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Last but not least, we thank all the authors for their excellent contributions to these proceeding and all the participants for their contributions to this successful conference, either in the form of oral presentation, posters, or in the form of their attention to the presentations and contributions to the discussions.

We hope that everyone enjoys reading the proceeding and that the papers inspire you in your research or other professional activities.

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PRELIMINARY STUDY ON FALOAK BARK POTENCY FOR PREVENTION OF MICROBIAL INFECTION

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Abstract. Faloak (*Sterculia quadrifida* R.Br.) is an indigenous plant originated from East Nusa Tenggara, the eastern part of Indonesia. The reports on its pharmacological activity is still scarce regardless that the bark has widely used by the local people as part of traditional remedy against various health complaints. In an attempt to provide scientific support, we have explored for the faloak bark potencies for radical scavenging, immunomodulator as well as antimicrobial activities by *in vitro* techniques. The bark was macerated in ethanol followed by evaporation to yield ethanolic extract. The extract was subjected to DPPH radical scavenging activity assay to gain the IC₅₀ value. Immunomodulatory assay was performed on mice macrophages and lymphocytes. Antimicrobial activities were evaluated against the planktonic growth and biofilm of *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli* by using microdilution techniques. Crystal violet was used to stain the formed biofilm. Results showed that the bark contains 34.43%GAE of total phenolics (equivalent gallic acid), 1.55% QE of total flavonoid (equivalent quercetin) and 84.07 µg/mL of DPPH radical scavenging activity (Vit C: 74.72 µg/mL). The stimulation index (SI) on mice lymphocyte was less than 2 suggesting no stimulation which intended to show a repression by higher concentration tested. The extract enhanced macrophage phagocytosis activity which was in correlation to the concentration tested. The extract was relatively not toxic to Vero cells, however it also showed no potential antimicrobial activity against test microbes. As a conclusion, the faloak bark is a potential immunomodulator and antioxidant.

Keywords: *Sterculia quadrifida*, immunomodulator, DPPH radical scavenging

INTRODUCTION

Sterculia quadrifida R.Br (Sterculiaceae) is an indigenous plant of the East Nusa Tenggara (NTT), Indonesia which has been used traditionally against various ailments such as liver disturbances, kidney disease, anemia, rheumatic, and as post partum remedy (Siswady, 2015). Regardless its wide therapeutic usages among the local people in the NTT, pharmacological activity reports on the plant is still lacking. Several reports on the closely related plants which has the same local name, faloak have been found, although the taxonomy was considered to may be interchangeable. Infusion of *Sterculia urceolata* Smith. has been reported to have hepatoprotective activity on CCl₄ pretreated male mice which was given one a day for peroral

for 6 days ED₅₀ 5.24 g/kgBW (Rambung, 2002). The acetone extract of *Sterculia comosa* showed antibacterial activity against *Staphylococcus aureus* dan *Bacillus cereus* with inhibition zone of 15.17 mm and 16.17 mm, respectively (Ranta, 2011).

Toyo (2003) also reported that the bark of *Sterculia urceolata* contains flavonoid, anthraquinone, saponin, cardenolide, and tannin (Toyo, 2003), while Ranta (2011) reported the presence of saponin and triterpenoid in the acetone extract of *S. comosa* bark. Epicatechin, and procyanidin B2 have been isolated from the bark of *Sterculia tragacantha* (Orisakeye and Olugbade, 2014); while lupeol, a triterpenoid has been isolated from the bark of *Sterculia striata* (Costa *et al.*, 2010). Epicatechin and procyanidin B2 stimulated the lymphocyte proliferation (Zhao *et al.*, 2007), while lupeol isolated increased the macrophage phagocytosis activity (Sutomo *et al.*, 2013).

In order to provide scientific supports for Faloak bark as traditional medicine, we screen the ethanol extract for possible bioactivities in relation to the traditional usage by using *in vitro* techniques. This research is a preliminary step aiming to find a potential bioassay guidance to direct the research of isolating bioactive marker from the Faloak bark.

METHODOLOGY

Materials

Samples were collected from Kupang, NTT on May-June 2014. Taxonomy identification was conducted in the Research Centre for Biology, Indonesian Institute of Sciences (Nr. 373/IPH.1.02/If.8/III/2012). Samples were sun-dried followed by oven dried and pulverized. The powder was macerated in ethanol in 1:5 and followed by remaceration. The combined macerates were evaporated to yield thick-solvent-free extract.

Laboratory materials used were as follows: 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).

Equipments

Oven (Memmert, Germany), freeze dryer (Benchtop Pro, USA), TLC scanner (Camag, Switzerland), laminar air flow (Labconco, Kansas, USA), micropipette (Socorex, Switzerland), vortex (Shimadzu, Japan), centrifugation (Sorvall, USA), hemocytometer (Neubauer, Germany), microplate 24 wells (Nunc, England), CO₂ incubator 5% (Heraeus, Germany), a light microscope (Olympus, Germany), microscope inverted (Olympus, Germany), and the cover slips (SPL, Korea).

Antimicrobial susceptibility testing, a microdilution method

Cultures of *S. aureus* and *E.coli* ($0.1 \div 10^8$ CFU ml⁻¹ in BHI) and *C. albicans* in BHI, were dilution until 10⁷ CFU ml⁻¹. Assays were taken place on sterile flat-bottom 96-well polystyrene micro titer plates and used Brain Heart Infusion (BHI) broth medium and incubated 90 minutes at CO₂ incubator. Controls were prepared as follows, negative controls (cells + media), positive

controls (cells + media + antibiotic – streptomycin or nystatin for *C. albicans*), and media controls. All plates were incubated overnight at 37 °C. Readings of the optical density were conducted at 595 nm. All tests were performed in triplicate. The formula from Pirbalouti *et. al.* was used to determine the MIC (11):

$$\text{Inhibition \%} = [(ODc - ODt) / ODc] \times 100$$

ODc is the OD₅₉₅ of the negative control at 24 h post-inoculation and Odt is the OD₅₉₅ for the tested samples tested at 24h post-inoculation. The essential oils concentration caused growth inhibition of microbes by at least 50% was considered as the MIC₅₀ (11).

Biofilm formation inhibition assay (7)

Cultures of *S. aureus* and *E.coli* ($0.1 \div 10^8$ CFU ml⁻¹ in BHI) and *C. albicans* in BHI, were dilution until 10^7 CFU ml⁻¹. Assays were taken place on sterile flat-bottom 96-well polystyrene micro titer plates and used Brain Heart Infusion (BHI) broth medium and incubated 90 minutes at CO2 incubator. Controls were prepared as follows, negative controls (cells + media), positive controls (cells + media + antibiotic – streptomycin or nystatin for *C. albicans*), and media controls. All plates were incubated overnight at 37 °C.

Following overnight incubation, the wells were poured off. After 3 times rinsing with distilled water, the plates were left to dry at room temperature for 45 min. Staining was conducted by adding 100 µL crystal violet 1%, left for 45 min. After the staining was being discarded and rinsed with tap water 350 µL to eliminate excess stain, 200µL ethanol 95% was added to the wells, incubated at temperature room 45 min. Optical density at 595 nm was measured and the results were used to determine the % inhibition and the minimum biofilm inhibitory concentration, MBIC values. The inhibition percentage was calculated as the average of OD of the control wells in comparison to that of the sample wells, as defined by the following formula:

$$[(OD_{\text{control}} - Odsample) / OD_{\text{control}}] \times 100$$

Effect on phagocytosis activity of macrophages (9)

Measurement of phagocytosis activity was conducted by using 3 µm latex beads (2.5×10^6 mL⁻¹ suspended in PBS). After 24h incubation, culture of peritoneal macrophages in wells equipped with cover slips was washed twice with RPMI 1640 and then added to a serial dilution of samples ranging from 10-40 µg/mL. Incubation was taken place in a 5% CO₂ incubator at 37 °C for 4h. After rinsing the cells three times with PBS, suspension of latex beads (200 µL/well) was added. Further incubation was performed in 5% CO₂ incubator at 37 °C for 30 min. The latex beads were removed by rinsing the cells three times with PBS. Following fixation with 300 µL/well methanol for 1 min and then poured off, the cover slip was allowed to dry, and 300 µL Giemsa dye 10% v/v was added and left for 20 minutes. After the dye was being discarded, rinsed with distilled water and dried, the macrophage phagocytic index was counted under light microscope. The phagocytic index was calculated according to the following formula (12):

$$\left(\frac{\text{total number of active macrophage in each 100 macrophages}}{\text{number of macrophages engulfed cells or beads}} \right)$$

The animal handling has been approved by the Ethical Clearance Commission for Preclinical-Studies of the LPPT-UGM.

Cytotoxicity assay (6):

Vero cells were cultured at 37°C under a humidified atmosphere containing 5% CO₂ in 25 cm² plastic culture flask containing M199 medium of which 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin were supplemented. Culture medium was removed when the cell reached 90% confluence. Trypsin EDTA was dispensed to the cell cultures for detaching cells from the flask. The cells suspension having density of 1×10^4 cells/well in 100µl medium and incubated overnight in a CO₂ incubator at 37°C. Afterwards, 100µl of each serial dilution samples (unformulated massoia oil and its nanoemulsion) was dispensed into each well. After 24 h of incubation period, the media were removed from the plate. Cell viability was identified using MTT reagent [3 (4, 5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide]. One hundred micro-liters MTT was dispensed onto each well. Following 4 h incubation, 100µl stopper solution (10% SDS) was added. After 24 incubation, the optical density was measured by micro titer plate reader at a wavelength of 595 nm and calculated as follows:

Percentage of viable cells = [(OD Treatment– blank) / (OD control-blank)] x 100%
IC₅₀ : (50-a)/b which is a : intercept, b : slope from Ln C vs percentage of viable cells curve.

RESULTS

The faloak extract showed high phenolic content (34.43% GAE) of which some are determined as flavonoid (1.55% QE). The phenolic content may contribute to a prominent DPPH radical scavenging activity as shown by IC₅₀ value of 84.07 µg/mL which was only slightly lower than the IC₅₀ shown by the positive control (Vitamin C) i.e. 74.72 µg/mL.

The extract showed a promising effect on the non specific immune response, as described by a concentration dependant macrophage phagocytosis stimulatory effect (table I). However, it was observed a negative corelation of the sample concentration to the lymphocyte stimulatory index (Fig. 1).

TABLE 1. Macrophage phagocytosis effect

No	Treatment (µg/mL)	Phagocytosis capacity	% Active macrophage	Phagocytosis Index
1	1000	11.84 ±0.44	68.00 ±1.76	270.00 ±17.15
2	700	11.20 ±0.12	31.00 ±0.67	115.67 ±2.12
3	500	10.15 ±0.80	28.67 ±1.35	96.67 ±8.83
4	300	11.31 ±0.27	21.33 ±1.07	81.00 ± 6.08
5	100	10.94 ±0.10	18.33 ±1.07	67.00 ± 4.33
Negative control		11.16±0.33	9.67±0.19	36.00±1.53

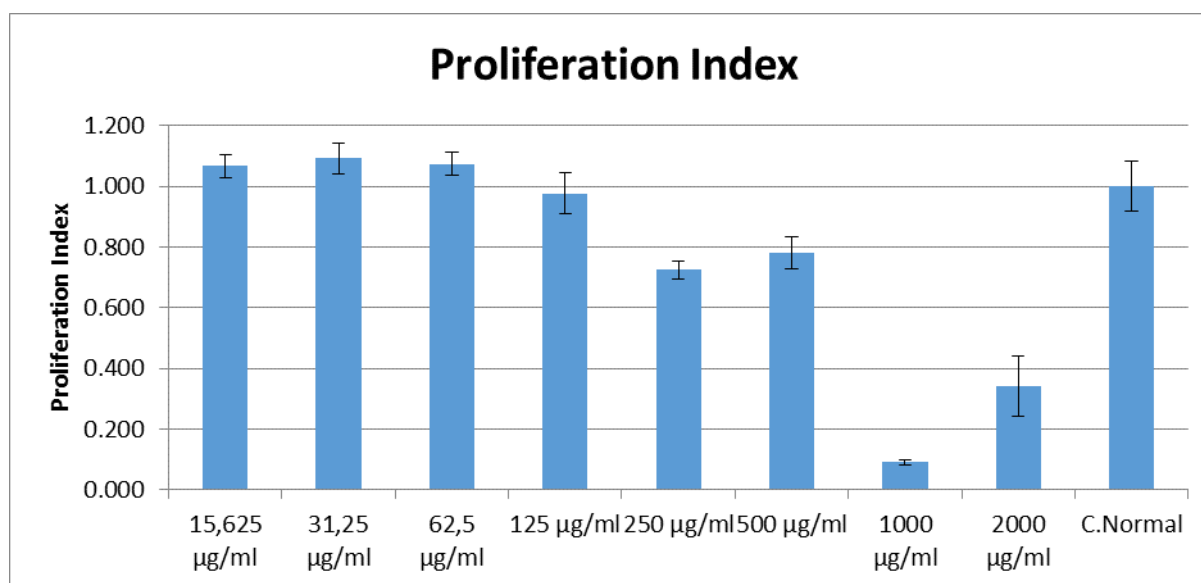


FIGURE 1. Proliferation Index

In order to evaluate to toxicity, the extract was subjected to Vero cell lines. It was observed lower inhibitory effect of the cell proliferation in comparison to negative control, and significantly lower than doxorubicin as positive control. Table II shown that IC50 extract

(462.99 ppm) lower than doxorubicin (137.15) which has indicated the extract more safety than doxorubicin.

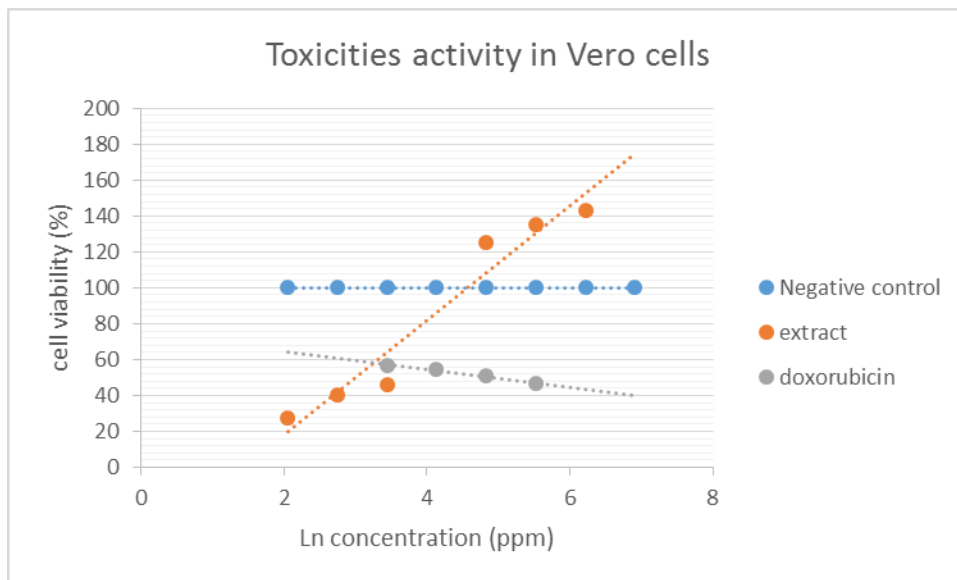


FIGURE 2. Toxicities activity in Vero Cells

TABLE 2. Toxicity in Vero Cells

No	Sample	Concentration (ppm)	cells viability (%)	IC50 (ppm)
1	Doxorubicin	31.25 – 500	46.4 – 56.6	137.15
2	Extract	15.625 – 2000	27.2 – 143.2	462.99

We also evaluate the extract potential to be develop as antiinfective. Therefore the extract was subjected to antimicrobial testing covering antibiofilm and anti planktonic growth. Model organisme from Gram positive and Gram negative microbes (*Staphylococcus aureus* and *Escherichia coli*) as well as a yeast, *Candida albicans* was tested. Unfortunately, no promising effect observed on the tested organisms (Table 3).

TABLE 3. Planktonik and Biofilm Inhibition Percentage with several concentration

No	Consentration (ppm)	Microbe Inhibition percentage (%)			Biofilm Inhibition percentage (%)		
		<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>
1	312.5	32.52	12.94	-19.76	-6.62	4.79	-4.74
2	625	22.63	18.96	-27.18	-6.06	6.23	-32.00
3	1250	45.48	2.75	-41.74	18.14	-1.06	-31.11
4	2500	-5.86	-2.71	-55.11	33.37	3.65	19.31
Positive control		74.85	88.33	80.57	51.64	61.07	33.48

DISCUSSION

Faloak extract have antioxidant activity from flavonoid compound which is one of extract compounds. Flavonoid can be reduce DPPH with hydrogen transferred into molecules as phenolic compounds³. Antioxidant activity can stimulate innate immune system, such as macrophage. Our research have been found increase in macrophage activity which dependent to sample concentration. Macrophage has been activated by endogenous compounds such as T helper 2 (Th2) cytokines, interleukin 4 (IL-4), interleukin 13 (IL-13) and interferon- γ (IFN- γ)⁶. In complex immune system, macrophage activity could been stimulated by lymphocyte T cells. Which it can be produce IFN- γ , ILN-2. IFN- γ which is released would be induce the expression of MHC class II and activated macrophage. Otherwise, IL-2 played a role in other immune cells proliferation, such as NK cells and B cells². However, index proliferation value from Lymphocyte cells did not correlate with macrophage activity. Thus the compounds in the extract were possible only affect for increase innate immune.

We also found that faloak extract did not cause normal cell toxicity from the value of IC50. The higher IC50 value indicated that extract have lower ability to inhibit normal cells proliferation.

CONCLUSION

The extract has been known to have antioxidant activity which may increase the innate immune system (macrophage) but has not been able to improve the adaptive immune system (lymphocyte cells). In addition, the extract has also known to be not toxic to Vero cells, however antimicrobial activities were not exerted.

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