent to 1.5×10^8 CFU (colony forming units)/mL. The turbidity of the McFarland standard was checked using a spectrophotometer. A McFarland standard of 0.5 had an absorbance reading of 0.08 to 0.1 at 625 nm.[11]

2.3.4. Preparation of Test Animals

Healthy male mice (*Mus musculus*), aged 3-4 months and weighing 20-40 g, were selected based on their behavior and acclimated for seven days at the Pharmacology Laboratory, Pharmacy Study Program, Universitas Mandala Waluya, Kendari. The experiment was conducted following the approval of the protocol Ethical Clearance issued by the Ethics Committee of Universitas Mandala Waluya under protocol No. 0172419018. After acclimatization, the mice were weighed to determine the appropriate dosage for treatment. The test animals were divided into six groups, each consisting of three mice, including treatment groups receiving doses of 100 mg/kg BW, 200 mg/kg BW, 300 mg/kg BW, and 400 mg/kg BW, along with a positive control group (Stimuno®) and a negative control group (0.5% Na-CMC).

2.3.5. Testing of Immunomodulatory Activity

a. Preparation of Bacterial Suspension

Staphylococcus aureus bacteria grown on nutrient agar media were suspended in a 0.9% NaCl solution. Then, the bacterial count was determined through spectrophotometry (λ = 580 nm, 25% transmittance), yielding a bacterial count equivalent to 108 cells/mL. The bacteria were prepared for injection into test animals. This process aimed

to induce a controlled animal infection, thereby simulating an immune challenge. The induced infection allowed researchers to assess the immunomodulatory effects of Arenga pinnata leaf extract by evaluating the immune response of infected animals, explicitly focusing on macrophage activity and phagocytosis in response to the bacterial infection.[12]

b. Testing the Immunomodulatory Effect of Enau Leaves (*Arenga pinnata*)

The immunomodulatory effect testing was conducted using the following procedure: Test animals were divided into 6 groups, each consisting of 3 test animals. Test animal treatment was administered orally once daily for 7 days according to the specified volume. Group I received enau leaf extract at 100 mg/kg BW. Group II received enau leaf extract at 200 mg/kg BW. Group III received enau leaf extract at 300 mg/kg BW. Group IV received enau leaf extract at 400 mg/kg BW. Group V received Stimuno® at 0.13 mg/kg BW, and Group VI received 0.5% Na-CMC as the negative control. The positive control, Stimuno®, was administered at 0.13 mg/kg BW daily for 7 consecutive days.[12]

c. Measurement of macrophage phagocytosis activity in experimental animals

On the eighth day, each mouse was infected with 0.5 mL of *Staphylococcus aureus* suspension via intraperitoneal injection and allowed to rest for one hour before surgery. The mice were then anesthetized with ether and dissected.[12] The value of phagocytosis activity is the percentage of active macrophage cells engaged in phagocytosis out of 100 macrophage cells.[13] The data obtained were statistically analyzed using One-Way ANOVA.

$$\%\ \text{activity} = \frac{\text{Total active macrophages}}{\textit{Total observed macrophages}} \times 100\ \%$$

3. Result

3.1. Enau (*Arenga pinnata*) leaf extraction

The result of *Arenga pinnata* leaf extraction was 500 grams weighed and extracted using a maceration method with a 96% ethanol solvent, yielding 78 grams of concentrated extract with a 15% yield (Table 1).

3.2. Phytochemical Screening of Ethanol Extract from Enau (*Arenga pinnata*) Leaves

The results of the phytochemical screening of Arenga pinnata leaf extract are presented in Table 2. The phytochemical screening results indicate that *Arenga pinnata* leaf extract contains flavonoids, saponins, tannins, and triterpenoids, while alkaloids and steroids were not detected.

3.3. Phagocytosis activity of ethanol extract from Enau (*Arenga pinnata*) leaves

Table 3 shows the naive and active macrophages in the ethanol extract of *Arenga pinnata* leaves. Figure 1 shows the average percentage of macrophage phagocytosis activity. The results presented in Table 3 show the percentage of naive and active macrophages across all treatment groups. The negative control (0.5% Na-CMC) exhibited the lowest macrophage activity, with active macrophages ranging between 20-31%. The positive control (0.013 mg/kgBW Stimuno®) demonstrated significantly higher activity, with active macrophages ranging from 67-80%. Among the treatment groups, the ethanol extract of *Arenga pinnata* leaves showed varying activity based on the dosage. The 100 mg/kgBW dose had 89-91% active macrophages, while the 200 mg/kgBW dose showed lower activity (26-49%). The 300 mg/kgBW dose had the highest macrophage activity, with 92-93% active macrophages, followed by the 400 mg/kgBW dose, which showed activity between 81-87%. These findings indicate dose-dependent variations,

Table 1. Result of Enau (Arenga pinnata) leaf extract

Weight of dried Enau Leaf (g)	Weight of extract (g)	Yield
500	78	15%

Table 2. The phytochemical screening in Enau leaf extract

Compound Screening	Result
Alkaloid	-
Flavonoid	+
Saponin	+
Tannin	+
Steroid	-
Triterpenoid	+

+: Indicates the presence of the compound, -: Indicates the absence of the compound.

with the 300 mg/kgBW dose being the most effective in increasing macrophage activity.

4. Discussion

In this study, the ethanol extract of Arenga pinnata leaves was utilized and processed using the maceration method. This method was chosen as it effectively preserves active compounds during extraction. The process involves breaking down the cell wall and membrane due to pressure differences between the interior and exterior of the cells, enabling secondary metabolites to dissolve in the ethanol solvent used. Ethanol, widely employed in extraction processes, was chosen for its low toxicity compared to acetone and methanol, cost-effectiveness, versatility in various extraction methods, and safety in pharmaceutical and food applications.[14,15]

The study used *Staphylococcus aureus* to simulate an infection model. During infection, T lymphocytes secrete lymphokines that attract and activate macrophages at the site of infection. Activated macrophages release various substances, including enzymes, lysozymes, elastase, collagenase, complement, and cytokines, to combat the infection. *Staphylococcus aureus* was chosen due to its classification as a Gram-positive bacterium, which binds the Giemsa stain well for clear microscopic observation. Moreover, *Staphylococcus aureus* lacks

Table 3. The average percentage of macrophage activity of ethanol extract from enau (*Arenga pinnata*) leaves

No	Group	Mice	Results	
			Naïve Macrophages (%)	Active Macrophages (%)
1	Na-CMC (Negative control)	I	69	31
		II	80	20
		III	77	23
2	Stimuno (Positive control)	I	20	80
		II	25	75
		III	33	67
3	100 mg / Kg BW Enau Extract	I	11	89
		II	17	83
		III	9	91
4	200 mg / Kg BW Enau Extract	I	74	26
		II	51	49
		III	74	26
5	300 mg / Kg BW Enau Extract	I	7	93
		II	8	92
		III	8	92
6	400 mg / Kg BW Enau Extract	I	13	87
		II	49	51
		III	19	81

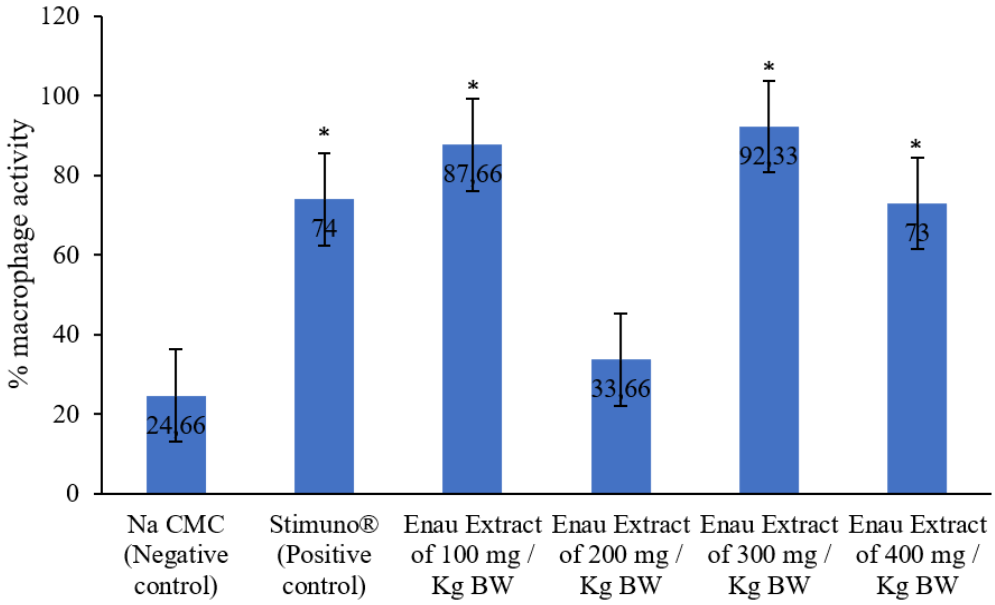

Figure 1. Average percentage of macrophage activity of ethanol extract from enau (Arenga pinnata) leaves. Data was presented as average ± SD; asterisks (*) indicate a significant difference compared to the negative control ($p < 0.05$).

protein A, an antiphagocytic protein, ensuring that macrophages can effectively perform phagocytosis.[16,17]

Dosage variations in this study were based on prior research on the immunomodulatory potential of macrophage phagocytosis.[12] Four *Arenga pinnata* ethanol extract dosage levels were tested: 100 mg/kgBW, 200 mg/kgBW, 300 mg/kgBW, and 400 mg/kgBW. Na-CMC (0.5%) was used as the negative control. At the same time, Stimuno® (0.013 mg/kgBW) served as the positive control, as it has been previously proven as an effective immunomodulator containing Andrographis paniculata extract that stimulates immune cell receptors.[13]

Phytochemical analysis of the ethanol extract revealed the presence of flavonoids, saponins, tannins, and triterpenoids. Flavonoids stimulate lymphocyte proliferation, increase T-cell numbers, and enhance interleukin-2 secretion, thereby boosting phagocytic activity in macrophages. This aligns with findings by Yusuf et al., where flavonoid-rich plant extracts demonstrated enhanced macrophage phagocytosis and lymphocyte proliferation. Additionally, tannins, saponins, and triterpenoids further contribute to the extract's immunomodulatory effects by stabilizing immune cell membranes and promoting cytokine production.[12,13]

Statistical analysis using the LSD test showed significant differences between the treatment groups and the control groups. The ethanol extract at 300 mg/kgBW and 400 mg/kgBW exhibited the highest macrophage phagocytosis activity, outperforming the negative and positive controls. This indicates that these doses effectively enhance immune responses by increasing macrophage activity.

Additional analysis using the Tukey HSD test confirmed that all extract doses significantly differed ($p < 0.05$) from the negative control, further validating the extract's immunomodulatory properties. The study's findings suggest that the ethanol extract of Arenga pinnata leaves effectively modulates specific and non-specific immune systems, with doses of 300 mg/kgBW and 400 mg/kgBW identified as optimal for macrophage activation.[13]

The bioactive compounds identified, including flavonoids, saponins, tannins, and triterpenoids, are crucial for the immunomodulatory activity observed. Flavonoids enhance immune responses by stimulating lymphocytes and macrophages.[18] Saponins act as natural adjuvants, boosting cytokine production and phagocytosis. Tannins provide antioxidant and immunomodulatory benefits, strengthening immune defenses. Lastly, even in small amounts, steroids

can stabilize immune cells and support the inflammatory response, complementing the extract's overall activity.[19,20]

5. Conclusion

The ethanol extract of *Arenga pinnata* leaves demonstrates significant immunomodulatory activity by enhancing macrophage phagocytosis in male mice (*Mus musculus*). The phytochemical screening confirmed the presence of bioactive compounds such as flavonoids, saponins, tannins, and triterpenoids, which are known to play critical roles in stimulating immune responses. Among the tested dosages, 300 mg/kgBW and 400 mg/kgBW were the most effective in increasing macrophage activity. These findings support the potential of *Arenga pinnata* leaf extract as a natural immunomodulator. Further research is necessary to explore its action mechanism and develop its pharmaceutical applications.

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