Immunomodulatory Effect of Massoia Bark Extract and the Cytotoxicity Activity Against Fibroblast and Vero Cells in Vitro

Permanasari P¹, Hertiandi T²*, Yuswanto A³

¹Pharmaceutical Sciences Master Program, Faculty of Pharmacy, Universitas Gadjah Mada
²Phytochemistry Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada
³Medicinal Chemistry Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada

ABSTRACT
Background: Bark of Massoia aromatica Becc. (Lauraceae) has been widely used in Java, Bali and Sumatra as a fragrance ingredient, seasoning and traditional medicine. C-10 Massoia lactone is a major constituent of its essential oil which has been proven as the antibacterial principle and has the ability to increase the phagocytic index of mice macrophage. Objective: to find out the immunomodulatory effects of the Massoia aromatica bark extract against Candida albicans as well as to explore the potential toxicities against fibroblast and vero cells. Material and methods: The extract was obtained by maceration of the pulverized barks with ethanol 96%, followed by solvent evaporation. The Massoia lactone contain was analysed by TLC-Densitometry. The immunological assay was based on the effect on SD mice lymphocyte proliferation and macrophage phagocytosis activities against latex and Candida albicans. Samples concentration tested were in the range of 30 – 2.5 μg/mL and done in triplicate. Cytotoxicity assays were done on fibroblast and vero cells by using the MTT assay. ANOVA followed by Tukey-HSD was used for statistical analyses. Results: The ethanolic extract was separated into two layers, i.e. solid and oily phases. All samples exhibited no effect on lymphocyte cell proliferation which indicated by the stimulation index proliferation value of less than 2. However, all samples showed activation of the macrophage phagocytosis both on latex and Candida albicans in a concentration dependant manner. IC₅₀S of oily and solid extracts on fibroblast were 46.13 and 53.03 μg/ml respectively, while on Vero cell were found at 43.59 and 153.05 μg/ml, respectively.

Keywords: Massoia aromatic, immune system, Candida albicans, cytotoxicity

INTRODUCTION
Immunocompromised is any condition of which the response to foreign antigen is subnormal. One of the material that stimulates the immune response is antigen molecule in the inside or surface of microorganisms (bacteria, fungi, viruses)¹. In the case of immunocompromised, the body is unable to produce an adequate immune response to overcome invading microorganism which may worsens the infection. Macrophages and lymphocyte cells play important roles in the immune response. Macrophages is responsible to eradicate microorganisms and dead cells by phagocyte the microorganisms and break it down into smaller parts². On the other hand, lymphocytes is responsible for the cell-mediated immunity³. Evaluating effects of substances on those particular agents, may predict the effects on the immune system. Massoia aromatica Becc. is a species of the family Lauraceae. Massoia bark contains essential oils of which the massoia lactones are the major components⁴. High lactone content on this essential oils may responsible for several negative effects like skin sensitization, irritation, and cytotoxicity⁵,⁶. Nevertheless, several lactone compound has been reported to have immunomodulatory effect, for example homoserine lactone⁷, and macrolides lactone antibiotics⁸. The C-10 massoia lactone was also reported to be as the antimicrobial principal of the essential oil, both towards planktonic and biofilm states⁹,¹⁰. Some herbal crude extracts are often preffered for medication over isolates because they have more complex chemical constituents which might perticipate in exerting biological activities and at the same time eliminate the side effects. The Massoia ethanolic extract has been investigated as antimicrobial against Staphylococcus aureus, Bacillus subtilis, E. coli¹¹ and Candida albicans (Hertiandi et al, unpublished data). The massoia lactone was found to be responsible for that particular activity, suggesting its potency to be developed as a broad spectrum antimicrobial agent. In order to support the anti-infective effect, a research on immune system was conducted. At the same time, a cytotoxicity to ensure the safety of sample was applied on normal cells i.e., vero and fibroblast cells. Cultures of human skin cells are used as a model for skin irritation testing. The use of human skin fibroblast cells used as one of the models for in vitro skin toxicity¹².

MATERIALS AND METHODS
**Materials:** Massoia bark purchased from traditional herbal medicine market at Yogyakarta, Indonesia, *Candida albicans* (collection of the Laboratory of Microbiology, Faculty of Pharmacy, UGM), Vero cell lines, Fibroblast (LPPT-UGM), DMEM medium, RPMI medium, PBS, FBS qualified (Gibco, USA), trypsin EDTA, DMSO, MTT, ammonium chloride buffer solution (Sigma, USA), SDS (Merck, USA), ketamine, latex beads (3μm). C-10 massoialactone as isolate was kindly given by Ms. Sylvia U.T.P.

**Equipments:** coverslips (SPL, Korea), Microplate reader (Bio-Rad, Japan), Haemocytometer (Neubauer, Germany), CO₂ incubator (Heraeus®, Germany), inverted microscope (Olympus, Japan).

**Methods:**

*Sample Preparation:* Ethanolic extract was prepared by macerating the dried pulverized bark in ethanol 96% for 24 h, repeated once. The filtrate was combined and evaporated to yield thick constituent.

*Citotoxicity assay*

Cell was cultured in DMEM, supplemented by 10% FBS 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured at 37°C under a humidified atmosphere containing 5% CO₂ in 25 cm² plastic culture flask. Culture medium was removed when the cell reached 90% confluence. Trypsin EDTA was added to the cell culture for detaching cells from the flask. The cells were seeded in 96-well plates at a density of 6 × 10⁴ cells/well in 100μl medium with 10% FBS and incubated in CO₂ incubator at 37°C for 1 h. Then, 100μl of each testing material (Massoia oily and solid extract) added to each well with different concentration (1 - 80 μg/ml) in media. After 24 h of incubation period, the media were removed from the plate. Cell viability was identified using reagent [3 (4, 5- dimethylthiazol-2-yl) -2.5-diphenyl tetrazolium bromide] MTT. One hundred micro-liters MTT was added to each well. Following 4 h incubation, 100μl stopper solution (10% SDS) was added. After 24

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**Figure 1:** Macrophages were observed under a light microscope

**Figure 2:** correlation between the C-10 massoia lactone content in samples Vs % cell viability

![Graph 1](image1.png)

![Graph 2](image2.png)
incubation, absorbance was measured by micro titer plate reader at a wavelength of 540 nm\textsuperscript{13,14}. Cytotoxicity effect was measured by using the equation\textsuperscript{15}:

\[
\text{Percentage of viable cells} = \frac{Abs (treatment–blank)}{Abs (control–blank)} \times 100\%
\]

**Immunological assay**

**Animal**
Male mice Balb/C of 8 – 12 week old were obtained from the laboratory animal studies, Faculty of Pharmacy Gadjah Mada University. The animal handling has been approved by the ethics committee LPPT-UGM number : 271/KEC-LPPT/II/2015.

**Lymphocyte Proliferation**
Mice were euthanized by ketamine intra muscular. Spleens were removed aseptically and placed in a sterile petri dish containing RPMI medium. Cells were extracted by injecting a syringe containing RPMI to the spleen. Red blood cells were lysed by buffered ammonium chloride solution, shaken until the suspension turns white, then centrifuged to separate debris\textsuperscript{16}. Lymphocytes were counted and diluted to a final concentration of 1.5 × 10\textsuperscript{5} cells/ml. Cells were plated into 96-well plates in a volume of 100 μl and 100μl of the testing material with different concentration (2.5 - 30 μg/ml) was added in to each well for 72 h at 37°C in a 5% CO\textsubscript{2} incubator. After incubation, media was removed carefully and 100μl MTT was added. Following 4 h incubation, 100μl stopper solution (DMSO) was added. Cell proliferation is proportional to the optical density read using ELISA reader at 540 nm\textsuperscript{17}. Proliferation index was measured by using the following equation:

\[
\text{SIProliferation} = \frac{Abs (treatment–blank)}{Abs (control–blank)}
\]

**Macrophages Phagocytic Activity**
Mice were euthanized by ketamine intra muscular. Into the peritoneal cavity, was added ± 10 ml of cold RPMI. The fluid was aspirated from peritoneum, and transferred into a centrifuge tube and centrifuged at 1200 rpm for 10 min. Cell number was calculated by a Neubauer haemocytometer and diluted to a final concentration of 2.5 × 10\textsuperscript{5} cell/well. Then the cell suspension was inoculated on 24 wells microtiter plates equipped with cover slips (5 × 10\textsuperscript{3} cell/well, 200 μL / well)\textsuperscript{18}. Measurement of the phagocytosis activities were conducted in two different experiments, i.e., towards 3 μm latex beads and C. albicans suspended in PBS. The 24 h culture of peritoneal macrophages was washed twice with RPMI 1640 and then added to the samples with various concentrations ranging from5 - 30 μg/ml (500 μL / well). Incubation was performed in a 5% CO\textsubscript{2} incubator at 37°C for 4 h. The cells were washed three times with PBS. One serial plates was added with a suspension of latex beads (5 × 10\textsuperscript{6} cell/well, 500 μL / well) and the other were added with the suspension of C. albicans (2 × 10\textsuperscript{6} cell/well, 500 μL / well). Incubation was taken place in the 5% CO\textsubscript{2} incubator at 37°C for 1 h. The cells were washed three times with PBS to remove the non-phagocyted latex beads (for non specific assay) and C. albicans (for specific assay). Following fixation with methanol for 10 min, the cover slip was allowed to dry, and then 20% v/v Giemsa dye was added and left for 20 minutes. After washed with distilled water and left to dry, the number of macrophages which phagocyted the cell, amount of cell phagocyted, were counted under a light microscope to count the macrophage phagocytic index\textsuperscript{19,20}. The phagocytic index was calculated according to the following formula:\textsuperscript{21}:

\[
\text{PI} = \frac{\text{total number of engulfed cells}}{\text{macrophages engulfed cells}} \times \frac{\text{number of macrophages engulfed cells}}{\text{counted macrophages}} \times 100
\]

**Statistical analysis**
Data were expressed as mean ± SE. Statistical significance was analysed using the one-way analysis of variance (ANOVA) test, followed by the Tukey-HSD multiple intergroup comparison test. Probabilities of <0.05 were considered as significant.

**RESULTS**

**Citotoxicity assay**
Figures 1 describes the correlation of sample concentrations against the percentage of vero and fibroblast cells viabilities in concentration dependant manners. Fibroblast was slightly more susceptible than Vero cell which might be due to its mortality properties. The solid extract showed lower toxicity than the oily extract (table 1).

**In Vitro Lymphocyte Proliferation Assay**
The lymphocyte proliferation stimulation index of all test groups shows less than 2, which means that treatment with various concentrations of Massoia oily extract and solid extract have no effect on lymphocyte cell proliferation (table 2).

**In Vitro Macrophage Phagocytic Test**
Non-specific testing refers to the activity of macrophages against 3 μm latex beads (figure 2), as the control cells showed no phagocytic activity of the macrophages against latex. Resident tissue macrophages should receive external stimulation to become the activated macrophages\textsuperscript{22}. Therefore, the untreated macrophages (control) were unable to engulf the latex beads. On the other hands, C. albicans activates macrophages through various kind of stimuli including the recognition by TLRs and C-type lectin receptors\textsuperscript{23}. This was expressed by a high value of
the phagocytic index shown by controls *C. albicans* (Table. 3). Massoia oily and solid extracts were able to activate the macrophages both on specific and non-specific testing in concentration dependent manners.

**DISCUSSION**

High content of oil extracted from the massoia bark by ethanol 96% caused a sporation of extract into two different phases, i.e., oily and solid phases which were evaluated separately in this research. In the previous study, both oily extract and solid extract showed similar GC-MS patterns which may be due to similar content of volatile constituents. Nevertheless, the TLC profiles were different. Measurements by TLC densitometry showed that the oily extract had 33.65% w/v and the solid extract has 10.25% w/v of C-10 Massoia lactone content. The massoia ethyl acetate extract was reported to have a LC50 of 12.12 ppm on Brine Shrimp Lethality Test34. Furthermore, our previous study on the oil and Massoia lactone showed toxicity against Vero and fibroblast cells as well. The Massoia lactones were also reported to be a potential cytotoxic due to its α,β, unsaturated lactone content25. Therefore we hypothesized that the oily extract would have a higher cytotoxicity than the solid due to a higher C-10 massoia lactone content. Our result confirmed the argument. The two testing covering both specific and non-specific assays of macrophage showed the high value of the phagocytic index. The comparison phagocytic index of each treatment was reduced by the control. It showed that phagocytic index macrophage against *C. albicans* remained higher when the index was compared with macrophages against latex. Thereby, the pretreatment of macrophages with extracts will be more responsive against microbiological agents such as *C. albicans*. Endogenous compounds which can activate macrophages are immune complexes, T helper 2 (Th2) cytokines, interleukin 4 (IL-4), interleukin 13 (IL-13) and interferon-γ (IFN-γ)26. Surprisingly, the macrophages activity were not dependent on C-10 Massoia lactone content. Treatment with oily and solid extract showed also significant increases of the phagocyte activities in comparison with controls. Despite the fact that the massoialactone itself also has stimulation activity on the macrophage phagocytosis (Hertiani, unpublished), this finding suggests the role of other constituents which also contribute to the macrophages activation. Beside the C-10 Massoia lactone, Massoia bark extract was reported to also contains flavonoids, tannins, steroids, terpenoids, and coumarin24. Further investigation on the role of other constituents is worth explored.

**CONCLUSION**

The Massoia bark extracts showed cytotoxicity against normal cells of which the C-10 Massoia lactone plays the major role for the activity. The massoia bark extracts showed a potential immunomodulatory activity which were indicated by stimulation of the macrophage phagocytosis activity both against latex beads and the *C. albicans* in a concentration dependant manner. However, both extracts were unable to affect the mice lymphocyte proliferation in *vitro*.

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