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Research Article
In vivo Immunomodulatory Activity of Faloak Bark Extract (*Sterculia quadrifida* R.Br)

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Abstract

Background and Objective: Faloak (*Sterculia quadrifida* R.Br) is widely used as traditional medicine in Indonesia to improve stamina (reduce tiredness for heavy workers). However, no scientific reports so far on the immunomodulatory effect. The aim of this study was to determine the effect of the bark of faloak as immunomodulatory agents by evaluating their effect on BALB/c mice lymphocytes proliferation, the activity of macrophage, nitric oxide production and the immunoglobulin G titer by *in vivo* techniques. **Materials and Methods:** Decoction of the faloak bark was used for the *in vivo* assay. BALB/c mice were divided into 5 dose groups, each consisting of 5 mice. One group was chosen as the baseline, 3 groups were used for the group treated with the test substance at doses of 7.5, 11.75 and 17.5 g kg^{-1} of body weight of mice (p.o) and a positive control group was treated with *Phyllanthus niruri* Linn. (PN) extract (Stimuno®) 0.585 g kg^{-1} b.wt., (p.o). The test samples were given every day. All mice were induced by hepatitis B vaccine at day 7 and 14. The activity of *in vivo* assay was determined at day 19. The activity of immunomodulatory effect is expressed in phagocytic capacity, phagocytosis index, nitric oxide, OD of lymphocyte proliferation and IgG titers. **Results:** The macrophage phagocytic capacity and phagocytosis index were significantly increased ($p<0.05$), nitric oxide production were altered significantly ($p<0.05$), but OD of lymphocyte proliferation and production of IgG titers were unchanged ($p>0.05$). **Conclusion:** This study showed that the faloak bark could increase the macrophages phagocytic activity, but no effect on lymphocyte cells and therefore did not influence the adaptive immune response.

Key words: Faloak bark, immunomodulatory, *in vivo* study, macrophage, lymphocytes, nitric oxide, IgG

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The use of potential natural medicines as immunomodulators is a new concept in medicine used as traditional medicine[1]. An immunomodulator is a compound that can improve the body's defense mechanism. The importance of the body's defense system to health is to prevent or protect the body from infection and also the immune system into a physiological system to regulate body homeostasis[1-3]. Herbs have been used extensively in ethnopharmacology as a potential immunomodulatory effect[4], example: semprot ginger rhizome (*Zingiber officinale*), lempuyang rhizome (*Zingiber zerumbet*)[5,6] and meniran (*Phyllanthus niruri*)[7-9].

Faloak (*Sterculia quadrifida* R.Br) is one of the herbs that has potential as a traditional medicinal ingredient but no sufficient scientific data to support the usage. In East Nusa Tenggara, bark faloak has been used for many medicinal purposes, such as typhoid, ulcers and liver diseases[10]. Based on the experience, consuming decoction of faloak bark on a regular basis can increase stamina (reducing fatigue for the heavy worker). During this time people use faloak bark for various purposes of treatment, which contain chemical compounds such as alkaloids, steroid, triterpenoid, phenolic and saponin. The content of the compound serves to treat various diseases caused by bacteria such as dysentery, diarrhea with bloody stools, gastroenteritis and typhoid (typhoid fever), sacroiliitis, rheumatism, hypertension and liver function disorders[11,12].

The content of phenolic compounds from plants has been known to have antioxidant and anti inflammatory activity. Phenolic compounds derived from plants are able to selectively interfere with the production of cytokines. Phenolic compounds have the capacity to modulate the immune response and have the potential for anti-inflammatory activity[13,14].

The preliminary *in vitro* research has found out that faloak bark extracts have the potential to increase mice macrophage phagocytosis activity[15]. However, the effect can not elevate lymphocyte proliferation. Water extract of faloak bark has the highest activity *in vitro* study. In order to evaluate *in vivo* effect, this research use decoction of faloak bark extract to analyze the effect on BALB/c mice lymphocytes proliferation, the activity of macrophage, nitric oxide production and the immunoglobulin G titer.

MATERIALS AND METHODS

Materials: Faloak barks (*S. quadifrida* R.Br) were collected from Kupang, East Nusa Tenggara, Indonesia. Material used for immunomodulatory assay was RPMI (Rosewell Park Memorial Institute) 1640 (Sigma-Aldrich, Germany), DMSO, MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide] (Merck, Germany), Phosphate Bovine Serum (PBS), Fetal Bovine Serum (FBS) (Gibco, Amerika Selatan), reagent stopper Sodium Dodecyl Sulphate (SDS) (Merck, Germany), latex (3 mm) (Sigma-Aldrich, Germany), Giemsa (Merck, Germany), hepatitis B vaccine (Biofarma, Indonesia), Aquabidest sterile, 25 BALB/c mice 8 weeks of age with 30-40 g body weight.

Materials used for nitrite oxide assay were sodium nitrite, Griess reagent and ELISA kit (Mouse IgG total Ready-SET-GO 8850400 Affymetrix eBioscience) was used to determine the immunoglobulin G titer.

Sample preparation: Faloak barks were identified in laboratory Pharmacognosy, Pharmaceutical Biology Department, Faculty of Pharmacy UGM, Indonesia under plant identification Nr. 00083/04/LPPT/IX/2016. After drying in an oven at 50°C followed by pulverization, faloak bark was decocted and then adjusted according to the desired dosage.

Experimental design: The animals adapted to the experimental cages for 1 week before the treatment. BALB/c mice were divided into 5 dose groups, each consisting of 5 mice. One group was designed as a baseline, 3 groups group were tested with substance at doses of 7.5, 11.75 and 17.5 g kg^{-1} of mice body weight (p.o) and the one group was given *Phyllanthus niruri* L. (PN) extract (Stimuno®) 0.585 g kg^{-1} b.wt., (p.o). The test sample was given every day for 18 days. All mice were induced by hepatitis B vaccine at day 7 and 14. The activity was determined[16] at day 19.

This research was conducted for 4 months in 2017. The dosage of faloak for mice was adjusted to the amount of consumption in the local people by drinking 1 cup (200 mL) faloak stem bark a day. A total of 0.5 kg of faloak bark were boiled in 1 L water. Faloak concentration obtained 100 g/200 mL of water and converted into mice dosage 17.5 g kg^{-1} b.wt.

Macrophage isolation and phagocytosis activity assay: Macrophages are isolated from mice peritoneal fluid by adding 10 mL of cold RPMI 1640. About 1 mL complete media was added. The cells were calculated and then suspended in complete media to achieve 2.5×10^6 cells/mL. The cell suspension inoculated on 24-well microtiter plates which were covered by coverslips and incubated in 5% CO_2 at 37°C for 30 min afterward, 1 mL complete medium was added into each well and incubated for 2 h. After medium decanted, cells

were washed twice with RPMI 1640 and then 1 mL complete medium was added followed by 24 h incubation. Latex beads were suspended in PBS. Peritoneal macrophage culture was washed twice with RPMI 1640. Latex beads (200 µL well^{-1}) and samples (200 µL well^{-1}) were added and incubated in 5% CO_2 incubator at 37°C for 60 min. After fixation with methanol for 30 sec, the methanol was evaporated. Afterward, the coverslips were left to dry followed by Giemsa 2% (v/v) was used as staining for 30 min and then washed with distilled water and left to dry[8]. The activity of macrophage phagocytosis is expressed in phagocytic capacity and phagocytosis index. Amount of macrophages phagocytes latex beads, as well as the number of latex beads phagocyted by the macrophage, were counted under a microscope[17,18].

Lymphocytes isolation and proliferation assay: Lymph tissue was aseptically isolated from the mice and subsequently fed into 50 mm petri dish containing RPMI 1640. The suspension was centrifuged at 3200 rpm, 4°C for 4 min. Clumps were separated from the supernatant and suspended Tris Buffered Ammonium Chloride to lyse erythrocyte and left at room temperature for 15 min. RPMI was added to obtain suspension and centrifugation was repeated for 4 min and the supernatant was discarded. After being washed twice with RPMI, it was suspended in a complete medium. Cells were incubated 5% CO_2 at 37°C. After suspending the cells in complete medium, each 100 µL suspension was added into 96-well microtiter plate. The plates were incubated on the CO_2 incubator, at 37°C for 72 h. After incubation was added 10 mL of 5 mg mL^{-1} MTT solution in sterile PBS, incubated for 4 h. Afterward, 100 µL stop solution (10% SDS solution in 0.01 N hydrochloric acid) was added. Incubation is continued during overnight than read with Microplate Reader at 550 nm wavelength[19].

Nitric oxide assay: Sodium nitrite of 69 mg was dissolved in 100 mL aquabidest to produce standard nitrite 2000 µM stock solution and stored at 0-4°C. Then make a series of concentration between 0-100 µM. Standard nitrite solutions with various concentration series were then incorporated into 96 microtiter plates Duplo 100 µL each, as well as macrophage cultures that had been incubated overnight, then 100 µL of the Griess reagent consisting of Griess A and Griess B (1:1) in each well, incubated at room temperature for 15 min then read its absorbance with microplate reader at 550 nm wavelength[20,21].

Antibody titers assay: Blood samples (0.5 mL) were taken from the orbitalis retro plexus (vein orbital eye). After being centrifuged at 5000 rpm for 10 min the blood samples were stored at -20°C until use. Further antibody titer measurements were performed with ELISA (Total IgG Mouse Ready-SET-GO 8850400 Affymetrix eBioscience) and then measured using ELISA at 450 nm wavelength (protocol by supplier).

Statistical analysis: Results are described as a mean±standard error. Statistical analysis was performed by one-way ANOVA, of which, $p<0.05$ was considered statistically significant.

RESULTS

Phagocytosis activity assay: Assay on the macrophage phagocytosis exhibited the immunostimulatory effect of the decoction of faloak bark as shown in Fig. 1 and 2. The administration of a dosage of 7.5, 11.75 and 17.5 g kg^{-1} b.wt., had an activity to activate phagocytosis of macrophages compared with baseline and showed significantly increased ($p<0.05$), in phagocytosis index value and percent of phagocytosis.

Nitric oxide assay: Activation of NO production in mice given treatment with positive control (Stimuno® extract) and treatment group was significantly higher ($p<0.05$) than baseline (Fig. 3). While NO production activity between groups of mice treated with a decoction of faloak bark had the same NO production as Stimuno® extract, this shows that the potential of NO production of mice treated with a decoction

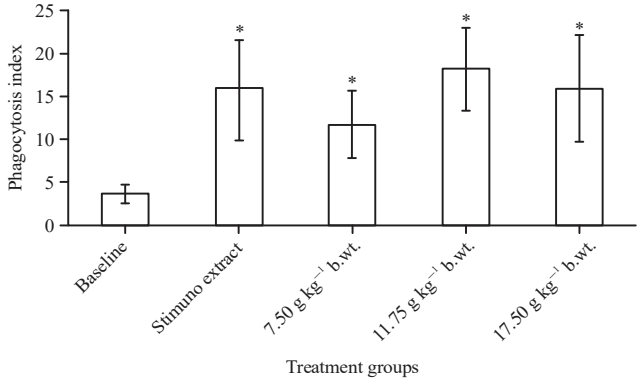

Fig. 1: Phagocytosis index of macrophages towards latex beads following sample application (decoction of faloak bark)
Mean±SD, *Significant different to control ($p<0.05$)

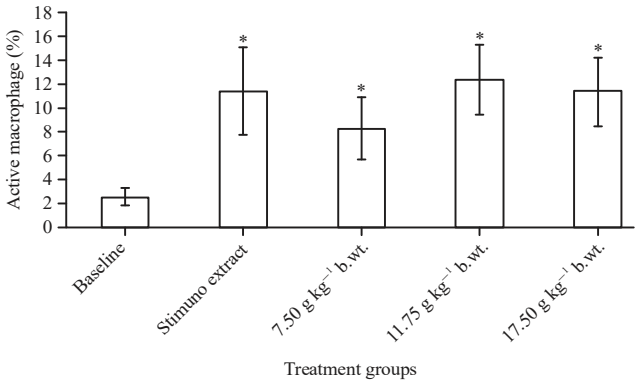

Fig. 2: Active macrophage (%) against latex beads following sample application (decoction of faloak bark)
Mean±SD, *Significant different to control (p<0.05)

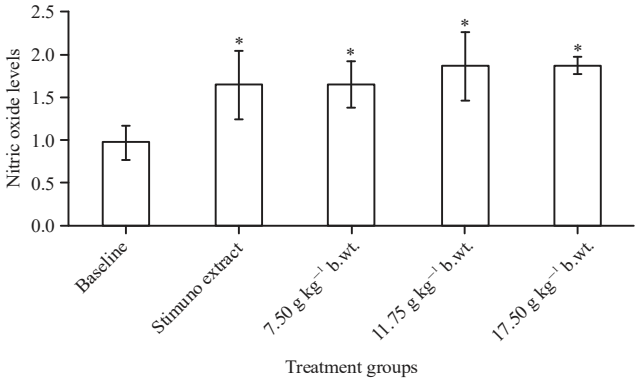

Fig. 3: Levels of nitric oxide following sample application (decoction of faloak bark)
Mean±SD, *Significant different to control (p<0.05)

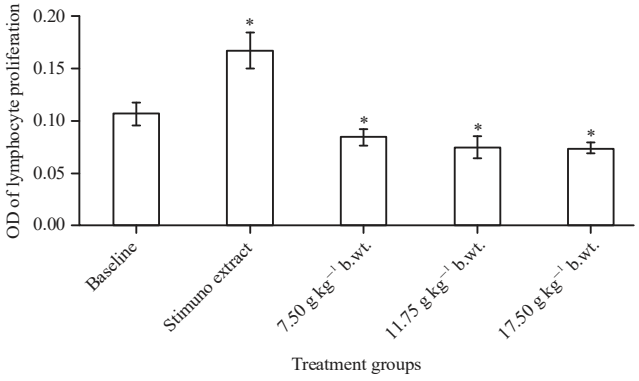

Fig. 4: Optical density of lymphocyte proliferation following sample application (decoction of faloak bark)
Mean±SD, *Significant different to control (p<0.05)

of faloak bark dose 7.5, 11.75 and 17.5 g kg^{-1} b.wt., equivalent to the production activity of mice treated with Stimuno®.

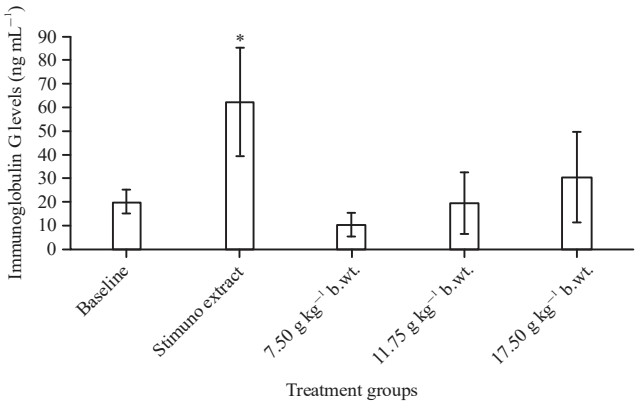

Fig. 5: Immunoglobulin G levels of lymphocyte proliferation following sample application (decoction of faloak bark)
Mean±SD, *Significant different to control (p<0.05)

Lymphocytes proliferation assay: Data in Fig. 4 showed a decrease in the number of OD lymphocytes occurred in all groups treated with faloak decoction when compared with the positive control group (Stimuno® extract). This result indicates that there is suppression of lymphocyte production due to the decoction of the bark of faloak. Statistical analysis showed that the amount of OD of lymphocytes in the group treated with faloak decoction was significantly decreased ($p<0.05$) than baseline and positive control.

Antibody titers assay: Figure 5 showed that there is no significant difference in antibody production between the treatment group and baseline group.

DISCUSSION

Faloak bark extract have potential to stimulate non specific immune response. The activity of phagocytosis occurs in the number of activated macrophages as well as at the activity level of each macrophage unit. In addition to increasing the number of activated macrophages in the body of mice, the administration of test compounds also increased individual macrophage capacity against latex. Many studies have reported than faloak contained flavonoids, alkaloids and terpenoids[10-12,22]. Flavonoid have pharmacological effect including immunomodulatory activity, anti-inflammatory and anti-cancer activity. There effect can modulated the macrophage activity[23,24].

The response of immune system can be modulated by any alteration involve induction, expression and inhibition of some part phase in immune response[25]. The content of secondary metabolites herbal medicine

can be affect of immunomodulatory activities such as polysaccharide, sterols, cannabinoids, alkaloids, flavonoids and lectin[23,25-26].

Positive control (Stimuno®) that contain *Phyllanthus niruri* Linn. extract had an equal activity of macrophage compared with the treatment group. Several studies reported that immunomodulatory activity of *Phyllanthus niruri* Linn. extract could increase phagocytosis of macrophage, the formation of lysosomal enzymes and release of TNF-α[7]. Other research showed that *in vivo* study of *Phyllanthus niruri* Linn. extract can increased activity and phagocytosis capacity[8].

Immunomodulatory activity of faloak bark decoction is consistent with studies of several another genus of *Sterculia* plants capable of enhancing macrophage phagocytosis activity. A study published by Das[21] reported that *Sterculia villosa* extracts could induce in killing parasites through modulation of cytokine expression and NO release compared with untreated macrophages. The macrophage phagocytosis activity of treatment group and positive control is relatively high, but it does not cause excessive production of NO. This effect may be due to the compound content in the sample being able to maintain the function of immune cells by protecting it from the excessive phagocytic activity[27]. According to Victor *et al.*[28] the process of oxidative stress induced by endotoxin, peritoneal macrophages showed increased attachment and phagocytosis function and decreased chemotaxis. The change is followed by the production of superoxide anions and high TNF-α release. Thus, an oxidant-antioxidant equilibrium is critical to immune cell function because it maintains cellular integrity and controls signal transmission and gene expression[28].

The lymphocyte cell used originated from the spleen organ of mice previously exposed to hepatitis B vaccine. The addition of antigen (hepatitis B vaccine) was performed to recognize the memory cells that had formed during the treatment. The result of OD Lymphocyte was suspected that the administration of the compound did not affect the increased activity of lymphocyte proliferation of mice. On the other hand, the positive control group showed an increase in lymphocyte proliferation activity. Nworu[7] reported that *P. niruri* extract could increase the expression of surface activation marker (CD69) and the proliferation of B and T lymphocyte.

The decoction of faloak showed the activity of suppression lymphocyte cell proliferation. Several studies related to the effect of plant extract on lymphocyte cell proliferation showed that suppression of lymphocyte cell proliferation, as Kanjwani demonstrated immunosuppressant activity in both cellular and humoral immune responses from *Piper betel* L. extract. IFN-γ, a cytokine involved in T lymphocyte activation, differentiation and maturation for cytotoxic T cell precursors, probably mediates this immunosuppressant activity[29]. In this study, faloak bark extract did not affect the proliferation of lymphocyte cells. flavonoid therefore faloak bark decoction is more effective in non-specific immune systems (phagocytosis), but less effect on specific immune responses.

The production of antibodies different among groups was possible because the compounds in the faloak bark decoction did not affect the cells that play a role in the production of antibodies. The amount of compounds in the extract was also thought to affect cells that work antagonistically together. As a result, the production of antibodies was not significantly different. The compounds contained in the faloak bark decoction do not affect the cells that play a role in the production of antibodies. These results reinforce that faloak bark extract does not affect the adaptive immune response.

CONCLUSION

Faloak bark extract showed that have potential to stimulate non specific immune response. The activity of phagocytosis occurs in the number of activated macrophages as well as at the activity level of each macrophage unit. This study showed that the bark faloak could increase the macrophages phagocytic activity, but less effect found towards the adaptive immune response. The compounds contained in the faloak bark decoction do not affect the cells that play a role in the production of antibodies.

SIGNIFICANCE STATEMENTS

This study discovered faloak extract can increase the activity of innate immune response (macrophage and NO activity) but less effect on the adaptive immune responses (lymphocyte activity) that can be beneficial for development of traditional medicinal plants and drug discovery. This study will help the researchers to uncover the critical areas to explore the effect of faloak, increased the activity of innate immune response but less effect on the adaptive immune responses that many researchers were not able to explore. Thus a new theory on immunomodulatory activity from faloak may be arrived at.

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