

# The Use of Local Kelor (Moringa Oleifera) Leaves Powder from West Nusa Tenggara to Increase the Neutrophile Cell Phagocyte Index and its Function on Rats with PEM Infected by Staphylococcus Aureus

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## **The Use of Local Kelor (*Moringa Oleifera*) Leaves Powder from West Nusa Tenggara to Increase the Neutrophile Cell Phagocyte Index and its Function on Rats with PEM Infected by *Staphylococcus Aureus***

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**Abstract**--Indonesia is reportedly ranked third in the prevalence of acute malnutrition in the Asia Pacific. Protein Energy Malnutrition (PEM) has a high prevalence in the West Nusa Tenggara (NTB) Province with over 600 cases reported by the Health Department from January to October 2015, especially among children under five of which 31 cases led to death. Meanwhile, the nutrition status is accurately determined through blood biochemical and hematology tests. The Neutrophil index is an important biomarker in combating infectious agents, such as *Staphylococcus aureus* which is often the cause of contagious diseases. Individuals suffering from Protein-Energy Malnutrition are most vulnerable to these types of infections. Fortunately, kelor (*Moringaoleifera*) has been found to have nutritious contents, especially protein and iron (Fe), but its potential has not been extensively explored in the West Nusa Tenggara. This study aims to confirm the effect of the local kelor (*Moringa oleifera*) leaves powder in increasing the Innate Immune Response of sample rats with Protein Energy Malnutrition (PEM). A post-test randomized control group design was used with 20 rats grouped into five with a period of 56 days.

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**1**  
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## Introduction

**1**  
Based on the 2019 UNICEF Eapro report, Indonesia ranks **third in the prevalence of acute malnutrition** among Toddlers in the Asia Pacific (UNICEF, 2020). Aside from the high malnutrition status, the prevalence of infectious diseases is also significant in NTB. According to WHO, the high incidence of infectious disease leading to the death rate of 45% in the ASEAN countries is caused by the infection of *Staphylococcus aureus* (Tong et al., 2015). Moreover, reports from the Health Departments of the regencies in the West Nusa Tenggara Province (NTB, 2014) between January and **October 2015**, stated that more than 600 malnutrition cases were reported **especially among children under five of which 31 cases led to death. Meanwhile**, several studies have been conducted on the clinical effects of *kelor* leaves in Indonesia (Luthfiyah, 2012). A study that examined the antibacterial activity of the extract on the *Streptococcus aureus* growth (Dima, 2016), found that the higher the concentration, the bigger the light zone or bacteria-free zone formed. A person's nutritional status is determined based on the assessment of food consumption including anthropometry, laboratory/biochemistry, and clinical (Agichtein et al., 2008). (Gibson, 2005), Among these methods, laboratory or biochemical measurements are relatively more rigorous and are an absolute examination carried out primarily to establish the diagnosis and screen for infectious diseases (Budiono, 2013).

Complete blood tests are also performed to establish the diagnosis of the disease, but these types of tests for nutrition are still rarely performed. This is due to a lack of information and studies for monitoring nutritional status and risk of infectious diseases in children. Through complete blood tests, an individual's immune response is often identified. Several studies have been conducted on the ability of the immune system to increase, for example, the ability of neutrophil cells to destroy microbes particularly staphylococcus aureus in the treatment namely kencur compound in the sequence of (1-100 ug/ml) (Parawansah et al., 2018). Also, the administration of ethanol extracts of johar leaves to increase the activity of macrophage phagocytosis has been reported (Kusmardi S., & Wulandari, 2006).

Activated macrophage cells kill infectious agents by increasing the phagocytic activity and developing microbicidal mechanisms through oxidative systems and producing Reactive Oxygen Intermediates (ROIs). Moreover, the interactions between receptors on the surface of macrophages and the ligands cause spikes in respiration which lead to a change in the oxidation complex activity on the membrane and reduction of oxygen to superoxide. There are 14 species of *kelor* in the world but the local variety native to West Nusa Tenggara has supposedly unique nutrition compositions which need further investigation on its medical effects (Upadhyay et al., 2015). In India, Moringa has been used as medicinal plants (Indian Herbs) for several years and the bioactive substances, as well as functions have been analyzed. One of the 49 phytonutrients is beta carotene which has phagocytotic activity (Udikala et al., 2017). Previous studies in

Indonesia on the clinical effects of Moringa leaves have also been carried out. Based on the antibacterial activity test results on the growth of *Streptococcus aureus* (Suriaman & Khasanah, 2017), the higher the concentration of leaf extract, the greater the light zone formed namely areas that are not overgrown with bacteria and a clear zone was gradually formed at a concentration of 5%. Furthermore, the nutritional quality of moringa plants depends on the soil nutrient elements in which the plants live. This is interesting as moringa from the west certainly has unique nutritional elements that need to be examined for its profound effect on health. Therefore, this study was conducted to examine the effect of local kelor leaves powder of NTB on hematological parameters such as Hb, leucocyte, lymphocyte, and monocyte levels, as well as the phagocyte index of neutrophil cells and the function in relation to Protein Energy Malnutrition in rats infected by the *Staphylococcus aureus* bacteria (Harahap et al., 2021; Sidiartha et al., 2018; Lakonawa et al., 2020).

## **Material and Methods**

### **Source materials and sample preparation**

Moringa leaves as part of the study materials were obtained from the garden in the city of Mataram, based on information from the Institute for Seed Control and Certification of Food Crops and Horticulture (BPSBTPH) Department of Agriculture Province of West Nusa Tenggara Number 521/279BPSBTPH, dated February 17, 2015. The moringa trees planted in this land were of two types namely white and red, both were taken as the study sample. The leaves were taken from several trees, each was separated from the trunk, then, the moringa leaf red group was processed separately into powder as well as the white type. Furthermore, the macronutrient and micronutrient compositions were examined by checking five times with a replication.

### **Designing a rat model of protein energy malnutrition**

Experimental animals used were 20 white male rats of Wistar strain *Rattus Novergicus* 5-6 weeks old weighing 100-150 g and obtained from the Physiology Laboratory, Faculty of Medicine, Brawijaya University. This study was approved by the ethical committee of Integrated Research and testing laboratory Brawijaya University (Approval Number: 0193/EC/KEPK-S2-JK/07/2018). The subjects were adapted for 7 days, grouped into 5, and were treated for 56 days. The treatments were as follows: P-0 = Negative control group fed with the normal diet; P-1 = positive control group fed with a low protein diet; P-2 = group of rats given low-protein diet + Moringa leaf powder dose I (0.18 g / day); P-3 = group of rats given a diet low in protein + powder Moringa leaf II dose (0.36 g / day); P-4 = Group of rats given a diet low in protein + powder Moringa leaf III dose (0.72 g / day). The model rats with EPM were given a low protein diet for 56 days, and the other groups were given kelor leaf powder per oral with sonde, which was given once in 30 days. During treatment, the rats were fed ad libitum (Van de Giessen et al., 2009; Yuan et al., 2014).

## Implementation

The materials for a complete blood examination include reagents for neutrophil and macrophage phagocytosis activity in-vitro (NurulUlum et al., 2016). Blood, peritoneal fluid, bacteria and bacterial preparations were dissolved with a Staphylococcus aureus concentration of 108 bacteria. Staphylococcus aureus was used because these microbes are readily available and frequently used in different studies (3). Meanwhile, the materials and equipment to test secretion of ROI are RPMI, Nitroblue tetrazolium (NBT) (Sigma chem.co), Neutral Red Solution (Sigma chem. co), and Methanol absolute. The bacteria used to test the phagocytosis activity was *S. aureus* by initially grew in broth Todd Hewitt Brott (THB) for 24 hours at a temperature of 37°C and disentrifused at 1000x g with a temperature of 37°C for 10 minutes. The precipitate was dissolved in HBSS, while the Optical Density (OD) was determined with 10% spectrophotometric transmission at a wavelength of 620nm to obtain a solution of 108 bacteria/ml for the phagocytosis test (McMillan et al., 2018; Panneerselvam & Govindasamy, 2003).

Furthermore, the neutrophil and peritoneal macrophages' phagocytic activity against *S.aureus* were determined by isolating a culture of mononuclear Peritoneal Adherent Cells (Www.invitrogen.com/cellculturebasic, 2010) taken in the form of macrophages. After the mice were euthanized, the abdominal wall was sterilized with 70% alcohol, then, it was opened to visualize the living peritoneum covering the abdominal contents. To obtain peritoneal macrophage cells, a medium M + 10 ml was injected with a syringe into the Abdominal area and was shaken for 5 minutes, the medium was taken back to the syringe. The cell viability was determined using trypan blue (Fatmawati et al., 2011), while the Phagocytosis and Secretion of Functional Test ROI peritoneal macrophages were tested in-vitro according to Breedveld et al. (1986), using gram-positive bacteria (*S. aureus*) solution. The peritoneal macrophages' ability to secrete ROI was measured by Nitroblue Tetrazolium (NBT) reduction assay according to Breedveld et al. (1986). With the existence of superoxide anions (O<sub>2</sub>O) NBT was reduced to form colored precipitates that are insoluble formazan. Cell cultures containing *S. aureus* are expected to induce secretion of superoxide anions

## Results

### Sample hematology description

Table 1  
The descriptive statistical distribution on the sample hematology description

Hematology Parameter	Standard*	Normal	EPM	EPM + Kelor EPM+dose1	EPM+dose2	EPM+ dose3	p - value
Hemoglobin (g/dl)	11.1 -18	12 + 0.9 <sup>b</sup>	5.2 + 0.5 <sup>a</sup>	13.00+ 1 <sup>b</sup>	12.00+0.1 <sup>b</sup>	12 + 0.9 <sup>b</sup>	0.000
Leukocyte (10 <sup>3</sup> /mm <sup>3</sup> )	3-15	15.27+2.4 <sup>c</sup>	24.46, + 4.6 <sup>a</sup>	8.34+3.08 <sup>b</sup>	6.75+3.71 <sup>b</sup>	5.17+1.18 <sup>a,b</sup>	0.000
Neutrophyl(10 <sup>3</sup> /mm <sup>3</sup> )	1,10-4,00	3,6+1,7	7,2+3,1 <sup>a</sup>	5,41+2,1 <sup>b</sup>	4,36+3,2 <sup>b</sup>	3,4+1.06 <sup>a,b</sup>	0,00
Albumin (g/dl)	2.7-5.1	3.17+0.17 <sup>d</sup>	2.27+0.09 <sup>a</sup>	2.32+0.05 <sup>a,b</sup>	2.57+0.12 <sup>b</sup>	2.65+0.12 <sup>c,b</sup>	0.000

The results showed that the kelor leaves powder treatment for dosages 1, 2, and 3 differed significantly, including the intaking groups had a higher level of leucocyte, lymphocyte, segment, hematocrit, Hb and erythrocyte, and thrombocyte than the group with EPM.

### The peritoneum and neutrophile phagocytoticmacrophage ability

The phagocytotic macrophage ability was stated as a phagocyte index, it was measured in 30 and 60 minutes.

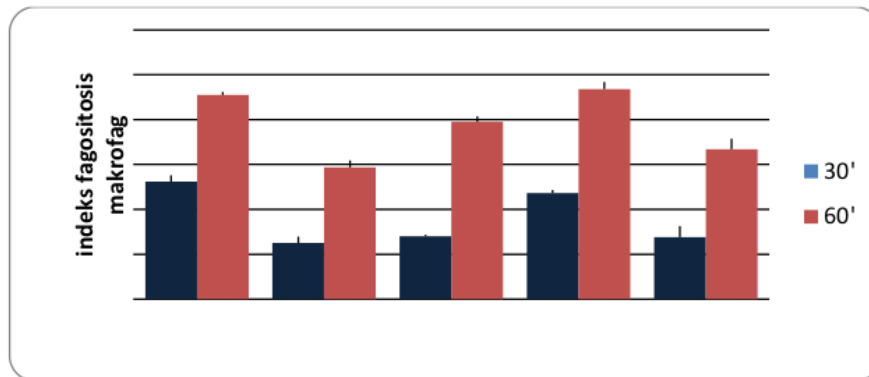


Figure 1. Peritoneal macrophage phagocyte index on each treatment group in 30 and 60 minutes

Note : index of fagositosis makrofag each group ; Normal (30' = 5,23+0,28; 60'=9,1+0,14); KEP (30'=2,51+0,28;60'=5,87+0,3); dosis 1(30'=2,80+0,06;60'=7,89+0,24); dosis 2(30'=4,72+0,13;60'=9,36+0,3); dosis 3(30'=2,76+0,5;60'=6,67+0,47)

Figure 1 shows that there is an increase in the phagocytotic index of all treatment groups in 30 and 60 minutes. The increase also occurred in the EPM group with dosage 2 of kelor treatment. Subsequently, there were significant differences among the normal treatment group ( $p=0.000$ ), EPM ( $p=0.000$ ), EPM+dosage1 ( $p=0.001$ ), dosage 2 ( $p=0.000$ ) and dosage 3 ( $p=0.000$ ) of ( $p<0.05$ ). Therefore, kelor leaves powder increased the phagocytotic macrophage ability as demonstrated by the treatment which had higher results than the EPM and almost equal with the normal group.

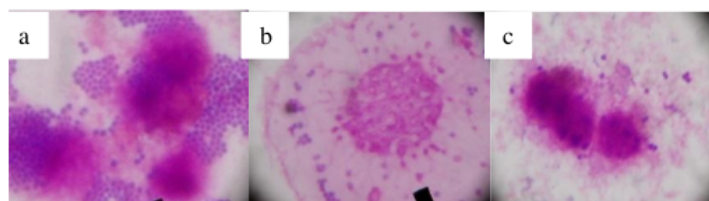


Figure 2. Peritoneum phagocytotic macrophage: a. Normal group, b. EPM Group, c. EPM group+moringa

Figure 2 shows that the ability of the peritoneal phagocytotic macrophage with various moringa leaves powder treatments was higher and differed significantly compared to EPM rats without the treatment. The phagocytotic index of the EPM group appears to decrease ( $2.5 \pm 0.2$ ), while the macrophage phagocytotic ability decreased significantly ( $p=0.000$ ) compared to the normal group (Katayon et al., 2006; Choudhary et al., 2013; Widana et al., 2021).

The *kelor* leaves powder treatment for the EPM rats infected by *S.aureus* tends to activate macrophage which is indicated by the increase in the phagocytotic macrophage ability compared to the normal rats. In this study, the phagocytotic macrophage ability increased significantly ( $p<0.05$ ) for all treatment groups. The highest index occurred on the EPM+dosage 2 ( $0.36 \text{ mg/day}$ ), hence, the treatment from  $0.16 \text{ mg/day}$  to  $0.36 \text{ mg/day}$  significantly increases the phagocytotic index.

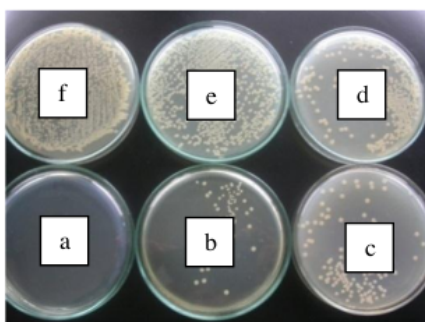


Figure 3. The growth of *S. aureus* bacteria colony. Figures a to f show obvious differences. Figures a&b (Normal group in 30 and 60 minutes), c&d (EPM group with *kelor*), e&f (EPM group)

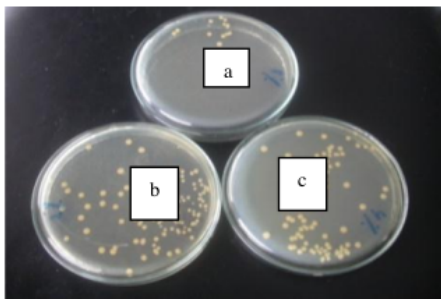


Figure 4. The growth of *S.aureus* bacteria colony. Figure a (rat group with EPM with dosage 3) Figure b (rat group with EPM with dosage 2) and Figure c (rat group with EPM with dosage 1)

These results show that the neutrophil phagocytotic ability of rats treated with dosage 1 of moringa leaves powder was higher and differed significantly from others with dosage 2 and dosage 3.

## Discussion

### Sample hematology description

The result shows that in the EPM+dosages 1, 2 and 3 of moringa leaves, the hematology description increased and approached the normal group. The increase on every testing parameter differed significantly with the one-way ANOVA statistical test result showing a p-value of  $0.000 < p=0.05$ ). This result proves that the moringa leaves powder treatment increased the sample hematology description in the EPM condition. Meanwhile, the nutrition composition of *kelor* leaves powder of NTB has been studied and showed that the protein value corresponds to the variety from African countries (Luthfiyah, 2012). The other essential nutrition substances are vitamins A, C, and betacarotene. According to Sidharaju and Becker (Amaglo et al., 2006), in India, *kelor* has been used as a herbal plant (Indian herbs) for decades and its bioactive substance contents along with its functions have been analyzed. The hematology description, such as Hb level, Leucocyte, Monocyte, Segment, and Lymphocyte, are directly associated with the Innate Immune Response of the body (Fahey, 2005).

The decrease in dosages 2 and 3 is not as sharp compared to the EPM group due to the low protein diet which decreased the red blood cell ability to form hemoglobin, hence, the Hb level decreased sharply. The 0.18 g/day treatment for 30 days tends to increase the protein intake in the EPM rat's body as demonstrated by the increase in the Hb level which corresponds to the normal condition.

When the hemoglobin level is less than 14g/dl and the blood erythrocyte is less than 41% in men, this leads to anemia. Similarly, women with a hemoglobin level less than 12 g/dl and blood erythrocytes less than 37%, are at risk of having anemia. In the rats used for this study, the standard hemoglobin level ranges between 11.1-18 g/dl. The EPM group became anemic because the hemoglobin rate was below the standard. In the EPM+moringa treatments, all groups have Hb level in the normal range hence, the anemic condition in EPM was overcome. The low Hb levels in dosage 2 and 3 do not differ significantly compared with the normal condition, indicating that a decrease in Hb does not cause anemia. This result is almost similar to another study which stated that there was an association between hemoglobin level and all-cause mortality in hemodialysis patients, the link with inflammation and malnutrition. Subsequently, this shows that the Hb level increases along with the decrease of infection and malnutrition risks (Ferraz & Monteiro, 2019; Collins et al., 2006; Zarosylo et al., 2021). When these two conditions are overcome, then the Hb level tends to always be at a normal limit.

The result also shows that the leucocyte level increased in the EPM group ( $24.46 \pm 4.6 \times 10^3/\text{mm}^3$ ) and decreased very sharply in the EPM+dosage1 *kelor* group ( $8.34 \pm 3.08 \times 10^3/\text{mm}^3$ ). The level further decreased slightly in the EPM+dosage2 group ( $6.75 \pm 3.71 \times 10^3/\text{mm}^3$ ) and EPM+dosage3 group ( $5.17 \pm 1.18 \times 10^3/\text{mm}^3$ ). The decrease in dosage 1, 2, and 3 approached towards the normal condition and the standard reference basic value. In this condition, the body increases its defense ability endogeneity to actively improve the malnourished



condition. This normally occurs in a relatively short time, depending on the malnutrition level.

This is in line with the previous studies which reported the important role of the leucocyte protector for defense against infection (Luthfiyah, 2012). The biochemical process during the identification and destruction by neutrophil polymorphonuclear is affected by several factors. However, another study suggests that lysosomal enzymes (Fahey, 2005), decrease its contents in the malnourished condition. Moreover, oxidative metabolism change such as stimulation in the absorption of oxygen and hexose monophosphate is reported to have a close relationship with the intracellular bactericidal ability of the phagocyte cells (Henningham et al., 2015), while glucose oxidation through hexose monophosphate shunt increases NADPH production. This is oxidated by bound-particle enzymes, NADPH to form peroxide hydrogen, which increases the leucocyte content during phagocytose (Ciliberto et al., 2005; Hirayama et al., 2018).

The increasing leucocyte in malnutrition along with peroxide hydrogen combined with the lysosomal enzyme, myeloperoxidase, and halide ion form a strong bactericidal system (Sigler et al., 1999). A previous study reported that the mortality of patients with severe malnutrition is usually due to bacterial infection, although the leucocyte content contains digested bacteria by high phagocytose (Sigler et al., 1999). The malnutrition condition causes leucocytes' failure to produce NADPH oxidate and respond to phagocytose. This is indicated by the decrease in the amount of bacteria particles caught and swallowed during dosage3 phagocytose after the macrophage was infected by *S.aureus*. The failure of normal NADPH oxidate stimulation during phagocytose is indicated by the small hydrogen production. This is further supported by the decrease in the release of phosphate acid from the lysosome into the supernatant fraction during phagocytose, leading to a reduction in bactericidal potential.

The decrease of NADPH oxidate in the EPM group leucocyte is possibly due to the direct effect of protein deficiency or the increase of cortisol concentration in the circulation which has been found to take place in malnutrition (Dos-Santos et al., 1997). Cortisol hinders the NADPH oxidase of leucocytes (Golden, 1998), and polymorphonuclear (PMN) leucocyte mobility slightly decreases in malnutrition. The PMNs chemotactic migration is suppressed and correlates more with the presence of infection, rather than with malnutrition (Golden, 1998).

When the leucocyte content is less than 3000/mm<sup>3</sup> which is the basic reference standard for rats), it indicates *leucopenia*, while levels higher than 15000/mm<sup>3</sup>, indicate infection. The EPM group demonstrated infected conditions as the leucocyte content was higher than the standard. In EPM+kelor groups, all treatments have leucocyte content in the normal range, hence, the leucocyte content increased towards the standard value. Besides, the kelor leaves powder extract has been shown to have antibacterial properties. The specific components have also been reported 2 anticancer and antibacterial. Although this compound is relatively unique 2 for Moringa family, it is also rich in several vitamins and minerals, such as carotenoids including  $\beta$ -carotene or pro-vitamin A (Upadhyay et al., 2015).

### Lymphocyte level and peritoneum phagocytotic macrophage ability

The lymphocyte measurement shows a significant difference among the normal, EPM, and EPM+kelor groups. This is indicated by the increasing lymphocyte level in the EPM group ( $8.2 + 3.09.10^3/\text{mm}^3$ ) compared with normal ( $6.2+3.82.10^3/\text{mm}^3$ ), while in the EPM+kelor groups, it increased in dosages 1 and 2 ( $7.15+6.23.10^3/\text{mm}^3$  and  $7.6+5.77 10^3/\text{mm}^3$ ), but slightly decreased in 3 ( $6.57+6.84.10^3/\text{mm}^3$ ). Lymphocyte B functions to form antibody and lymphocyte T functions to attack and kill germs.

Although EPM was found as the cause of immunity deficiency, this is not consistent with the study entitled *Flow Cytometry Study of Lymphocyte Subsets in Malnourished and Well-Nourished Children with Bacterial Infections* (Nájera et al., 2004) which evaluated the impacts of infection and malnutrition on the nutrition status of non-bacterial infected good nutrition (WN), bacteria-infected good nutrition WNI), and bacteria-infected malnutrition (MNI) in the children with flow cytometry. The result shows a significant decrease in the lymphocyte levels among the infected malnutrition (MNI) group. Inadequate nutrition and essential nutrient conditions are the cause of the lymphocyte levels decrease, while the phagocytotic ability as the Innate Immune Response is also in line with the adaptive immune response mechanism. Lymphocytes play an important role in increasing the adaptive immune response (10), hence, the increasing lymphocyte level in EPM might be caused by the increase in the activated polymorphonuclear cells' cytokine. Neutrophil produces several cytokines which cause the activation of B cell and T cell lymphocyte. This shows the synergy between the Innate and Adaptive Immune Responses in fighting infection (Lee et al., 2017; Barkema et al., 2009; Wilson, 2019).

### Conclusion

The local kelor leaves powder of NTB increased the complete blood performance including the hematology description of rats with EPM. Also, it significantly affected the increase in the neutrophil cell phagocyte index and its function on the rats with EPM infected by the *Staphylococcus aureus* bacteria. The ability of Moringa leaf powder to increase the immune system might potentially help in fighting other viral infections such as COVID-19. Therefore, there is a need to conduct a more in-depth study to assess the hypothesis of the supportive effect of Moringa leaf in fighting COVID-19.

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