Abstract

**BACKGROUND:** *Aquilaria malaccensis* has been consumed as herbal medicine, and *in vitro* study showed that the leaf extract possesses high antioxidant activities. A brief preliminary study indicated that *A. malaccensis* showed a promising immunomodulatory activity when evaluated using latex beads. This current study aimed to evaluate the immunomodulatory activity of *A. malaccensis* leaf extract on the macrophage, which was challenged with pathogenic bacteria *Staphylococcus aureus*.

**METHODS:** Bioactivity was determined by evaluating the phagocytic capacity of macrophages isolated from *Mus musculus* against *S. aureus*. First, the cytotoxicity of extracts on macrophages was evaluated using MTT assays, and the IC₅₀ value was used to determine the dose of immunomodulatory assays. The highest toxicity was observed on chloroform extract with an IC₅₀ value of 111.4 µg/mL. Therefore, the treatment was 100 and 50 µg/mL. Two parameters, including the phagocytic activity and phagocytic capacity of macrophages infected with *S. aureus*, were used to evaluate immunomodulatory activity.

**RESULTS:** Chloroform and ethanol extracts at a 50 µg/mL concentration showed the best results with the phagocytic activity of 82.33±9.61% and 80.33±1.53%. The ethyl acetate showed lower phagocytic activities of 70.67±1.53. All extracts significantly increased phagocytic activity and phagocytic capacity, and the results differed significantly between negative and positive controls. Thin-layer chromatography indicated that the extract contained terpenoid, flavonoid, phenolic, and tannin.

**CONCLUSION:** *A. malaccensis* leaf extracts showed immunomodulatory activity. Both chloroform and ethanol extracts showed comparable activity, while the ethyl acetate extract was lower. The extracts contained diverse bioactive compounds that may support activating macrophage cells for immunomodulatory activity.

**KEYWORDS:** *Aquilaria malaccensis*, immunomodulator, phagocytosis, macrophages, *Staphylococcus aureus*

Introduction

The use of plants as an alternative medicine for some diseases in Indonesia has been carried out for generations. (1,2) Herbal medicines are also widely used to increase the immunity of the body, which are commonly referred to as immunomodulators. The ability of herbal medicine as an immunomodulator can be determined based on its secondary metabolite content. For example, flavonoids found in the leaves of *Sonchus arvensis* can increase the secretion of interleukin (IL)-2, trigger the proliferation of lymphocytes, and also increase the number of T cells. (3) Immunomodulators are divided into three types based on their functions, namely immunostimulants, immunosuppressants, and immunoadjuvant. (4) Immunosuppressants suppress or reduce the side effects of excessive inflammatory activity. (5) By contrast, the immunoadjuvant is a substance...
that can induce an immune response toward specific antigens.(6)

The principle of bioactive compounds in plants as immunomodulators is to activate macrophages that can secrete molecules to activate other immune cells.(7) Macrophage is an effector in the innate immune system that phagocytizes microbes and secrete pro-inflammatory and antimicrobial compounds. In general, macrophages phagocytize and degrade dead cells, debris, tumor cells, and foreign material.(8) The main target of plants as immunomodulatory agents is macrophages, as the immune system defends and then facilitates the formation of other cells of the immune system.

Agarwood plants are naturally distributed in some countries such as Bangladesh, India, Indonesia, Malaysia, Myanmar, and Vietnam.(9) Agarwood leaves have been studied for their application potential as an antioxidant, antibacterial (10), antiviral (11), and antihyperglycemic (12). The leaf extracts contained secondary metabolites with anticancer activity.(13) In addition, the stem has been reported to have anticancer activity.(14) Traditionally, agarwood plants are used for treating asthma, infectious wounds, fractures, and digestive diseases.

Aquilaria malaccensis contains phytochemicals, such as saponins, alkaloids, flavonoids, terpenoids, tannins, steroids, glycosides, coumarin, emodin, anthraquinone, and resins. (15) These phytochemicals can synergize with one another to improve the human immune system.

In our preliminary study, the immunomodulatory activity of agarwood was compared between two species of Agarwood, A. malaccensis, and Gyrinops versteegii. The result showed that A. malaccensis extract had a better immunomodulatory performance than G. versteegii when evaluated using macrophage cells challenge with latex beads. (16) Hence, this study tested the immunomodulatory activity of A. malaccensis leaf extract on the macrophage, which was challenged with pathogenic bacteria Staphylococcus aureus. Moreover, the potential compounds as an immunomodulator of A. malaccensis were investigated.

**Methods**

**Extraction of A. malaccensis Leaves**
The leaves were collected from a smallholder agarwood orchard in Karanganyar, Central Java, Indonesia. The 6th and lower leaves were collected as the sample, then dried at a maximum temperature of 40°C. Extraction was conducted by performing soxhletation using three solvents from different polarity indexes: chloroform (Merck, Kenilworth, NJ, USA), ethyl acetate (Merck), and ethanol (Merck). Ten grams of simplicia were extracted with 150 mL of solvent. Extraction was conducted at approximately 50–60°C. Finally, the solvent was evaporated until it became paste form.

**Extract Preparation**
Extracts for cytotoxicity test and immunomodulatory activity assay were prepared by dissolving 5 mg of extract in 50 μL of dimethyl sulfoxide (DMSO) (Invitrogen, Waltham, MA, USA). Afterward, concentration variations were made by diluting the stock using an RPMI 1640 medium (Gibco, Waltham, MA, USA). Finally, extract for thin-layer chromatographic (TLC) was prepared by dissolving 50 mg of extract in 1 mL of respective solvent.

**Macrophage Cell Culture**
This research used 8-week-old male Mus musculus. This study had been approved by the Medical and Health Research Ethics Committee of Faculty of Medicine, Public Health and Nurse, Universitas Gadjah Mada, Indonesia (No. KE/KF/0244/EC/2021).

Macrophages were isolated from the intraperitoneal cavity. Ten milliliters of cold RPMI medium were injected into the intraperitoneal cavity and massaged gently for 3 min. NaCl physiological salts were administered periodically on the surface to prevent bleeding. The solution was collected using a syringe in cavities far from the intestines to prevent erythrocytes and adiposes from being taken. The solution was centrifuged at 1200 rpm for 10 min at 4°C, and the supernatant was discarded. Then, a complete RPMI medium (containing phosphate-buffered saline and penicillin-streptomycin) was added until 1 mL of solution was reached. Macrophages were cultured in 96-well and 24-well microplates (Iwaki, Tokyo, Japan).

**Cytotoxicity Test**
The cytotoxicity test was conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The series concentration of each extract was 25, 62.5, 75, 125, and 150 μg/mL. All macrophages were incubated for 24 h. After the MTT reagent and stopper solution was administered, formazan absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) reader at 595 nm. The cytotoxic results were expressed in half-maximal inhibitory concentration (IC50) values, calculated based on linear regression equations from the log concentration against cell viability.
Preparation of Bacterial Suspension of *S. aureus*

A pure isolate of *S. aureus* was dissolved with nutrient broth in a sterile flask and then incubated in a shaker at 200 rpm, and 37°C overnight resulting in bacterial suspension of 4.047 (4 McF = 12×10⁸ CFU/mL) measured using UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 625 nm. Next, the bacterial density was diluted with 5 mL RPMI medium to reach a bacterial density of 10⁸ CFU/mL. A volume of 200 uL of the bacterial suspension was then distributed into 24-well plates for the following study.

Measurement of Phagocytosis in Macrophages

Macrophages with a density of 4×10⁵ cells/mL (200 μL per well of 24-well plate) were incubated for 24 h to attach cells to the coverslip. Afterward, macrophages were given several treatment groups, including negative control, positive control, chloroform extract, ethyl acetate, and ethanol with concentrations of 50 and 100 μg/mL overnight. Next, cells were challenged with 200 μL/mL of *S. aureus* at a density of 4×10⁶ CFU/mL. Then, they were incubated for 4 h at 37°C with 5% CO₂. Phagocytosis in macrophages was observed using dyeing techniques using 3% Giemsa, which was presented in purple in the cell nucleus, particularly a transparent light purple on the cytoplasm. In addition, the bacteria were shown in purplish pink on the cytoplasm.(17)

The phagocytic activity of macrophages was observed using a light microscope at 400× magnification, whereas the phagocytic capacity was observed under 1000× magnification. The phagocytic activity and phagocytic capacity were calculated using the formula in this reference.(18)

TLC

The bioactive compounds in *A. malaccensis* leaf extract were identified using TLC. Silica Gel GF254 (Sigma-Aldrich, St. Louis, MO, USA) with a size of 1.5 cm × 10 cm was used as the stationary phase, and 1.5 cm from the bottom was marked for sample location. The eluent used in chloroform and ethyl acetate extracts was n-hexane and ethyl acetate in a ratio of 7:3. In addition, ethanol extract used the ethyl acetate phase of motion, acetic acid, toluene, and water in a ratio of 5:2:3:0.5. Silica gel plate was observed using UV and visible-light transilluminators at 254 and 356 nm, respectively. Representative diagrams were based on positive spots observed after spraying with standard visualization reagent for terpenoid, phenolic, flavonoid, tannin, and alkaloid.

Statistical Analysis

The experiment was conducted in triplicates for data sets. First, data were analysed using Two-way ANOVA with the IBM SPSS Statistics 26 (IBM Corporation, Armonk, NY, USA) application. Then, the Duncan post hoc test was used to determine the groups showing significant differences. The significance indicative was specified into *p*-value<0.05. Data were expressed as mean±SD. Values with different superscript letters are significantly different between groups and concentration (*p*<0.05).

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC₅₀ (μg/mL)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>111.4</td>
<td>0.9539</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>485.9</td>
<td>0.9932</td>
</tr>
<tr>
<td>Ethanol</td>
<td>246.4</td>
<td>0.8724</td>
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Table 1. Cytotoxicity test of *A. malaccensis* leaf extract with a concentration of 25–150 μg/mL against macrophages.
Figure 1. Macrophage morphology observed in a light microscope with 400× magnification. A: activated macrophage; B: dilated cytoplasm; C: inactivated macrophage; D: filopodia.

Figure 2. Macrophage cell morphology observed in a light microscope with 1000× magnification. A: macrophages with bacterial phagocytosis; B: colonies of bacteria found in the cytoplasm of cells; C: bacteria that are not phagocytosed by macrophages.

the chloroform tended to have slightly higher phagocytic activity than the ethanol extracts, with an activity value of 82.33±9.61%, and 78.33±2.08%, for 50 and 100 μg/mL, respectively. However, both extracts were failed to be significantly different (p<0.05). The extract that demonstrated the lowest phagocytic activity was ethyl acetate at 100 μg/mL with a value of activity 57.33%±16.04% (Table 2).

Phagocytic Capacity
Phagocytic capacity was calculated based on the number of S. aureus bacteria exposed by 50 macrophages. The morphology of cells that actively phagocyte the bacteria S. aureus was presented in Figure 2.

The largest phagocytic capacity was observed in both chloroform extract at a concentration of 50 μg/mL with an activity value of 594.33±40.92. By contrast, the smallest phagocytic capacity was observed in ethyl acetate extract at a concentration of 100 μg/mL with an activity of 213.00±6.23 Extract Concentration (µg/mL) Average (%)

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration (µg/mL)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>50</td>
<td>82.33±9.61a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>78.33±2.08a</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>50</td>
<td>70.67±1.53a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>57.33±16.04b</td>
</tr>
<tr>
<td>Ethanol</td>
<td>50</td>
<td>80.33±1.53a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>76.67±4.04a</td>
</tr>
<tr>
<td>Positive control</td>
<td>100</td>
<td>37.00±6.00c</td>
</tr>
<tr>
<td>Negative control</td>
<td>27.00±9.00c</td>
<td></td>
</tr>
</tbody>
</table>

Different letters following the numbers indicate a significant difference between groups and concentration (p<0.05).

Table 2. The phagocytosis activity in macrophages against S. aureus after treated with A. malaccensis extracts.

(Table 3). The concentration in this study did not show a different effect on the phagocytic capacity demonstrated by no significant difference observed between 50 and 100 μg/mL, but it was not the case for ethyl acetate extract in which 50 μg/mL had a higher phagocytic capacity than 100 μg/mL. In this study, both positive and negative control showed lower capacity than all types of extract treatment except for the ethyl acetate at 100 μg/mL.

Bioactive Compound as Immunomodulator
The most positive terpenoid spots were the chloroform extract, with five purple spots followed by ethanol and ethyl acetate extracts with three spots. Although ethanol and ethyl acetate extract produced similar spots, the Rf and the color developed after being sprayed with vanillin sulfate were also different (Figure 3A). The flavonoid detection was

Table 3. The capacity of macrophage phagocytosis after treated with A. malaccensis extracts.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration (µg/mL)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>50</td>
<td>594.33±40.92a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>573.00±89.87a</td>
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<tr>
<td>Ethyl acetate</td>
<td>50</td>
<td>356.67±12.66b</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>213.00±46.23c</td>
</tr>
<tr>
<td>Ethanol</td>
<td>50</td>
<td>545.00±43.28a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>569.00±89.45a</td>
</tr>
<tr>
<td>Positive control</td>
<td>100</td>
<td>202.00±19.47c</td>
</tr>
<tr>
<td>Negative control</td>
<td>152.33±16.86c</td>
<td></td>
</tr>
</tbody>
</table>

Different letters following the numbers indicate a significant difference between groups and concentration (p<0.05).
done by reacting the extracts with the Sitroborate reagent. All extracts contained flavonoids, but each of the extracts showed a different profile. The chloroform extract showed five yellow spots, indicating positive results to flavonoids. The ethanol and ethyl acetate extracts had less number of spots, two and three spots, respectively (Figure 3B). The phenolic contents of extracts were detected by spraying the TLC plates with FeCl₃ reagent. The chloroform produced five spots, while the ethanol and ethyl acetate only one spot each. The three extracts showed different phenolic profiles (Figure 3C). In line with the other detection for the bioactive class, chloroform extract showed the most positive results for tannin compared to the other two extracts. Chloroform developed four positive spots, while the ethanol and ethyl acetate extracts had one positive spot (Figure 3D). Detection for alkaloids showed that all extracts did not contain alkaloids (Figure 3E).

**Figure 3. Representative diagram of TLC results for the secondary metabolites detection of *A. malaccensis* extracts.** A: Terpenoid; B: Flavonoid; C: Phenolic; D: Tannin; E: Alkaloid. Note: K: chloroform extract; EA: ethyl acetate extract; Et: Ethanol extract; TB: Thymol Blue; Q: Quercetin; F: Folin; Ag: Gallic acid; Caf: Caffein.

**Discussion**

The immunomodulatory activity of agarwood *A. malaccensis* leaf was evaluated by phagocytic analysis and phagocytic activity of macrophage cells, known as the best-specialized cells charges for clearing pathogen and dying cells.(19) Three different solvents extracted the agarwood leaf representing three different polarities. This is expected to include all secondary metabolites contained in agarwood leaves. However, first, the toxicity of each extract should be determined, which is then used as a concentration treatment.

The toxicity result was consistent with a previously reported study, showing that agarwood *A. malaccensis* leaf chloroform extracts had the highest toxicity compared with ethanol and water extracts with IC₅₀ values of 166.73 μg/mL against T47D breast cancer cells.(20) In this study, the IC₅₀ values were determined to evaluate the potential of agarwood leaf extracts as a potential anti-cancer agent.
value in the cytotoxicity assay of *A. malaccensis* against macrophages was 111.4 μg/mL. This concentration is considered safe and not toxic to macrophages. Based on this result, the highest concentration applied in the phagocytic analysis was 100 μg/mL. However, in this study, only two 50 and 100 μg/mL concentrations were used because of the limited number of macrophages obtained from one mice.

Both phagocytic activity and phagocytic capacity in this study showed a similar result, in which the chloroform extract provided the best effect, followed by ethanol and ethyl acetate extracts. However, chloroform extract data did not significantly differ from the ethanol extract. This may be explained by the bioactive detection result in this study, showing that several positive spots of ethanol for terpenoids, flavonoids, and phenolics were also present in the chloroform extract. This was not the case for the ethyl acetate extract, which has a very different metabolite profile from chloroform. Furthermore, considering the toxicity of the ethyl acetate assay with an IC₅₀ close to 500 μg/mL, it is likely that ethyl acetate requires a concentration of more than 100 μg/mL to produce optimum immunomodulatory activity.

In contrast to the active extract, consistently lower concentrations. Fifty μg/mL tend to give better results (although statistically not significantly different) than the 100 μg/mL. It is very likely that the concentration of 100 μg/mL is too close to the IC₅₀ value of the extract's toxicity to macrophage cells; therefore, the cells did not show a linear dose-response with the phagocytic activity. Thus, the study indicates that chloroform and ethanol extracts of *A. malaccensis* leaves have excellent application potential as immunomodulators. The immunomodulatory activity of both extracts was considered better than the ethanolic extract of the fruit of *Momordica charantia* L., which reported significant activity at a concentration of 1,200 μg/mL. The study used a different species, *Staphylococcus epidermidis*, but it is still in the same genus as *S. aureus*. (21) The potency of ethanol leaf extract of other herbal, *Spondias pinnata*, has been reported to improve the phagocytic activity of macrophage cells challenged with latex beads. (22)

The different visualization between the active and inactive macrophages showed phagocytosis activity. Before phagocytosis, the receptors in macrophages will initially bind to ligands on the surface of pathogenic bacteria. The receptor will give a signal to regulate the actin cytoskeleton, thereby forming membrane protrusions in phagocytic pathogens. Phagocytes will explore the target phagocyte by forming membrane wrinkles, filopodia, and podosomes. (23) After entering phagocytes and becoming a phagosome, nitric oxide (NO) will kill pathogenic microorganisms that have been exposed. (24) This may explain the increased capacity of macrophage phagocytosis against pathogenic bacteria *S. aureus* of *A. malaccensis* leaf extract that can increase NO levels. However, further study should be done to prove this hypothesis.

The TLC detection for terpenoids, flavonoids, phenolics, and alkaloids showed that the three extracts, including chloroform, ethanol and ethyl acetate, differed in bioactive content.

In general, chloroform extract contained more bioactive for all detected secondary metabolite groups, followed by ethanol and ethyl acetate extracts. The main compounds in the chloroform extract were terpenoids, flavonoids, and phenolics, in which five spots were detected each. On the other hand, ethanol extract with immunomodulatory activity comparable to that of chloroform extract was detected to have fewer compounds than chloroform extract. This is possible because TLC as a detection analysis method has some sensitivity limitations and is influenced by the eluent combination used as the mobile phase. (25)

Terpenoids are secondary metabolites that tend to be semi-polar to polar and are commonly found in agarwood leaf extracts. (26) Terpenoids have shown potential as immunomodulators against macrophages infected with *Mycobacterium tuberculosis*, and their immunomodulatory activity can alert macrophage activation. In addition, the production of TNF-α and NO in macrophages treated with such compounds increases. (27) Another study reported that triterpenoids could inhibit the growth of *S. aureus* and *S. mutans*. (28)

Flavonoids, phenolics, and tannins are abundant in nature. Three types of flavonoids, namely, quercetin, naringenin, and naringin, which are found in human macrophages, can reprogram the pro-inflammatory macrophage cell metabolism system stimulated by lipopolysaccharide (LPS) and interferon (IFN)-γ. (29) Phenolics are serve as antioxidants, protecting against UV rays and plants pathogens. Phenolic compounds are reported to have health benefits such as antibacterial, anti-inflammatory, and antimutagenic. (30) Tannins also have biological activities such as antimicrobial, anti-parasites, antioxidants, antivirals, and anti-inflammatories. (31)

Several studies reported that alkaloids could stimulate the immune response of the body. (32) However, in this current study, we failed to detect the alkaloid content in all extracts. The absence of positive spots of alkaloids might be due to the low concentration of alkaloids contained in the extract; thus, such compounds are challenging to
detect. A lesser study confirmed the presence of alkaloids in the *A. malaccensis* leaves. Positive alkaloid detection was observed on the methanol extract of *A. malaccensis* leaves collected from Central Java, while plants from others were not detected.(33) In general, the alkaloid content in young plants is greater than that of adult plants. Moreover, the alkaloid content of the leaves and stems of tea plants decreases significantly with the age of the plant.(32,34)

### Conclusion

*A. malaccensis* leaf extracts showed immunomodulatory activity with chloroform extract, and ethanol extract showed a comparable activity followed by ethyl acetate extract. All leaf extracts of *A. malaccensis* contain terpenoids, flavonoids, phenolics, and tannins. The chloroform extract was the extract that was detected to contain the most bioactive, followed by ethanol extract and ethyl acetate extract.

### Acknowledgements

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### Authors Contribution

HYY conducted the phagocytosis analysis and wrote the manuscript. LH performed the extraction and TLC analysis. NW designed the study and supervised the phagocytosis analysis; TRN designed the study, wrote the manuscript, and supervised the study.

### References


