

Effect of *Massoia (Massoia aromatica* Becc.) bark on the phagocytic activity of Wistar rat macrophages

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Abstract

Massoia (Massoia aromatica Becc., Lauraceae) bark has been widely used as a component of traditional Indonesian medicine. The indigenous people boil or steam the bark for traditional applications. Our preliminary research revealed the potency of *Massoia* essential oil and its major compound, C-10 *Massoialactone* as potential immunomodulator in vitro. However, no scientific evidence regarding its in vivo effects is available. Therefore, this study evaluated the potential immunomodulatory effects of *Massoia* bark infusion on the nonspecific immune response (phagocytosis) of Wistar rats.

The aqueous extract of *Massoia* bark was obtained by boiling pulverized bark in water, and the C-10 *massoialactone* content of the extract was determined through Thin Layer Chromatography (TLC) densitometry. For the in vitro assay, macrophages were treated with the freeze-dried infusion at the concentrations of 2.5, 5, 10, 20, or 40 µg/mL media. For the in vivo assay, 2-month-old male Wistar rats were divided into 5 groups. The baseline group received distilled water at the dose of 1 mL/100 g BW with the immunostimulant herbal product "X" administered as the positive control at the dose of 0.54 mL/rat. The treatment groups received the infusion at a dose of 100, 300, or 500 mg/100 g BW. Treatments were given orally every day for 14 days. The ability of macrophage cells to phagocytose latex was determined as phagocytic index (PI) and was observed under microscopy with 300 macrophages.

The in vitro study revealed that the phagocytic activity of the infusion-treated macrophages significantly increased in comparison with that of the control macrophages in a concentration-dependent manner. Among all treatment concentrations, the concentration of 40 µg/ml provided the highest activity with a PI value of 70.51% ± 1.11%. The results of the in vivo assay confirmed those of the in vitro assay. The results of the present study indicate that *Massoia* bark can increase the phagocytic activity of rat macrophage cells. Its potential as a naturally derived immunomodulatory agent requires further study.

Keywords: Immunomodulatory, *Massoia aromatica* Becc., macrophage phagocytosis

1. Introduction

Immunomodulatory agents affect mechanisms related to the pathophysiology and etiology of various diseases by regulating the immune system. An immunomodulator is required to

activate the host defense mechanism in response to immune disruption (Inamdar et al., 2008; Mitra Mazumder et al., 2012; Raj and Gothandam, 2015) or to boost immune response against bacterial pathogens (Inamdar et al., 2008). *Massoia* (*Massoia aromatica* Becc, family Lauraceae) is a plant that originates from Papua and Maluku. Local people use parts of this plant, particularly its boiled or steamed bark and trunk, as a traditional treatment for fever and inflammation and to deter fungi and insects (Atmadja et al., 2015; Moestafa et al., 1999; Sa'roni and Adjirni, 1999). The aqueous infusion derived from boiled *Massoia* bark may contain C-10 massoilactone as an essential component. Sa'roni and Adjirni (1999) reported that the infusion exerts a mild anti-inflammatory effect in mice when administered at a dose of 300 mg/100 g BW. The analgesic activity of *Massoia* bark infusion when administered at the dose of 100 mg/10 g BW is comparable with that of acetosal administered at the dose of 0.52 mg/10 g BW (Widowati and Pudjiastuti, 1999).

The bark contains essential oil with C-10-massoilactone as the major constituent (Rali et al., 2007; Sa'roni and Adjirni, 1999). Our previous in vitro experiment revealed that *Massoia* essential oil and C-10 massoilactone enhance the phagocytic activity of mouse macrophages (Hertiani et al., 2016). However, no study has evaluated the effect of *Massoia* bark infusion as an immunomodulator on the nonspecific immune response (phagocytosis) of Wistar rats.

2. Methodology

2.1 Equipment: Oven (Memmert, Germany), freeze dryer (Benchtop Pro, USA), TLC scanner (Camag, Switzerland), laminar air flow hood (Labconco, Kansas, USA), micropipette (Socorex, Switzerland), vortex (Shimadzu, Japan), centrifuge (Sorvall, USA), hemocytometer (Neubauer, Germany), 24-well microplates (Nunc, England), 5% CO₂ incubator (Heraeus, Germany), light microscope (Olympus, Germany), inverted microscope (Olympus, Germany), and cover slips (SPL, Korea).

2.2 Materials: *M. aromatica* bark was collected from Sorong, Papua. Taxonomy identification was performed in Laboratory of Pharmacognosy, Dept. of Pharmaceutical Biology, Faculty of Pharmacy, UGM and registered as Nr. BF/3507/Ident/I /2016. Precoated TLC silica gel F254 plates (Merck, Germany), ethyl acetate, toluene (Pro Analyse, Merck, Germany), RPMI (Roswell Park Memorial Institute) (Sigma-Aldrich, Germany), fetal bovine serum (FBS) (Gibco, South America), Fungizone (Gibco, South America), penicillin–streptomycin (Pen-Strep) (Sigma-Aldrich, Germany), latex (3 μm) (Sigma-Aldrich, Germany), Phosfat Buffer Saline (PBS) (Gibco, South America), and Giemsa (Merck, Germany).

2.3 Animal testing: Male Wistar rats with an age of 2 months old were bred in the Integrated Research and Testing Laboratory (LPPT) Universitas Gadjah Mada. The handling of laboratory animals was approved by the Commission of Ethical Clearance for Preclinical Research LPPT UGM with Nr. 315/KEC-LPPT/VIII/2015.

3. Methods

3.1 Sample preparation

Bark samples were dried in an oven at 40 °C–60 °C, powdered, and infused in accordance with the Indonesian Pharmacopoeia (Sa'roni and Adjirni, 1999; Widowati and Pudjiastuti, 1999). In vitro tests were performed by using dried extract (WEM) prepared from freeze-dried 10% infusion. In vivo testing was performed using 20% infusion, which was prepared fresh daily.

3.2 Phytochemical analysis

WEM was fractionated with ethyl acetate–water (1:1 v/v) to obtain the ethyl acetate fraction (FEM). Following solvent evaporation, the FEM was analyzed by using

precoated silica gel F254 plates as the stationary phase and toluene:ethyl acetate (93:7 v/v) as the mobile phase. Qualitative analysis for determining the chemical profile of the extract was performed by detecting the eluted TLC plate under UV254 nm light and spraying reagents. Quantitative analysis was performed with TLC scanner at 211 nm by using C-10 massoialactone isolate as a standard (Nature et al., 2013 with modifications). TLC analysis densitometry was performed using TLC silica gel F254 plates measuring 20 cm × 10 cm. A total of 3 mL of sample was spotted on a TLC plate with a distance of 1 cm between spots, then eluted in a saturated twin-trough chamber for a distance of 8 cm.

3.3 Macrophage phagocytosis assay

Animals used for the in vitro assay were directly sacrificed without pretreatment. For the in vivo test, animals were divided into five groups. Each group consisted of 5 mice and received different treatments, namely: 1) 1 mL/100 g BW baseline distilled water; 2) 0.54 mL/rat immunostimulant herbal product “X”; 3) 100 mg Massoia bark infusion/100 g BW; 4) 300 mg Massoia bark infusion/100 g BW; and 5) 500 mg Massoia bark infusion/100 g BW. The infusion was given orally once a day for 14 days. The animals were sacrificed on the 14th day of treatment for analyses.

3.3.1 Macrophage isolation

Macrophages were isolated by injecting ±10 mL cold RPMI 1640 into the peritoneal cavity of each rat. Aspirates were centrifuged at 1200 rpm for 10 min. Then, 3 mL of complete RPMI media (containing 10% FBS) were added to each pellet. Cells were counted with a hemocytometer and suspended in complete media to obtain a cell suspension with a density of 2.5×10^4 cells. The cell suspension was placed in a 24-

well microtiter plate covered with round cover slips (5×10^3 cells/wells). The cells were incubated in a 5% CO₂ incubator at 37 °C for 30 min. Complete medium was added to each well. The plates were then incubated for 24 h (Sumardi et al., 2013).

3.3.2 Latex test for phagocytic activity

Phagocytic activity was tested using latex discs 3 µm in diameter and suspended in PBS. Macrophage cultures were incubated for 24 h and then washed twice with RPMI. Then, Massoia bark infusion was added at the concentration of 2.5, 5, 10, 20, or 40 µg/mL to each sample. The macrophage cultures were incubated again for 4 h in a 5% CO₂ incubator. The cells were washed thrice with PBS. Afterwards, the latex suspension (5×10^4 cells/wells, 200 µL/wells) was added to the cells. The plates were then incubated in a 5% CO₂ incubator at 37 °C for 1 h. The cells were washed thrice with PBS to remove excess latex. Cells were fixed with methanol for 10 min. The cover slips were allowed to dry and then stained with 20% v/v Giemsa for 20 min. The number of macrophages that phagocytosed latex, as well as the amount of latex phagocytosed by macrophages, was counted under microscopy to calculate the macrophage index (Sano et al., 2003). For the *in vivo* test, the latex suspension was added directly after the macrophage culture had been incubated for 24 h and washed twice with PBS. The rest of the assay steps were similar to those of *in vitro* testing.

4. Data analyses

Data were analyzed for homogeneity and normality using SPSS 16.0 to determine if parametric or nonparametric test statistical analysis should be conducted. Normally and homogeneously distributed ($p > 0.05$) data were analyzed through one-way analysis of variance. Data that were not normally or homogeneously distributed ($p < 0.05$) were analyzed

through the Kruskal Wallis test followed by Mann–Whitney test. $p < 0.05$ was considered significant.

5. Results

The Massoia bark infusion contained 8.14% (10% decocta) and 12.21% (20% decocta) C-10 massoialactone. Phytochemical screening detected only essential oil components, whereas phenolics, flavonoids, alkaloids, saponin, and tannin were absent. The *in vitro* assay showed that the aqueous Massoia bark extract can significantly increase the phagocytic activity of macrophages in comparison with the control treatment (Figure 3). Among all treatment dosages, treatment with 40 $\mu\text{g/mL}$ Massoia bark infusion showed the highest ability to enhance the phagocytic activity of macrophages. Concentration-dependent activity was observed.

The results of the *in vivo* assay corresponded with those of the *in vitro* assay. Groups that received the infusion at a dose of 300 or 500 mg/100 g BW exhibited a higher phagocytic index (PI) value than the baseline and the positive control groups. By contrast, the PI value of groups that received the infusion at a dose of 100 mg/100 g BW was almost similar to that of the positive control (Figure 4). Dose-dependent activity was also confirmed.

6. Discussion

The results of the *in vitro* assay supported the results of Hertiani et al. (2016), who reported that that increase in the phagocytic activity of macrophages is related to the increase in the content of C-10 massoialactone. The infusion method can be used to efficiently extract the active compound from the bark of the Massoia plant.

Several sesquiterpene lactones (Lopez-Anton et al., 2007) and lactones, i.e. zearalane (Edwards et al., 1989), can increase the phagocytic activity of macrophages. The active site

of C-10 massoialactone is mainly correlated with its β -unsaturated lactone moiety (Barros dkk., 2014). However, this compound is toxic to the Vero cell line and human fibroblast primary cells (Permanasari et al., unpublished data). Its toxicity may thus limit its application. Nevertheless, the active compound exhibits a broad-spectrum antimicrobial activity against planktonic and biofilm cultures (Hertiani et al., 2016). This activity may enhance the potential value of Massoia bark as an immunomodulator in responses to microbial infection.

7. Conclusion

In vitro and in vivo assay results showed that the aqueous infusion of Massoia bark can effectively increase macrophage phagocytic activity. Treatment with the 20% infusion administered at the dose of 300 or 500 mg/g BW significantly enhances phagocytic activity in comparison with treatment with the positive control.

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Figure 1. *Massoia aromatica* Becc. bark

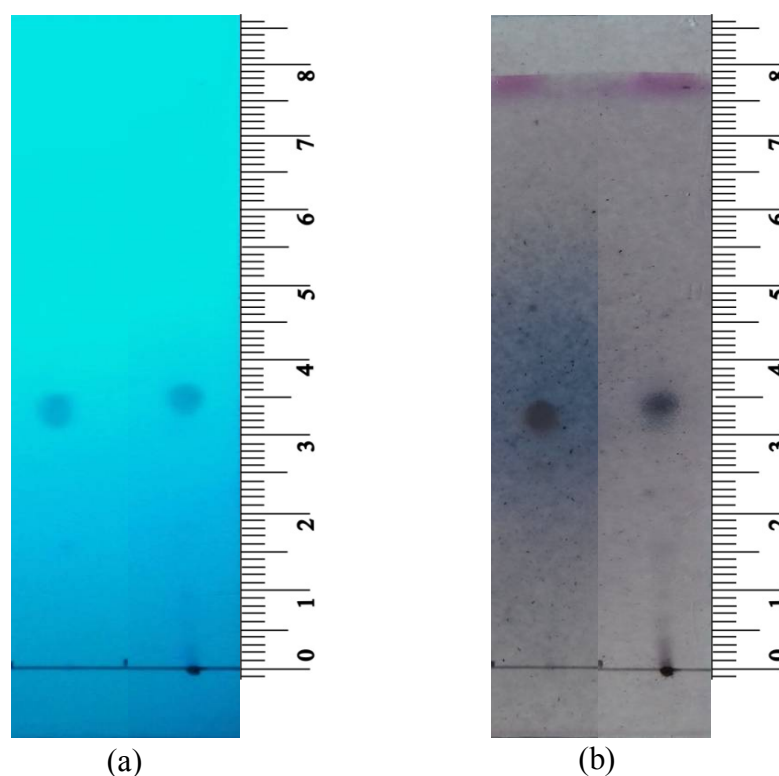


Figure 2. TLC chromatogram of *Massoia* bark fractions (a) under UV_{254} , (b) after spraying with anisaldehyd sulfuric acid and heating at $105\text{ }^{\circ}\text{C}$ for 10 min

TLC identification of FEM using toluene:ethyl acetate (93:7 v/v) as the mobile phase was performed in accordance with previous research. The TLC profile of the ethyl acetate fraction indicated that the massoialactone compound, which is shown as the massoialactone spot in Figure 2, has a R_f value of 0.38 attributable to C-10 massoialactone. The massoialactone spot exhibited a brown color after spraying with anisaldehyd sulfuric acid and heating $105\text{ }^{\circ}\text{C}$ for 10 min (Figure 4.b).

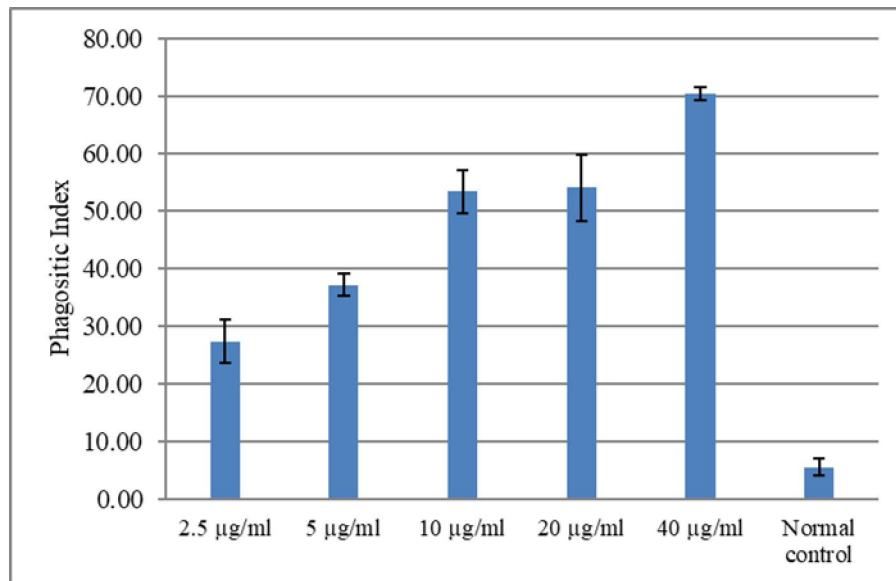


Figure 3. Phagocytic index of macrophages against latex after treatment with WEM *in vitro* (n = 3, $\alpha = 0.05$)

The phagocytic activity of macrophages under treatment with 2.5, 5, 10, 20, or 40 µg/ml media was analyzed in reference to previous research. Results showed that treatment with the Massoia bark infusion significantly increased macrophage PI compared with treatment with the control. The highest increase in PI was observed under treatment with 40 µg/ml Massoia bark infusion (PI = 70.51 ± 1.11), followed by that under treatment with 20 µg/ml Massoia bark infusion (PI = 53.97 ± 5.77), then by that under treatment with 10 µg/ml Massoia bark infusion (PI = 53.28 ± 3.69), that under treatment with 5 µg/ml Massoia bark infusion (PI = 37.17 ± 1.82), and finally by that under treatment with 2.5 µg/ml Massoia bark infusion (PI = 27.28 ± 3.68). PI increased in a dose-dependent manner.

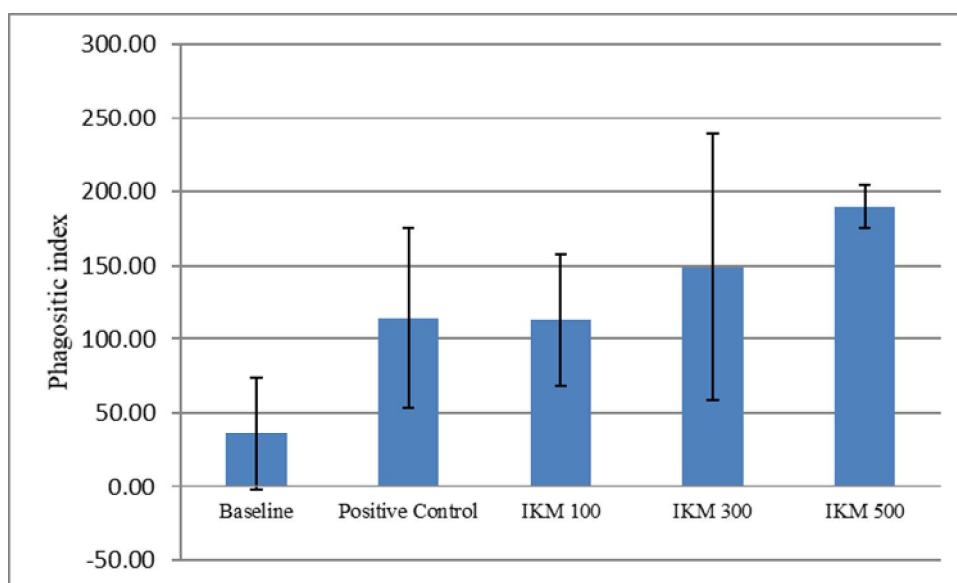


Figure 4. Phagocytic index of macrophages against latex after treatment with massoia bark infusion *in vivo* (n = 5, $\alpha = 0.05$)

The in vivo phagocytic activity of macrophages under treatment with 20% infusion administered at the dose of 100, 300, or 500 mg/100 g BW was studied. The phagocytic activity of groups treated with 20% infusion administered at the dose of 100, 300, or 500 mg/100 g BW increased compared with that of the baseline and positive control groups. Under treatment with 100 mg infusion/100 g BW, phagocytic activity increased to levels not significantly different from those under treatment with the positive control.

Table 1. C₁₀ massoialactone content of massoia bark infusion

Samples	C ₁₀ massoialactone content of sample (mg/ml)				C ₁₀ content massoialactone of sample (%)					
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
FEM 10%	0.30	0.30	0.27	0.29	0.02	8.49	8.49	7.43	8.14	0.61
FEM 20%	0.71	0.71	0.67	0.70	0.02	12.27	12.27	12.09	12.21	0.10

Note. FEM = ethyl acetate fraction of 10% or 20% massoia bark infusion

The C-10 massoialactone content of the ethyl acetate fraction of the Massoia infusion was measured through TLC densitometry. The results are shown in Table 2. The C-10 massoialactone content of 10% and 20% FEM was 0.29 and 0–70 mg/ml (8.13% and 12.21%), respectively.