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ORIGINAL RESEARCH

# The immunostimulant effect of jamblang stem bark (*Syzygium cumini* L.) ethanol extract against mice macrophages phagocytosis activity and capacity

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***Abstract.*** This study aims to determine the effect of administering Jamblang Stem Bark Ethanol Extract (*Syzygium cumini* L.) (JSBEE) on the activity and phagocytosis capacity of macrophages in mice (*Mus musculus*) infected with *Staphylococcus aureus* bacteria. The research method used a completely randomized design (CRD) with five treatments and five repetitions, conducted in-vitro and in vivo test. The treatments for in vitro involved administering distilled water (T0), Stimuno (T1), and JSBEE at concentrations of 10 ppm (T2), 100 ppm (T3), and 1000 ppm (T4). Subsequently, in vivo treatment was conducted using distilled water (T0), stimuno (T1), JSBEE at 10 mg/kg (T2), 100 mg/kg (T3), and 1000 mg/kg (T4). Initially, mice underwent in vivo administration of JSBEE, administered orally via catheter tip, with a dosage of 1 mL per 10 g of body weight. JSBEE was administered orally for 10 days, followed by infection with *S. aureus* on the 11th day. The in vitro tests were conducted by isolating macrophage cells from the intraperitoneal fluid, to which *S. aureus* and JSBEE were added. Intraperitoneal fluid collected from the mice was used to prepare smears using the thin blood smear method and Giemsa staining. Macrophage phagocytosis activity was observed and assessed based on the percentage activity formula, and the phagocytic capacity of macrophages was measured by the number of *S. aureus* cells phagocytosed. The results indicated that JSBEE significantly affected ($P<0.05$) both the activity and phagocytic capacity of macrophages in vivo and in-vitro. The best concentration of JSBEE for increasing both the activity value and phagocytic capacity of macrophages was 100 ppm (T3).

**Keywords**: Blood smear, immunostimulan, in vivo, in vitro, *Staphylococcus aureus*

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## INTRODUCTION

Most Indonesian people still commonly use traditional medicine to improve their health. Recently, the use of plants for traditional medicine has become a preferred treatment option compared to modern medicine, primarily because traditional medicine is relatively more cost-effective and safer when used correctly and appropriately. However, most of the traditional medicinal plants, including those that act as immunostimulants, have not been scientifically studied for their efficacy [1]. Immunostimulants are substances that can stimulate the immune system [2,3]. The general mechanism of substances that can become immunostimulant materials involves improving and increasing the function and activity of the immune system's components [4]. Flavonoids, tannins, saponins, and alkaloids are among the active metabolites in plants that are thought to have immunostimulant properties [5]. The presence of these chemicals in jamblang bark raises the possibility of immunostimulating properties. Several compounds found in jamblang bark have been explained by previous studies, including genins, flavonoids, and tannins; these include betulinic acid, friedelin, β-sitosterol, eugenin, and fatty acid esters from epifriedelanol, β-sitosterol, quercetin, kaempferol, myricetin, gallic acid, and ellagic acid [6]. Many health

benefits of the jamblang plant are well-known, including anti-inflammatory, anti-bacterial, anti-cancer, anti-hyperlipidemia, hepatoprotective, anti-allergic, anti-rheumatic, and antioxidant [7,8].

Previous studies on jamblang seeds as immunostimulants stated that saponins, triterpenoids, flavonoids, tannins, steroids, and alkaloids have activity in enhancing the immune system [9]. Several secondary metabolites, namely alkaloids, polysaccharides, lectins, polyphenols, flavonoids, anthocyanins, tannins, saponins, terpenoids, and sterols have potential as immunostimulating agents. Thus the active compounds contained in jamblang, namely flavonoids, tannins, and saponins have the potential to be biological immunostimulants. The mechanism of flavonoids and alkaloids as immunostimulants by increasing the activity of IL-2 (interleukin 2) and lymphocyte proliferation [9].

Given that jamblang seeds are only available during specific seasons when the plant bears fruit, it is necessary to identify alternative parts of the plant that possess the same content and can be obtained at any time. One notable example is the bark, which is available throughout the year. Despite the many beneficial properties of jamblang bark for maintaining a healthy body and its potential as a biological immunostimulant, complete information regarding its efficacy as an immunostimulant is lacking. Therefore, further research is necessary to determine the effects of the ethanol extract of jamblang stem bark on the capacity and phagocytic activity of macrophages.

## METHODOLOGY

### Research design

This study used an experimental method with a completely randomized design (CRD) comprising five treatment groups and five repetitions in both in vivo and in vitro tests. The research design and the determination of dose concentrations were based on the findings of research conducted by Hariyanti *et al*. (2015) [10] and were subsequently modified as follows: T0: Negative control treatment with the administration of aqueous; T1: Positive control treatment (administration of Stimuno Supplement) of 200 µl; T2: Jamblang bark ethanol extract administered at a concentration of 10 ppm; T3: Jamblang bark ethanol extract administered at a concentration of 100 ppm; T4: Jamblang bark ethanol extract administered at a concentration of 1000 ppm.

Samples were collected from community gardens in Keude Blang Jreun Village, Tanah Luas District, North Aceh Regency. Sampling involved cutting sections of the jamblang stem with a diameter of 4-5 cm (from mature trees). The outermost layer (periderm) was removed, the bark (pepagan) was peeled, and then cut into small pieces to be dried.

### Animal maintenance

The mice were housed and maintained in the vivarium at the Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Syiah Kuala University. Male BALB/c mice weighing 25-30 grams were used, with a total of 35 mice (25 for in vivo tests and 10 for in vitro tests). The mice were kept in cages with a base of rice husks and wire netting as a cover. They were provided with pellet food and water ad libitum. Cages and drinking equipment were cleaned, and husk bedding was replaced every three days. Before treatment, the mice underwent a one-week acclimatization period.

### Preparation of Jamblang bark extract test solution

The dried sample was ground using a blender to obtain a fine powder. A total of 1 kg of fine powder was macerated with 96% ethanol, ensuring that the entire sample was submerged and covered by approximately 2 cm of ethanol above the powder's surface. The container was sealed and left to sit for 24 hours, with periodic stirring. The mixture was then filtered, and the remaining pulp was re-soaked for another 24 hours. This process was repeated approximately five times until the filtrate became nearly colorless. All macerates were combined, and the extract was concentrated using a vacuum rotary evaporator at 50°C until no solvent remained, leaving a liquid extract. Phytochemical screening was then performed to identify the compounds in the ethanol extract of jamblang stem bark.

### *Staphylococcus aureus* (*S. aureus*) bacterial culture

*Staphylococcus aureus* bacterial culture was prepared by regenerating *S. aureus*, following the bacterial culture procedures. Bacterial density was measured using a UV-Vis spectrophotometer at a wavelength of 580 nm and suspended in 4 ml of physiological NaCl, which was slowly added until 25% transmission was achieved, resulting in a bacterial count of 109 cells/ml.

### In vivo test

Jamblang bark ethanol extract was administered once a day at 9:00 AM for 10 days, according to the specified treatment concentrations. The extract was given orally using a catheter tip, with a *dosage* of 1 mL per 10 grams of body weight. On the 11th day, all treatments were injected with 100 µl of *S. aureus* bacterial suspension intraperitoneally. After one hour, the mice were anesthetized using chloroform. The abdominal skin was then opened, and peritoneal fluid was aspirated using a 1 ml syringe, 3-4 drops of intraperitoneal fluid were aspirated.

### In vitro test

### Collection of peritoneal fluid from mice

Mice were killed by cervical dislocation and then placed on a surgical board. The abdominal area was cleaned with 70% ethanol, and the abdomen was dissected using sterile surgical instruments. If a small amount of peritoneal liquid was observed, 1-2 ml of sterile PBS solution was added, stirred carefully, and then collected using a micropipette [11]. The number of macrophage cells was counted using a hemocytometer until a concentration of 107 macrophage cells/ml was obtained.

### Viability test

The macrophage viability test was conducted using 0,4% trypan blue dye to assess cell viability. The number of viable (colored) cells and the number of dead

(uncolored) cells were calculated using a hemocytometer. Percent viability was calculated using microscope at 100x magnification [10].

**In vitro phagocytosis test**

The phagocytosis assay was performed by mixing 200 µl of bacterial suspension, 200 µl of macrophage cells, and 200 µl of the test solution with concentrations of 10 ppm (T2), 100 ppm (T3), and 1000 ppm (T4). A mixture of 200 µl bacterial suspension and 200 µl macrophage cells was used as a negative control (T0). As a positive control (T1) mixed 200 µl bacterial suspension, 200 µl macrophage cells, and 200 µl stimuno 1000 ppm. Each sample was incubated for 30 min at 37ºC then 50 µl of 0.2 M Na2EDTA solution was added and homogenized.

**Determination of the value of the activity and phagocytic capacity of macrophages**

The phagocytic capacity of macrophage cells was determined based on the number of *S. aureus* bacteria engulfed (phagocytes) by 50 active macrophage cells. Phagocytic activity was assessed by calculating the number of macrophage cells actively involved in the phagocytic process out of 100 macrophage cells [12].

**Data analysis**

The results for activity and phagocytic capacity of macrophages in both in vivo and in vitro experiments were analyzed for normal distribution using a normality test and for homogeneity using a homogeneity test. The data were then statistically analyzed using Analysis of Variance (ANOVA). Differences in treatment effects were further evaluated using Duncan's multiple-range test.

**Research ethics**

All experimental protocols used in this study were approved by the Ethics Committee for the use of experimental animals, Faculty of Veterinary Medicine, Syiah Kuala University (No 113/KEPH/VII/2021).

## RESULTS AND DISCUSSION

**Phytocemical test results**

Phytochemical testing of ethanol extracts from jamblang bark (Syzygium cumini) revealed the presence of various secondary metabolites with potential pharmacological activity. According to the tests as listed in Table 1, the extract contains phenolic compounds, flavonoids, terpenoids, saponins, and alkaloids, all of which yielded positive (+) results in qualitative tests.

Conversely, tests for tannins and steroids yielded negative results, indicating that neither type of compound was present in the jamblang bark ethanol extract.

**Results of the analysis of activity values and phagocytic capacity of macrophages in vivo**

The results of statistical analysis showed that the data for activity and phagocytic capacity of macrophages were normally distributed (P>0.05) and homogeneously varied (P>0.05), which were then analyzed using variance analysis (ANOVA). Based on the results of the ANOVA, it was found that there was a significant effect (P<0.05) of administering JSBEE on increasing activity and phagocytic capacity of macrophages in vivo, with a Pvalue=0.00. The results also showed that JSBEE has immunostimulant potential compared to the control group. Further analysis using Duncan's multiple range

**Table 1.** Phytochemical test of Jamblang bark ethanol extract

| Phytocemical test | Test Results |
|---|---|
| Phenolics | + |
| Tannins | - |
| Flavonoids | + |
| Terpenoids | + |
| Steroids | - |
| Saponins | + |
| Alkaloid (DD) | + |
| Alkaloid (Mayer) | + |
| Alkaloid (Wagner) | + |

Description (+) indicates a positive result and (-). indicates a negative result

**Table 2.** The analysis of the activity value and phagocytic capacity of macrophages in vivo

| Treatments | Average ± DS | |
|---|---|---|
| | Activity (%) | Capacity (cells/50 active macrophages) |
| T0 | $71^{a} \pm 2.4$ | $250.8^{a} \pm 3.47$ |
| T1 | $85.2^{b} \pm 1.5$ | $535.4^{b} \pm 7.71$ |
| T2 | $88.2^{b} \pm 2.3$ | $424^{b} \pm 6.97$ |
| T3 | $87^{b} \pm 1$ | $700^{c} \pm 8.73$ |
| T4 | $86.8^{b} \pm 3.7$ | $843.4^{d} \pm 8.18$ |

Description: T0 = Negative control treatment with the administration of aqueous; T1 = Positive control treatment (administration of Stimuno Supplement) of 200 µl; T2 = Administration of JSBEE with a concentration of 10 ppm; T3 = Administration of JSBEE with a concentration of 100 ppm; T4 = Administration of JSBEE with a concentration of 1000 ppm. Note: Numbers followed by different letters in the same column are significantly different according to Duncan's test with the significance level of 5%.

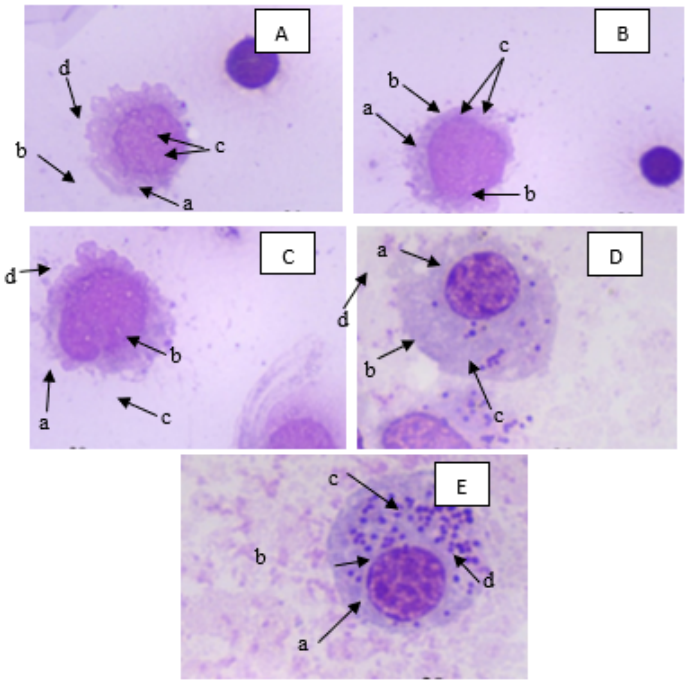


**Figure 1.** Peritoneal macrophage cells of mice after administration of JSBEE treatment and *S. aureus* bacterial infection. A.) Positive Control; B.) Negative control; C.) JSBEE concentration 10 ppm; D.) JSBEE concentration 100 ppm; E.) JSBEE concentration of 1000 ppm. a = cell nucleus; b = cytoplasm; c = phagosomes; d = pseudopodia.10x100 magnification

test showed that the administration of JSBEE at various concentrations was most effective in increasing both activity and phagocytic capacity of macrophages, as listed in Table 2.

The average activity value of macrophage cells activity without immunostimulant administration (T0) was 71%. These results are consistent with the previous studies, which found the value of macrophage phagocytic activity in control treatment with the administration of parijoto fruit extract (*Medinilla speciosa*) which is 61,5% and 74%. The value of macrophage phagocytic activity is higher than the value of phagocytic activity which is 53-61% [13].

The activity value and phagocytic capacity of macrophages possessed by the negative control treatment were lower than those in the positive control treatment. This is because the activity and phagocytic capacity of macrophages in the negative control treatments relied solely on natural immunity, whereas the positive control treatments' higher values were influenced by the active compounds found in Stimuno.

**Table 3.** Macrophages cell viability test results

| Repetitions | Living cells | Dead cells | Viability (%) |
|---|---|---|---|
| 1 | 82 | 3 | 96.4 |
| 2 | 80 | 2 | 97.5 |
| 3 | 83 | 3 | 96.3 |
| 4 | 79 | 4 | 95.1 |
| 5 | 81 | 3 | 96.3 |
| **Average** | | | **96.3** |

Further tests on the JSBEE-treated animals with concentrations of 10 ppm, 100 ppm, and 1000 ppm showed significantly higher activity and phagocytic capacities compared to the negative control treatments. The JSBEE treatment of the three concentrations had a higher phagocytic activity value of 88.2±2.3 (T2), 87±1(P3), and 86.8±3.7(T4) compared to the phagocytic activity value of the negative control treatment of 71±2.4(T0). The values of the phagocytic capacity in the JSBEE treatment from the three concentrations were successively, namely 424±6.97 (T2), 700±8.73 (T3), and 843.4±8.18(P4) higher than the phagocytic capacity values in the negative control treatment of 250.8±3.47(T0). Figure 1 showed that macrophage cells of mice after treatment.

These results suggest that JSBEE can enhance both activity and phagocytic capacity of macrophages, likely due to the effects of the secondary metabolites present in the extract. The presence of synergistic effects between secondary metabolite compounds from plants can produce pharmacological outcomes, and these compounds often exhibit polyvalent activity, making them effective against a variety of diseases [14]. The best concentration of JSBEE for increasing macrophage phagocytic capacity in vivo was 1000 ppm (T4).

Higher doses of flavonoids make leukocyte cells (phagocytes) more active in bacterial phagocytes and more bacteria that can be damaged by these leukocyte

**Table 4.** The analysis of the activity value and phagocytic capacity of macrophages in vitro

| Treatments | Average ± DS | |
|---|---|---|
| | Activity (%) | Capacity (cells/50 active macrophages) |
| T0 | $60.4^{b} \pm 6.8$ | $306.2^{a} \pm 3.46$ |
| T1 | $95.0^{d} \pm 1.5$ | $667^{d} \pm 5.04$ |
| T2 | $76.6^{c} \pm 1.8$ | $621^{c} \pm 7.30$ |
| T3 | $82.2^{c} \pm 2.7$ | $700^{d} \pm 7.17$ |
| T4 | $39.2^{a} \pm 6.4$ | $421^{b} \pm 5.83$ |

Description: T0 = Negative control treatment with the administration of aqueous; T1 = Positive control treatment (administration of Stimuno Supplement) of 200 µl; T2 = Administration of JSBEE with a concentration of 10 ppm; T3 = Administration of JSBEE with a concentration of 100 ppm; T4 = Administration of JSBEE with a concentration of 1000 ppm. Note: Numbers followed by different letters in the same column are significantly different according to Duncan's test with the significance level of 5%.

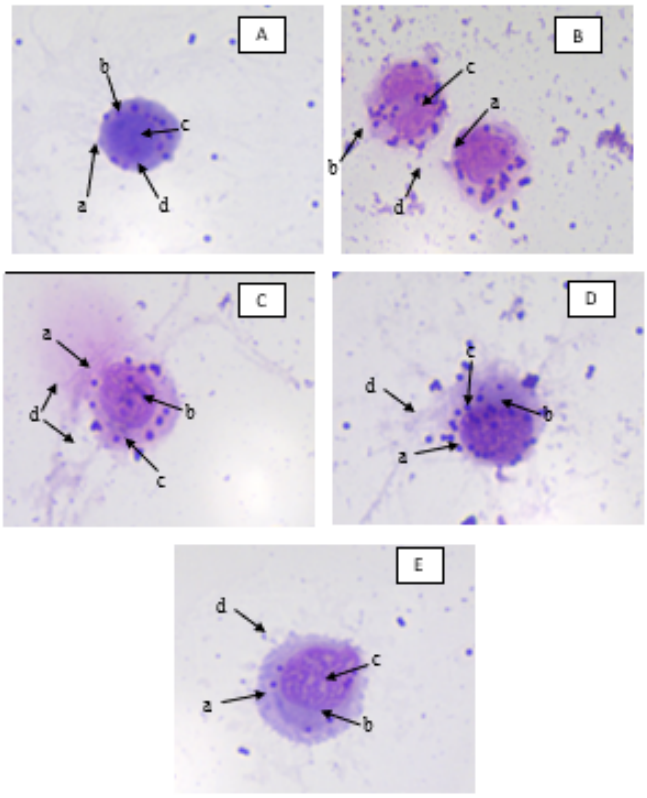


**Figure 2.** Peritoneal macrophage cells of mice after administration of JSBEE treatment and *S. aureus* bacterial infection. A.) Positive Control; B.) Negative control; C.) JSBEE dose 10 ppm; D.) JSBEE concentration 100 ppm; E.) JSBEE concentration of 1000 ppm. A = cell nucleus; b = cytoplasm; c = phagosomes; d = pseudopodia; e = inactive macrophage cell.

cells [15]. Increasing the dose of jamblang seed extract led to enhanced immunomodulatory activity through improvements in the humoral antibody response, cell-mediated immune response (DTH response), and white blood cell count [16].

**Macrophage cells viability test results**

Macrophage cell viability was determined using trypan blue solution to differentiate between living and dead cells. Living macrophage cells do not absorb color because they have good membrane integrity so dyes cannot penetrate the cell membrane. In contrast, dead macrophage cells absorb the trypan blue dye because their damaged membrane structure allows the dye to penetrate and color the cells (Figure 2). The results of the macrophage cell viability test are expressed as percentages and are shown in Table 3.

The average result of the macrophage cell viability from the test was 96.3%. Macrophage cell viability should not be less than 95%. Based on the results obtained in this study, the macrophage cells are considered viable and suitable for use in the in vitro phagocytosis test.

**Results of the analysis of activity values and phagocytic capacity of macrophages in In vitro**

The results of statistical analysis showed that the activity and phagocytic capacity of macrophages data were normally distributed (P>0.05) and homogeneously varied (P>0.05). The data were then analyzed using Analysis of Variance (ANOVA). The ANOVA results showed a significant effect (P<0.05, P-value = 0.00) of JSBEE administration on increasing activity and phagocytic capacity of macrophages in vitro. The results also demonstrate that JSBEE possesses immunostimulant potential. Further analysis using Duncan's test revealed that JSBEE dosing was most effective in enhancing activity and phagocytic capacity of macrophages, as shown in Table 4.

Based on Table 4 It is known that the average macrophage activity T0 (negative control) was 60.4±6.8 and the capacity value was 306.2±3.46. The differences in activity and phagocytic capacity of macrophages observed in this study compared to previous research are likely due to variations in the bacterial strains used [17,18].

The average activity and phagocytic capacity of macrophages in the negative control group (T0) were lower compared to the positive control group (T1), which used Stimuno containing green meniran extract (*Phyllanthus niruri*) as a comparative immunostimulant. The administration of JSBEE at concentrations of 10 ppm (T2) and 100 ppm (T3) significantly increased the activity and phagocytic capacity of macrophages compared to the control. This enhancement is attributed to the flavonoid compounds in JSBEE, known for their antioxidant activity, which improves the function of immune system component previous studies demonstrated that jamblang bark ethanol extract exhibits moderate antioxidant activity with an IC50 value of 164.3 ppm, as assessed by the DPPH method [19].

In addition, flavonoids also have antibacterial activity against *S. aureus*, flavonoids act as antibacterials through several mechanisms, including inhibition of nucleic acid synthesis, disruption of cytoplasmic membrane function, and interference with bacterial energy metabolism [20,21]. The 100 ppm concentration of JSBEE was the most effective at increasing activity and phagocytic capacity of macrophages in vitro. Further analysis revealed that the activity and phagocytic capacity of macrophages at this concentration were not significantly different from those in the positive control group, indicating that JSBEE at 100 ppm can enhance the immune system similarly to Stimuno in vitro.

However, unlike the effect observed in T2 and T3, the administration of JSBEE at 1000 ppm (T4), resulted in decreasing macrophage phagocytic activity, with an average lower of T0, even though the phagocytic capacity was higher. This reduction in activity is likely due to the immunosuppressant effect of the high dose (T4) on mouse macrophages in vitro. The immunosuppressants inhibit or suppress immune system activity by mechanisms such as inhibiting cytokine transcription, thereby weakening key immune responses, particularly IL-2 [22,23]. Effect of immunosuppressants is the inhibition of phagocytosis and the processing of antigens by macrophages.

According to previous studies, the immune effects of plant extracts show that the effects that appear are highly dependent on the test dose, whereas immunosuppression/ cytotoxic effects occur at higher doses [24]. Flavonoids have immunomodulatory properties, they can act as immunosuppressants at high doses, reducing activity and phagocytic capacity of macrophages [25,26].

## CONCLUSION

The administration of ethanol extract of jamblang bark (*Syzygium cumini* L.) (JSBEE) significantly affects the activity and phagocytic capacity of macrophages infected with *S. aureus*. The administration of JSBEE was shown to increase activity and phagocytic capacity of macrophages both in vivo and in vitro. The JSBEE concentration of 1000 ppm was the most effective in enhancing the phagocytic capacity of macrophages in vivo, while the concentration of 100 ppm was the most effective in increasing phagocytic capacity in vitro.

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