Research Article **Open Access**

Potential of *Pandan anggur* (*Sararanga Sinuosa* Hemsley) as immunomodulator

[1,2] Lefaan Paula Nancy, [1]Murdiati Agnes, [1] Fibri Dwi Larasatie Nur, [3,*]Nurliyani

[1] Department of Food Technology and Agricultural Products, Faculty of Agricultural Technology, Gadjah Mada University, Yogyakarta, Indonesia; [2] Department of Public Health, Faculty of Public Health, Cenderawasih University, Jayapura, Indonesia; [3]Department of Animal Product Technology Gadjah Mada University, Yogyakarta, Indonesia.

***Corresponding Author**: Nurliyani, Prof, [3]Department of Animal Product Technology Gadjah Mada University, Yogyakarta, Indonesia

Submission Date: May 21st, 2024; **Acceptance Date:** June 21st, 2024; **Publication Date:** July 8th, 2024

Please cite this article as: Nancy L. P., Agnes M., Nur F. D. L., Nurliyani. Potential of pandan anggur (sararanga sinuosa hemsley) as immunomodulator. *Functional Foods in Health and Disease* 2024; 14(7): 455-471. DOI: https://doi.org/10.31989/ffhd.v14i7.13768

ABSTRACT

Background: Pandan Anggur plant is an endemic plant of the Papua archipelago in Indonesia, and the fruit called Pandan Anggur Fruit (PAF) is freshly consumed or prepared as a juice. Based on previous studies on the phytochemical content of PAF, the ethanol and water extract contain flavonoids, which act as immunomodulators, to regulate the response of immunity.

Objectives: This study aimed to examine the potential of PAF as an immunomodulator in malnutrition cases, by evaluating six parameters which are phagocytosis capacity and nitric oxide (NO) production of peritoneal macrophage, spleen lymphocyte proliferation, IFN-γ and IL-4 in lymphocyte culture, and intestinal sIgA levels. By proving the potential of PAF as an immunomodulator, this research will lead to significant development of functional food products of Pandan Anggur.

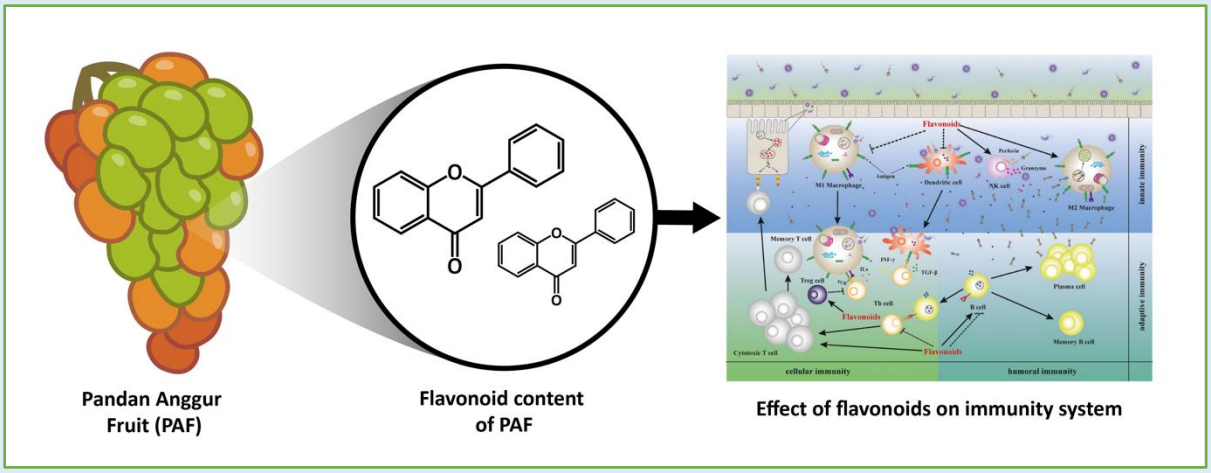
Methods: A total of 35 *Sprague Dawley* rats were used as animal models divided into seven different groups named standard group (healthy standard), malnourished (negative control), malnourished rats given Imboost Force (positive control), as well as PAF ethanol extract doses of I, II, III, and PAF water extract. The treatment lasted for seven weeks in total and the measurement of immunomodulator parameters was carried out at the end of the treatment period. The

immunomodulator parameters included phagocytosis capacity and nitric oxide (NO) production of peritoneal macrophage, spleen lymphocyte proliferation, IFN-γ and IL-4 in lymphocyte culture, and intestinal sIgA levels.

Result: The results indicated that the administration of PAF ethanol extract at a dose of 3.15 mg/mL provided an optimum immunostimulant effect, by increasing the macrophage phagocytosis capacity, nitric oxide, lymphocyte proliferation, IL-4, and sIgA levels in rat intestine. However, increasing the dose of extract did not produce a better effect, but led to suppression of immunity.

Conclusion: PAF extract at a dose of 3.15 mg/mL provides an immunostimulant effect.

Keywords: *In Vivo*, IL-4, IF-γ, sIgA, lymphocyte proliferation, macrophage, Pandan Anggur Fruit



INTRODUCTION

Plants are renowned for several benefits from both the nutritional content and secondary metabolites known as phytochemicals. These secondary metabolites can improve human health, in terms of prevention and cure. In recent years, several food crops and metabolites have been extensively investigated for their benefits and abilities, particularly as immunomodulators. Indonesia as one of the mega biodiversity countries, has a very large opportunity for the development of functional foods, specifically those with great potential as immunomodulators. Currently, Dr. Martirosyan and the FFC define functional foods as natural or processed foods that contain biologically active compounds, which, in defined effective non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers for prevention, management, or treatment of chronic/viral disease or its symptoms [1].

Pandan Anggur is an endemic species in Papua, the easternmost province of Indonesia. This plant is limited to the coast of Papua Island and the Philippines. Taxonomically belonging to the family Pandanaceae, with the genus *Sararanga*. There are only two species of the genus *Sararanga: Sararanga sinuosa* Hemsley, which is only found in Papua island and *Sararanga Philipinensis* native to the Philippines. Pandan Anggur plant has fruit characteristics similar to pandanus-equivalent groups, such as berries. As shown in Figure 1,

Pandan Anggur fruit (PAF) has a small size and shape similar to a kidney, with a small fruit flesh content and many seeds [2]. The group of berries is a flavonoid-rich foods [3- 4].

Numerous studies have demonstrated the benefits of flavonoids on cognitive health in humans [5,6,7]. Notably, a recent observational study found a link between the long-term intake of dietary flavonoids and a lower risk of cognitive decline [5]. A higher dietary intake of dietary antioxidants (e.g., anthocyanins) has been associated with a lower risk of Parkinson's disease [8], and a cohort study associated the long-term intake of dietary flavonoids with a lower risk of Alzheimer's disease and related dementia [9].

Flavonoids are one of the secondary metabolite compounds with great potential as immunomodulators, referring to synthetic or natural compounds that can modulate, suppress, or enhance the work of the immune system both adaptive and innate [10]. Flavonoid content of salicylic acid and resveratrol derivatives in cranberries showed anti-inflammatory activity in test animals [11]. Furthermore, polyphenolic compounds and derivatives are components that function as antioxidants, improving the immune system and increasing the cytotoxic ability of T cells [12]. A study guided by Middleton [13] showed that flavonoids act as an immunomodulator, enhancing the position of the immune procedure at certain doses. However, with excessive doses, these compounds might also act as immunosuppressants. A recent study assessed the effects of flavonoids on the immune system and then their impact on the mTOR pathway. Flavonoids can suppress mTOR activity and are consequently able to induce the T regulatory subset [14].

Figure 1. a. Pandan Anggur fruit (PAF) attached to the fruit stem. b. PAF has the form of a kidney.

The body's immune functions serve as vital defenders against pathogen attacks, playing a pivotal role in maintaining overall health. Yet, factors such as malnutrition, aging, physical and mental stress, or unhealthy lifestyles can disrupt these functions. Consuming foods with immune-modulating properties is recognized as an effective strategy to help sustain immune functions, thereby, mitigating the risk of infections and other illnesses[15].

MATERIALS AND METHODS

Materials: The secretory immunoglobulin A (sIgA) ELISA Kit brand Abbkine, IFN- γ ELISA Kit brand Abbkine, and IL-4 ELISA Kit brand Abbkine were purchased from PT. Kairos Jaya Sejahtera. In addition, the chemicals used during the analysis process including 70% alcohol, RPMI media, latex suspension, methanol, giemsa, solution griess A and B, as well as nitric oxide reagents and aquades, were purchased from the Integrated Research And Testing Laboratory of Gadjah Mada University.

Methods

Animal study design: This *in vivo* study was performed at the Integrated Research and Examination Laboratory Unit 4, Gadjah Mada University in the Special Region of Yogyakarta, Indonesia. The subjects were Sprague Dawley rats obtained from the laboratory and fed with AIN93 and a low-protein diet (40%) (Table.1).

Table 1. Compositions of standard diets (AIN93M) and low protein diets.

Ingredients	Ingredients concentration (g / Kg)	
	Standard*	Low Protein Diet
Cornstarch	620.7	696.7
Casein (85% protein)	140	50
Sucrose	100	100
Corn oil	40	44
Fiber	50	60
AIN-93M Mineral mix	35	35
AIN-93 Vitamin mix	10	10
L. Cystine	1.8	1.8
Choline bitartrate	2.5	2.5

*Resources: Barnard et al., 2003 [16].

A total of 35 rats being 8 weeks old (weighing 150-200 grams) each were split into seven groups, namely the standard, negative, and positive control, three PAF ethanol extract, and one PAF water extract treatment group (Table 2). Each group consisted of 5 rats. In the early stages of the study, all rats were conditioned to be Malnourished, except those in the healthy standard group. The state of malnutrition was created by low-protein feeding for three weeks, then the conditions were confirmed with weight loss and blood serum albumin levels.

Table 2. The design of animal test groups.

Groups	Treatment
Standard	Standard diet
Negative Control	Low protein diet
Positive Control	Low protein diet + Imboost Force
Treatment I	Low protein diet+ PAF ethanol extract 1.44 mg/ml
Treatment II	Low protein diet + PAF ethanol extract 3.15 mg/ml
Treatment III	Low protein diet + PAF ethanol extract 6.34 mg/ml
Treatment IV	Low protein diet + PAF water extract 6.34 mg/ml

After confirming malnutrition, all treatment groups received PAF ethanol extract of 1.44 mg/ml, 3.15 mg/ml, and 6.34 mg/ml, while the concentration of water extract given was 6.34 mg/ml. As a positive control, Imboost Force was used. PAF ethanol and water extract, as well as Imboost Force, were administered orally with a gastric syringe once a day for four weeks. The parameters used to measure the immune response were phagocytosis,

peritoneal macrophage, NO, lymphocyte proliferation in the spleen organs, IFN-γ, and IL-4, as well as sIgA levels in the intestines. In vivo experiments were carried out based on ethical approval from the LPPT UGM ethical commission with certificate number 00007/04/LPPT/III/2023. The animal study design can be seen in the following Figure 2.

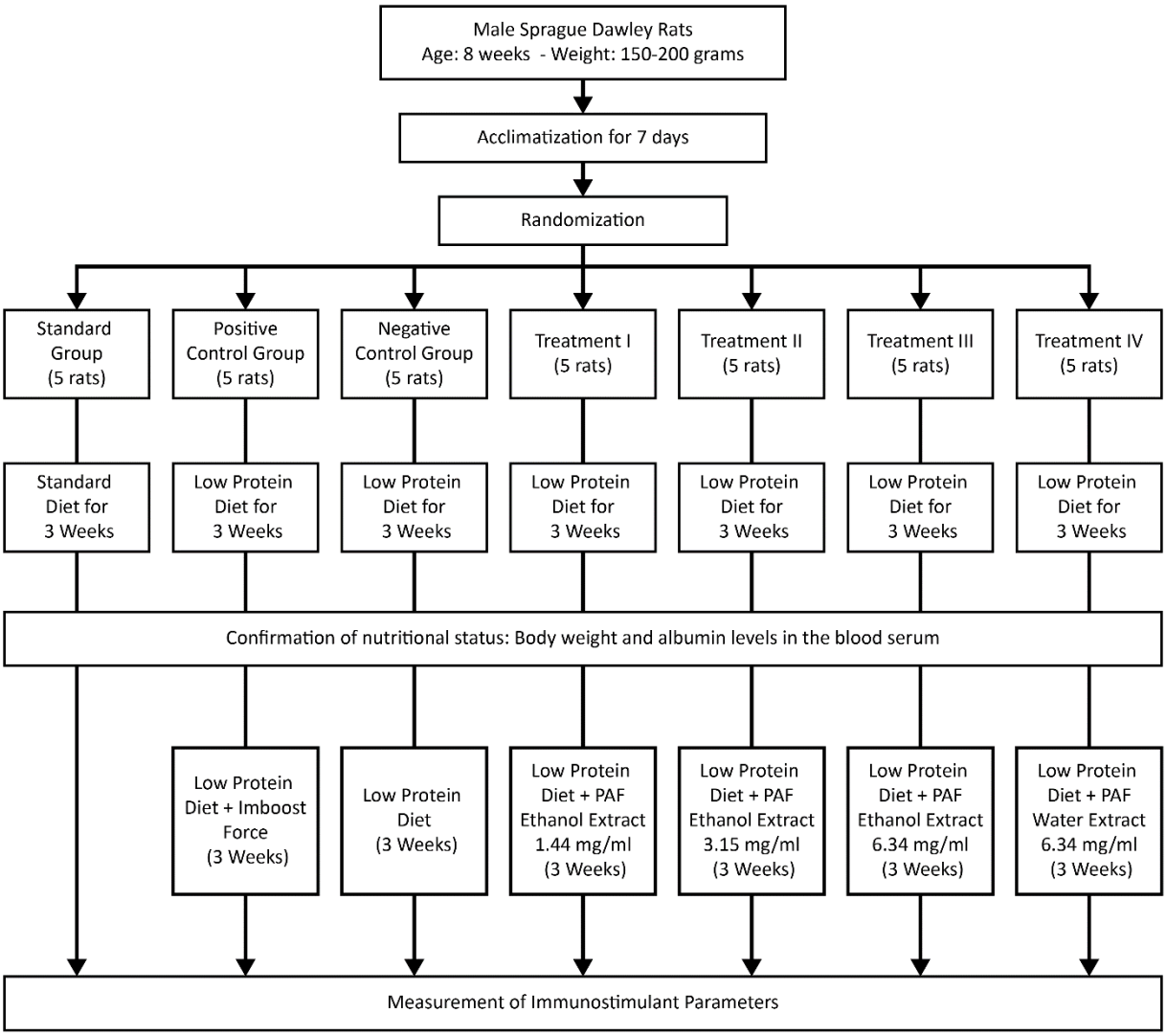


Figure 2. Animal study design.

There are 2 types of extracts used in this study, which are ethanol extract and water extract. Each extract has differences in flavonoid content. Ethanol extract had higher flavonoid content than water extract. The use of water extract in this study refers to the habits of indigenous who consume PAF as juice, so the addition of the treatment was done to confirm if the benefits of immunomodulators can still be obtained in a water base.

Isolation of macrophage: The rats were anesthetized, placed in a supine place, and then the belly skin was opened and spread with 70% ethanol for disinfection. About 10 mL of cold RPMI medium was infiltrated into the peritoneum cavity utilizing a syringe. After a gentle abdominal massage, about 30 mL of peritoneal liquid was extracted using the same syringe and transmitted into 50-mL sterile polypropylene tubes on ice, followed by centrifugation at 1200 rpm for 10 min at 4°C.

Supernatants were extracted and added with 1-2 mL full RPMI medium into the sediment (pellet). Furthermore, a 20 µL aliquot was agitated and added with 930 µL RPMI medium and 50 µL trypan blue (total 1.0 mL). The number of viable cells was measured with a hemocytometer. The counted cells were then resuspended to the attention of 106 cells/mL. Cells suspension (200 µL) were cultured into per well in a sterile microplate 24 wells, and set in 5% CO_2 at 37°C for 24 hours.

Measurement of macrophage phagocytosis capacity: Measurement of phagocytosis capacity was conducted according to Istini et al. [17] with slight transformations. Macrophages that had been set for 2 hours were washed using RPMI media 2x, and added with latex suspension of $2.5x10^7$ cells/mL, reaching 100 µL. The suspension was incubated at 37 °C and 5% carbon dioxide (CO_2) for 60 min. The cells were rinsed with saline buffer phosphate, dried, and fixated using absolute methanol, followed by drying at room temperature. About 20% of Giemsa solution was added and the mix was allowed to stand for 20 minutes. Subsequently, washing was carried out using aquades followed by drying. The number of macrophages and latex was observed and counted using a microscope. The phagocytosis capacity of macrophages was estimated utilizing the formula:

$$\text{Phagocytosis Capacity} = \frac{\text{Number of phagocytic macrophages}}{\text{Counted number of macrophages (100 cells)}} \times 100\%$$

Measurement of nitric oxide (NO) level: About 100 µL of NO reagent was placed in a microplate well and added with 100 µL of supernatant samples that had been incubated for 24 hours. Subsequently, 100 µL solution of Gries A and B was added into each well, and color changes in the microplate reader were observed at a wavelength of 550 nm. A change in color from red to purple shows a high level of NO.

Measurement of lymphocyte proliferation: Rats were sacrificed under anesthesia, placed in the supine position, and then sprayed with alcohol for sterilization. The outer skin of the left side of the abdomen was carefully opened using scissors and tweezers. Lymph was carefully removed, washed 2x using RPMI, and then inserted into a Petri dish. The cell rest was centrifuged at 1200 rpm, 4 ^{O}C for 5 min as well as the pellets obtained were suspended in 5 mL of tris ammonium chloride buffer at room temperature until the color turned yellowish after allowing to stand for 5 minutes. Subsequently, RPMI was counted until the volume advanced 10 mL and the suspension was centrifuged at 1200 rpm, 4 ^{O}C for 5 min. The supernatant was pulled and the pellets were washed using RPMI 2 times. Pellets containing lymphocyte cells were suspended with complete RPMI media and the number of cells was calculated using a hemocytometer, yielding 1.5 x 10 cells/mL. A total of 200 µL containing 1.5 x 106 cells/mL was distributed into the Microplate 96 well. Half of the treatment well per sample was added with 5 µg/mL PHA followed by incubation for 72 hours at a temperature of 37 °C and a carbon dioxide (CO_2) concentration of 5%. Furthermore, 10 µL Thiazoyl blue tetrazolium bromide (MTT) was added to each of the two incubated cell wells. Re-incubation was conducted for four hours at a temperature of 37 °C and a carbon dioxide (CO_2) concentration of 5%. About 50 µL Sodium Dodecyl Sulfate (SDS) (10% SDS in 0.01 N hydrochloric acid solution) was added and observed by incubation overnight at room temperature and dark conditions. The optical density (OD) value was read using a microplate reader at 550 nm wavelength.

Measurement of IL-4 level (Abbclonal rat IL-4 ELISA kit): About 100ul of standard or sample (lymphocyte culture of the spleen organ) was counted per well, incubated for 2 hours at 37°C, and then washed three times. Subsequently, 100 µL working biotin conjugate antibody was added, followed by incubation for 1 hour at 37°C, and washing three times. About 100µL working streptavidin-HRP was added, incubated for 30 mins at 37°C, and then washed three times. Approximately 100µL substrate solution was added and incubated for 15-20 mins at 37°C under dim conditions. Furthermore, a 50µL stop explanation was added and the optical density (OD) was resolved within 5 minutes at a wavelength of 450 nm.

Measurement of IFN-γ level (Abbclonal rat IFN-γ ELISA kit): All reagents were prepared including the working standard and sample (lymphocyte culture of the spleen organ). Excess microplate strips were released from the frame, produced to the foil pouch including the desiccant pack, and resealed properly. Wash buffer 350 µL/well was added and each well was aspirated after holding for 40 secs. The process was duplicated two times, for a total of three washes, then 100 µL standard or sample diluent was added in a blank well. About 100 µL different concentrations of normal or model were added in other wells, and covered with the adhesive strip provided. The suspension was incubated for 2 hours at 37°C followed by washing of the well three times. Subsequently, 100 µL working biotin conjugate antibody was added in each well, covered with new sealer, and incubated for 1 hour at 37°C. The well was washed three times and 100 µL working streptavidin-HRP was added to each, pursued by incubation for 30 mins at 37°C. The aspiration step was repeated, then 100 µL TMB substrate was added and the suspension was incubated for 15-20 mins at 37°C in dark condition. The 50 µL stop solution was added and the OD was determined at wavelength of 450 nm.

Measurement of sIgA level (Abbkine rat sIgA ELISA kit): About 50 µL standard and intestinal fluid diluent were counted to the well and the suspension was incubated for 45 mins at 37°C. Each well was aspirated followed by washing and the process was repeated four times. Subsequently, 50 µL HRP-conjugated detection antibody was added to each well and incubated for 30 mins at 37°C. The aspiration process was repeated five times, then chromogen solution A and B solution 50 µL was added to each well. The combination was gently mixed and incubated for 15 mins at 37°C in dark conditions. About 50 µL visit solution was added and the color in the wells should vary from blue to yellow. The OD was determined at a wavelength of 450 nm within 15 mins.

Statistical analysis: All immunomodulator parameters were subjected to an analysis of variance (ANOVA) using SPSS Version 26 to confirm the significant difference between the group, followed by the Duncan Multiple Region Test (DMRT) to describe means with a 95% confidence level to confirm the significant group that contribute to the parameter. In addition, all the experiments were conducted in triplets (n=3).

RESULTS AND DISCUSSION

Phagocytosis capacity of peritoneal macrophage: Macrophages play a crucial role in the immune reaction, both functioning in phagocytosis and as antigen-presenting cells (APC). To perform both functions, endogenous mediators such as cytokines are needed. However, the use of exogenous mediators such as carotene and flavonoids still requires in-depth investigation [18]. As an immunity parameter, macrophage capacity describes the percentage of macrophages that actively phagocyte latex, compared to the total number of cells counted (Figure 3).

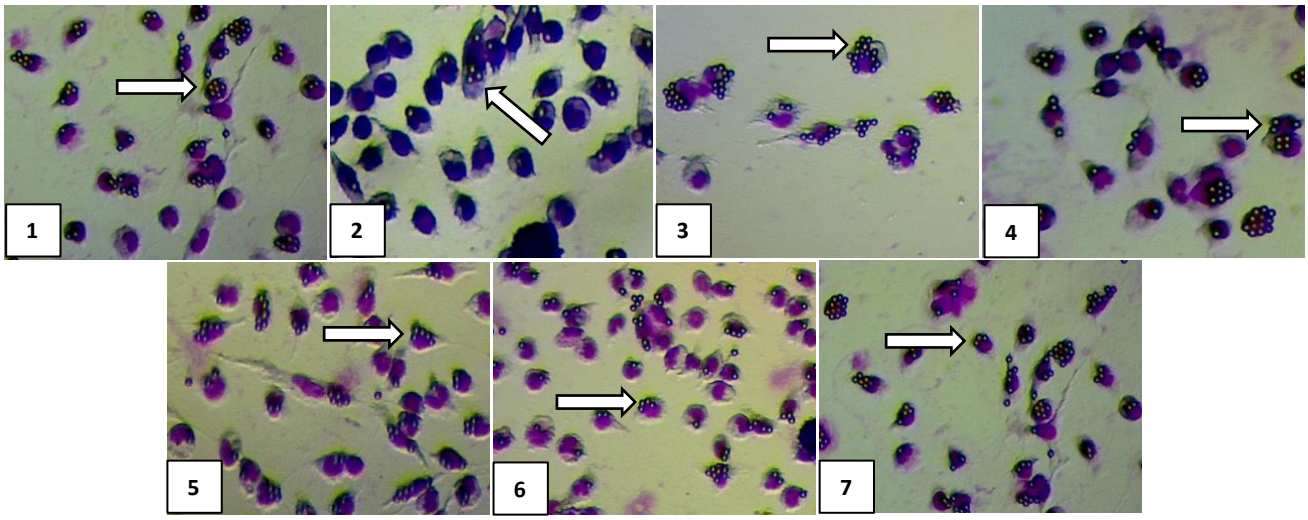


Figure 3. Microscopic image of Macrophage–with giemsa staining of each group. The arrows show active Macrophages phagocytic latex.

Figure 4 showed that malnourished rats given PAF ethanol extract at a dose of 3.15 mg/mL (treatment II) had the highest phagocytosis capacity among other groups, with a value of 85.3%. The value was even higher than in the standard group, where the rats were in healthy and well-nourished conditions. Based on the statistical test results, treatment group II differed significantly from negative control, standard group, and positive control (rats given Imboost Force). This confirmed that the management of PAF ethanol extract at a dose of 3.15 mg/mL could significantly increase the phagocytosis capacity of macrophages. Statistically, the water extract treatment had a similar effect to ethanol.

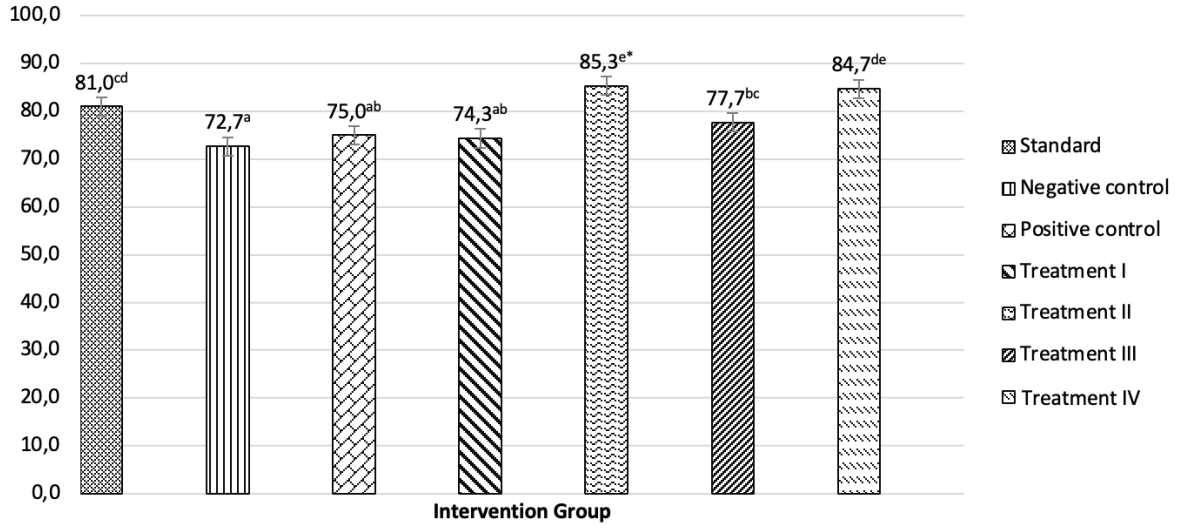


Figure 4. Percentage of rats peritoneal macrophage capacity in each treatment group. Various letters between the bars show important differences (p<0,05).

Table 3 displays the average value of the phagocytosis index of rats' peritoneal macrophage in each group. Rats treated with PAF ethanol extract at a dose of 3.15 mg/mL had the highest phagocytosis index of 2.97 among other groups, including moderately nourished rats with macrophage phagocytosis index of 2.89. Furthermore,

rats given PAF ethanol extract treatment had a higher phagocytosis index than those given an Imboost Force as a patent immunostimulator, in the positive control group. This implied that PAF ethanol extract could stimulate the function of macrophages to phagocyte pathogenic microorganisms or other foreign bodies.

Duncan's statistical test showed that the treatment of PAF ethanol extract at a dose of 3.15 mg/mL had a substantial difference from the group of malnutrition-deficit rats in the negative control group.

Table 3. Phagocytosis Index of Rats Peritoneal Macrophage Per Group.

Group	Phagocytosis Index
Standard	2.89 ± 0.07^{ab}
Negative Control	1.95 ± 0.23^{a}
Positive Control	2.14 ± 0.31^{ab}
Treatment I	2.12 ± 0.37^{ab}
Treatment II	$3,14\pm1,0^{ab}$
Treatment III	2.57 ± 0.92^{ab}
Treatment IV	2.97 ± 0.40^{ab}

Other letters within the column show significantly different ($p<0.05$)

Compounds with PI>1 value, classified as immunostimulants can stimulate or increase endurance, while those with IF<1 values are immunosuppressants capable of suppressing the body's resistance [18]. The highest phagocytosis index of 3.14 was found at an ethanol extract concentration of 3.15 mg/mL. Therefore, it was concluded that PAF ethanol and water extracts could act as immunostimulants.

The statistical test of ANOVA showed a marked difference between the treatment and managing groups. Based on Duncan's follow-up test, the phagocytosis index found in treatment group II differed significantly compared to the negative control group. This implied that giving PAF ethanol extract could increase macrophage phagocytosis index in malnourished rats.

According to previous studies, PAF extract contains flavonoids at 14.3% dry basis. These compounds have anti-tumour, immunostimulant, antioxidant, anti-inflammatory, analgesic, anti-viral, anti-fungal, and anti-bacterial effects. Flavonoids have been established to improve IL-2 and lymphocyte proliferation [19] which affect CD4+ cells, causing the activation of Th1 cells [20]. The activated Th1 cells stimulate SMAF (*Specific Macrophage Activating Factor*), which are multiple molecules. An example is IFN-γ (Interferon-γ) capable of activating macrophages, leading to increased phagocytosis activity [21]. Flavonoids also activate NK cells to promote the exhibition of IFN-γ, the main cytokine of MAC (*Macrophage Activating Cytokine*) which will start macrophages and spur increased phagocytosis activity [22].

Flavonoids in the right dose can act as immunomodulator by increasing the average phagocyte power index of macrophages. However, large doses may act as an immunosuppressant against the average phagocyte power index of macrophages. Flavonoids have IFN-γ-like activity that induces and activates macrophages and T lymphocytes. Activation of macrophages shows the secretion of cytokines (IL-1, IL-6, IL-12, and TNF-α) and stimulation of T cells, which

secretes IFN-γ, resulting in the inhibition of B cells antibody production differentiation. IFN-γ causes the activation of cell-mediated immune responses while suppressing humoral immune responses [23-26].

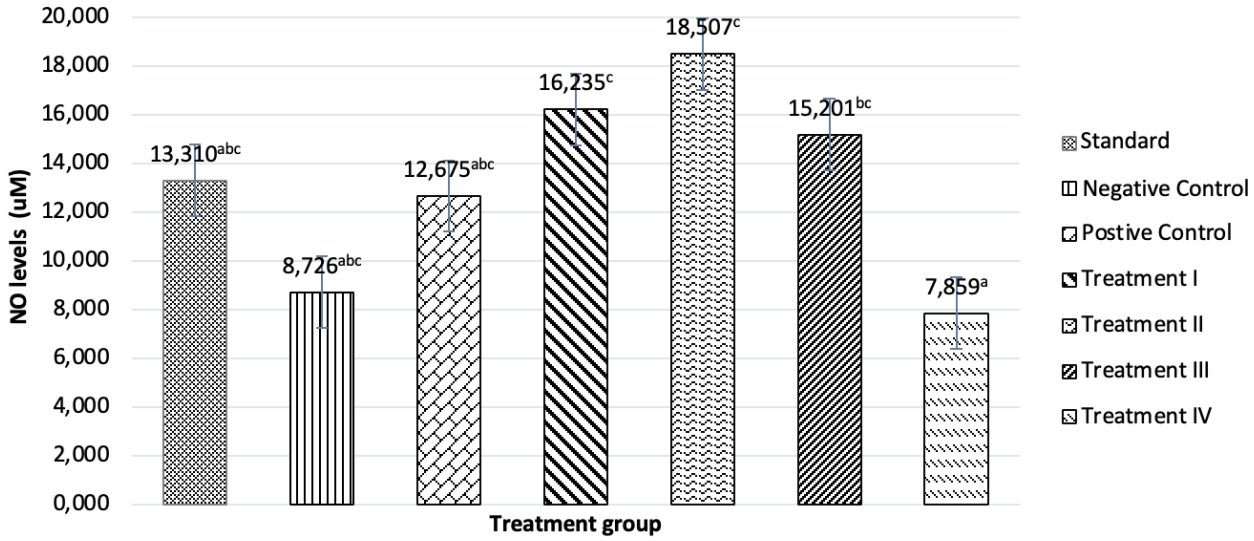


Figure 5. Nitric oxide levels of rats peritoneal macrophage at the end of treatment. Different letters between the bars show a significant difference (p<0,05).

NO levels of peritoneal macrophage: NO levels of peritoneal macrophage in rats given extract intervention for three weeks are shown in Figure 4. Based on the results, healthy and well-nourished rats had nitric oxide levels of 13,310 µM. This value was lower than the levels of NO in malnourished rats given ethanol extract at a dose of 3.15 mg/ml. The highest and lowest level of NO was encountered in the group of malnourished rats treated with PAF water extract, which did not differ significantly from those not given any treatment.

Macrophages can be triggered by LPS (lipopolysaccharides) bacteria, hurt body cells, or IFN-γ (*interferon gamma*). Activated macrophages feed on intracellular bacteria, producing nitric oxide (NO), lysozyme, complement, *hydrogen peroxide elastase* and *collagenase*, IL-1, IL-12, IL-10, as well as prothrombin [27]. When macrophages are activated, the transcription of genes that induce iNOS increases, leading to the production of more NO. Meanwhile, NO acts as an immunoregulator and immunosuppressor [28]. Treatment with PAF ethanol extract exceeding a concentration of 3.15 mg/mL led to lower NO levels.

Lymphocyte proliferation: Figure 6 displays the OD value representing the number of lymphocyte cells, with higher values showing increased lymphocyte cell proliferation. Based on the results, treatment I had the highest OD value, followed by group II, with respective values of 0.45 and 0.43. Both groups had OD values almost the same as that of the standard group, with a difference of 0.04 for I and 0.06 for II. Compared to the negative and positive control, these two groups also had higher OD values. Moreover, increasing the dose of the extract did not affect the OD value produced. PAF water extract treatment with an OD value of 0.38, showed no significant difference from water extract in malnourished rats.

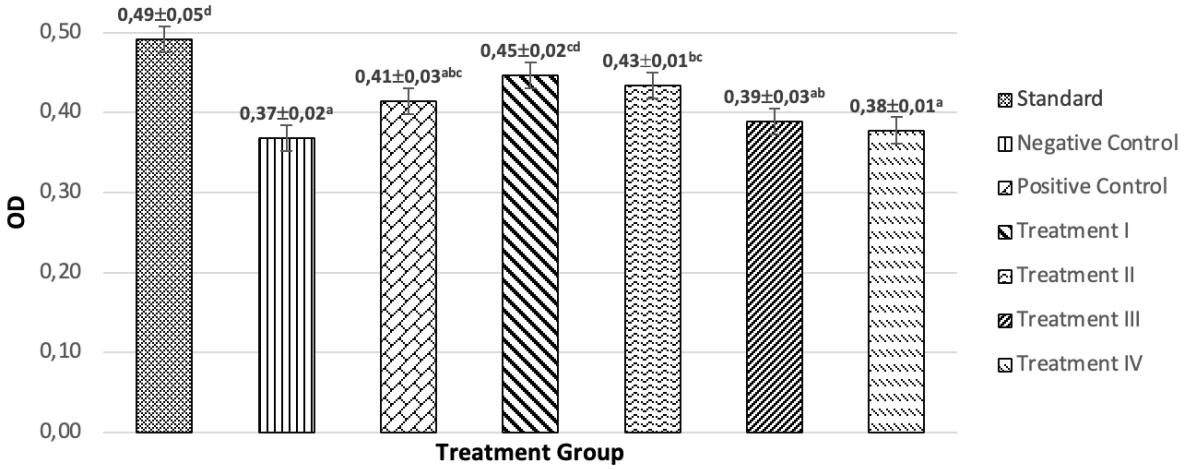


Figure 6. Average OD score per treatment. Different letters between the bars indicate significant differences (p<0,05).

Duncan's statistical test showed a significant difference between the group of rats given PAF ethanol extract with concentrations of 1.44 mg/mL and 3.15 mg/mL, reaching the negative control group. The OD value of rats given both doses of PAF ethanol extract had statistically similar effects as the healthy rats and the positive control.

Protein intake had a significant relationship with lymphocyte numbers showed by p = 0.004 and OR value of 4.09 suggesting that less protein intake posed a 4.09 times higher risk of causing low lymphocyte numbers. Subjects given less protein intake mostly had low lymphocyte numbers with a percentage of 62.07% while those given sufficient intake had a normal value of 71.43% [29].

Lymphocyte proliferation is one indicator of immune response capable of describing immune system functions in maintaining a healthy body. When antigens enter the body, lymphocyte cells react by proliferating to produce antibodies. Based on the results, the administration of ethanol PAF extract had a significant effect on increasing lymphocyte proliferation. PAF ethanol extract contains flavonoids capable of acting as antioxidants and immune stimulants. Antioxidant properties protect lymphocyte cells from oxidative stress to increase proliferation activity. This effect may arise from several mechanisms such as the ability of phenolic compounds to donate electrons, scavenge free radicals or ROS into non-reactive products, and chelate metal, thereby preventing the build of hydroxyl free radicals (OH-) which is highly reactive in damaging cells [30] . The high potency of flavonoids in inhibiting free radicals relates to their ability to transfer a hydrogen atom from a hydroxyl group to the free radical and ultimately stabilize it as follows [31]:

$$FLOH + R^{\circ} \rightarrow FLO^{\circ} + R.H.$$

According to Dykes and Rooney [32], phenolic components of sorghum such as ferulic acid, p-coumarin, and flavonoids bind easily to proteins. Tejasari [33] reported that phenolic combinations bind to lymphocytes through cell receptors, activating the G protein. Consequently, the enzyme phospholipase C is activated, which breaks down phosphatidyl inositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3) on the membrane. IP_3 diffuses from the membrane into the cytosol and binds to receptor proteins on the exterior of the cytoplasmic calcium-sequestering compartment. This binding leads to an

expansion in the concentration of cytosolic $Ca2^{+}$ ions. Diacylglycerol and expanded Ca2+ concentration activate the protein kinase C enzyme, which phosphorylates or transfers phosphate groups to specific serine or threonine residues in membrane proteins thereby activating Na+, and H+ exchange resulting in high pH. The increase in pH signals the cell to carry out proliferative activities. Activation of protein kinase C also promotes the presentation of interleukin-2 (IL-2) which activates the proliferation of B or T cells.

IFN-γ Levels: Interferon gamma (IFN-γ) is a cytokine that recreates a crucial role in the natural and adaptive immune system. It is a significant activator of macrophages and an inducer of MHC II expression, while also acting as an inhibitor of direct viral replication [34]

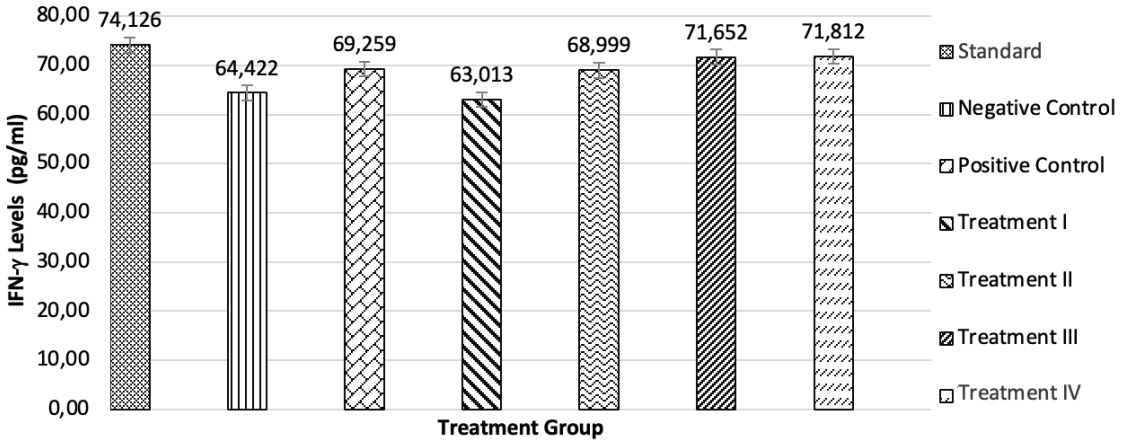


Figure 7. IFN- γ levels at the end of treatment. Different letters between the bars indicate significant differences (p<0,05).

As shown in Figure 7, IFNγ- levels in treatments II, III, and IV increased significantly compared to the negative control. Moreover, the malnourished rat group given ethanol and PAF water extract dose of 6.34 mg/mL produced higher IFN- γ levels than the positive control pack. The statistical tests showed no meaningful difference between the therapy and control groups, suggesting that the treatment did not have a significant effect on IFN-γ levels.

IFN-γ is a crucial element of the immune system, preventing indirect replication of viruses and stimulating certain proteins that destroy infected cells. It is associated with the rule of almost all phases of immune and provocative responses, both *innate and* adaptive including activation and differentiation of T, B, NK, macrophages, and other cells. Therefore, IFN-γ is often referred to as *a distinct immunoregulatory cytokine* [35].

In the case of malnutrition, the immune system is suppressed, characterized by low levels of IFN-γ. This was also illustrated in Figure 5, where IFN-γ levels in the malnourished rat group declined by 10% compared to the healthy ones whose nutritional needs were fulfilled. The administration of PAF ethanol extract effectively increased the percentage of IFN-γ levels in nutrient-deficit rats.

The increase in IFN-γ levels produced by CD4+ T cells following treatment with Moringa leaves was presumably caused by the active ingredients of the extract in the form of flavonoids that acted as Mitogen-Activated Protein Kinase (MAPK), triggering cell proliferation. According to Middleton et al. [13], flavonoids potentially trigger MAPK activity which causes the postpoliration of various protein transcription factors needed in the process of protein synthesis. Flavonoids in Moringa leaf extract increased the secretion of cytokine

IL-2 through the activation of Th cells [36]. Furthermore, IFN-γ plays an influential role both as an immunostimulator and immunoregulator. It differs in biochemistry and biology compared to IFN-α and IFN-β, both of which are made by virus-infected cells. IFN-γ is produced during the resistant response by the presence of specific antigens of T and NK cells collected by IL-2. The various effects include activating macrophages to increase phagocytosis and promote the growth of cytolytic T and NK cells. It also enhances antigen presentation, increases lysosomal activity, activates APC, and stimulates Th1 differentiation by regulation of T factor transcription [37].

IL-4 levels: Interleukin 4 (IL-4) is an anti-inflammatory cytokine derived from the Th2 response, which acts as a suppressor of the Th1 response.

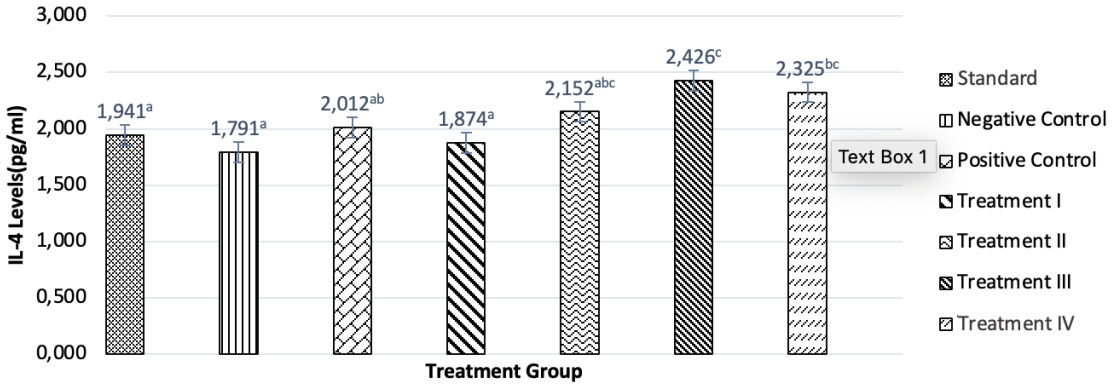


Figure 8. IL-4 levels in rats according to the treatment group at the end of the intervention period. Different letters between the bars indicate significant differences (p<0,05).

Figure 8 shows that the administration of ethanol and PAF water extract had a significant effect on increasing IL-4 level. The highest level of 2.426 pg/mL occurred in group III, namely rats given PAF ethanol section at a dose of 6.34 mg/mL. This value was significantly higher analogized to the positive control group given the Imboost Force patent immunostimulant and healthy rats.

The statistical test results showed that the treatment groups III and IV differed significantly from the standard and the positive control. This confirms that the administration of PAF ethanol and PAF water extract has the same effect at the same dose, significantly increasing IL-4 levels in malnourished rats. The results showed that IL-4 levels in rats treated with PAF extract were higher compared to those not given the treatment. High levels of IL-4 produced by Th-2 activate humoral immunity, which indirectly affects the activity of macrophages to phagocytosis. Increased T cell proliferation facilitates the secretion of IFN-γ (Th1) and IL-4 (Th2). The increase in IL-4 is related to the increase in the number of Th-2 cells due to the proliferation process. When compared with the insignificance of IFN- γ levels, it can be assumed that the proliferation process that occurs is dominated by Th-2 cells which have an impact on increasing IL-4 levels [38].

Secretory IgA levels in rat intestines: Secretory IgA plays a crucial role in facilitating the process of clearing antigens, as well as pathogenic microorganisms, from the intestinal lumen by securing access to epithelial receptors and facilitating disposal through the anus [39].

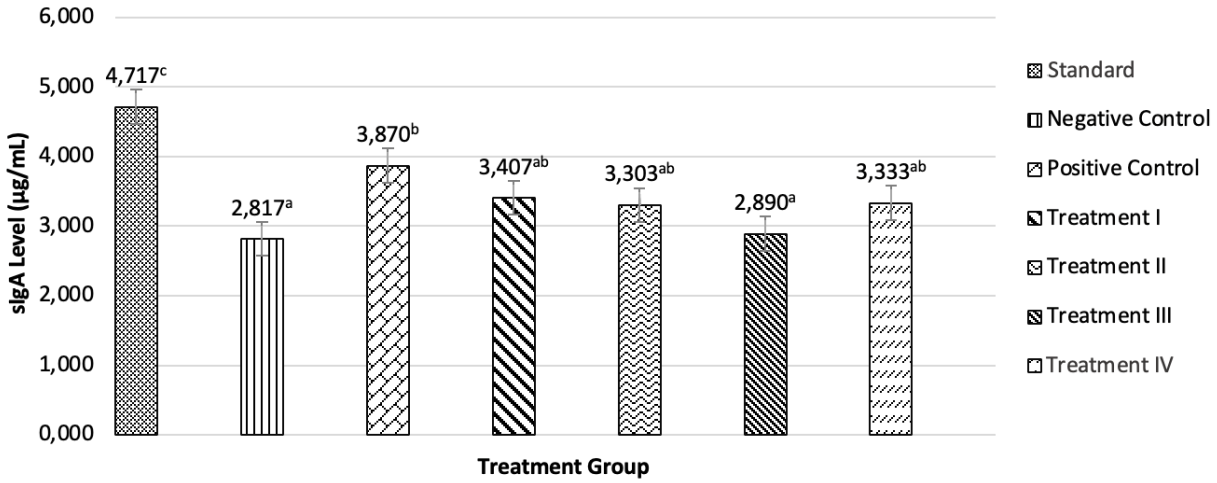


Figure 9. Level of secretory IgA in rats in each treatment group. Different letters between the bars indicate significant differences (p<0,05).

Figure 9 shows that the highest sIgA level of 4.717 µg/mL was found in the nutritionally adequate rat group compared to other treatments. Meanwhile, the group of rats conditioned for malnutrition and without treatment had the lowest level. The sIgA level of positive control became a reference for measurement in the treatment group. Rats given PAF ethanol extract at doses of 3.15 and 6.34 had sIgA levels of 3.407 µg/mL and 3.303 µg/ mL, respectivelly. A similar result was found in those given PAF water extract, with sIgA level of 3.333 µg/mL.

Secretory immunoglobulin A (sIgA), the numerous abundant types of immunoglobulin in the intestinal lumen, has an exhibition of effects that are crucial to mucosal immunity and homeostasis [40]. The secretory element protects sIgA from degradation, with standing proteolytic and digestive enzymes current in the gut lumen. In contrast to different antibodies, such as IgG present in nearly sterile systemic conditions, sIgA resides in the intestinal lumen significantly invaded with microbes. It plays crucial roles in steric hindrance, receptor blockade, or resistant exclusion, resulting in a less inflammatory reaction [41].

The Duncan statistical tests showed that sIgA level in the negative control was significantly different from the healthy and the positive control. Treatment with ethanol extracts of 1.44 mg/mL, 3.15 mg/mL, and water extract, showed no significant difference from positive control. This shows that giving PAF extract can increase sIgA levels in the intestines of malnourished rats. This increase can be influenced by the content of flavonoids as immunomodulatory compounds in PAF extracts given. increased sIgA can be caused by increased levels of IL-4. The secretion of sIga is the result of expression of pIgR. The expression of pIgR is regulated through IL-4 stimulation of the nuclear factor STAT-6, a member of the JAK/STAT signalling cascade [43]. STAT-6, in part, regulates luminal sIgA through regulation of the mucosal transport protein pIgR [42]. The results of the same study were also found by Pierre et al [43], where the addition of proanthocyanins to the diet given can increase the intestinal sIgA levels of rats.

CONCLUSION

In conclusion, the administration of PAF ethanol extract in malnourished rats exerted an immunomodulatory effect by inducing the capacity of macrophage to phagocyte the antigen, as well as increasing the level of NO, lymphocyte proliferation, IL-4, and sIgA in the intestine. The best dose to obtain an optimum immune response stimulation was found to be 3.15 mg/mL.

Administering higher doses might reduce the level of stimulation. The results indicate that the immunomodulator properties of PAF in undernutrition condition make it a promising new raw material for functional product development.

The Novelty of This Work: This research article is the first article which shows the immunomodulator effect of Pandan Anggur fruit in malnutrition case.

Acknowledgements: The authors are grateful to all LPPT UGM members for providing technical support, tools, as well as materials used in this study.

Conflict of Interest: The authors state that there is no conflict of interest.

Authors' Contributions: L.P.N. donated to the experiment, and data analysis, and wrote the manuscript; M.A., F.D.L.N, and N contributed to the methodology, investigation, design of the investigations, and supervised the manuscript.

REFERENCES

1. Leem C, Martirosyan D: The Bioactive compounds of probiotic food/ supplements and their application in managing mental disorders. Bioact Compd in Health Dis 2019, 2(10):206-220. DOI: https://doi.org/10.31989/bchd.v2i10.431
2. Purnamasari V, Nelly L, Simon BW: Morphometric characteristic, nutritional value and antioxidant activity of *Sararanga Sinuosa* Hemsley (Pandanaceae) during ripening: a native berries of Papua, Indonesia. Pak. J Bot 2020, 52:427-433. DOI: http://dx.doi.org/10.30848/PJB2020-2(1)
3. Waheed Janabi AH, Kamboh AA, Saeed M, Xiaoyu L, BiBi J, Majeed F, Naveed M, Mughal MJ, Korejo NA, Kamboh R, Alagawany M, Lv H: Flavonoid-rich foods (FRF): a promising nutraceutical approach against lifespan-shortening diseases. Iran J Basic Med Sci 2020, 23(2):140-153. DOI: https://doi.org/10.22038/IJBMS.2019.35125.8353
4. Liu J, Hefni ME, Cornelia MW: Characterization of flavonoid compounds in common swedish berry species foods 2020, 9(3):358. DOI: https://doi.org/10.3390/foods9030358
5. Yeh TS, Yuan C, Ascherio A, Rosner BA, Willett WC, Blacker D: Long-term dietary flavonoid intake and subjective cognitive decline in US men and women. Neurology 2021, 97:e1041–e1056. DOI: https://doi.org/10.1212/WNL.0000000000012454
6. Micek A, Godos J, Del Rio D, Galvano F, Grosso G: Dietary flavonoids and cardiovascular disease: a comprehensive dose-response meta-analysis. Mol Nutr Food Res 2021, 65(6):e2001019. DOI: https://doi.org/10.1002/mnfr.202001019
7. Fusi F, Trezza A, Tramaglino M, Sgaragli G, Saponara S, Spiga O: The beneficial health effects of flavonoids on the cardiovascular system: focus on K^+ channels. Pharmacol Res 2020, 152:104625. DOI: https://doi.org/10.1016/j.phrs.2019.104625
8. Talebi S, Ghoreishy SM, Jayedi A, Travica N, Mohammadi H: Dietary antioxidants and risk of parkinson's disease: a systematic review and dose-response meta-analysis of observational studies. Adv Nutr 2022, 13(5):1493–1504. DOI: https://doi.org/10.1093/advances/nmac001
9. Shishtar E, Rogers GT, Blumberg JB, Au R, Jacques PF: Long-term dietary flavonoid intake and risk of alzheimer disease and related dementias in the Framingham offspring cohort. Am J Clin Nutr 2020, 112(2):343–353. DOI: https://doi.org/10.1093/ajcn/nqaa079
10. Roy A, Khan A, Ahmad I, Alghamdi S, Rajab BS, Babalghith AO, Alshahrani MY, Islam S, Islam MR: Flavonoids a bioactive compound from medicinal plants and its therapeutic applications. Biomed Res Int 2022, 2022(1):1-9. DOI: https://doi.org/10.1155%2F2022%2F5445291
11. Pappas E, Schaich KM: Phytochemicals of cranberries and cranberry products: characterization, potential health effects, and processing stability. Crit Rev Food Sci Nutr 2009, 49(9):741–781. DOI: https://doi.org/10.1080/10408390802145377
12. Baratawidjaja KGB, Iris R: Basic Immunology 8th edition. Jakarta: Balai Publisher Faculty of Medicine; 2009.
13. Middleton E, Kandaswami C, Theoharides TC: The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000, 52(4):673–751.

DOI : https://pubmed.ncbi.nlm.nih.gov/11121513/

14. Hosseinzade A, Sadeghi O, Biregani AN, Soukhtehzari S, Brandt GS, Esmaillzadeh A: Immunomodulatory effects of flavonoids: possible induction of T CD4+ regulatory cells through suppression of mTOR pathway signaling activity. Front Immunol 2019, 10:51.
DOI: https://doi.org/10.3389/fimmu.2019.00051
15. Han L, Fu Q, Deng C, Luo L, Xiang T, Zhao H: Immunomodul atory potential of flavonoids for the treatment of autoimmune diseases and tumour. Scand J Immunol 2022, 95(1):1-19. DOI: https://doi.org/10.1111/sji.13106
16. Barnard DE, Lewis SM, Teter BB, Thigpen JE: Open- and closed-formula laboratory animal diets and their Importance to research. J Am Assoc Lab Anim Sci 2009, 48(6):709-713.
17. Istini, Dwiyati P, Nurliyani: The 6th International Conference on Sustainable Animal Agriculture for Developing Countries: 16-19 October 2017; Batu City, Indonesia. Edited by Subagyo I: Brawijaya University-East Jawa-Indonesia; 2017:355-359.
18. Kusmadi, Kumala S, Triana EE: Immunomodulatory effects of Chinese ketepeng leaf extract (*Cassia alata* L.) against the phagocytosis activity and capacity of macrophages. Makara Health 2007, 11: 50-53.
19. Kresno SB: Imunologi diagnosis dan prosedur laboratorium. Penerbit Fakultas Kedokteran Universitas Indonesia 2010.
20. Mendes LF, Gaspar VM, Conde TA, Mano JF, Duarte IF: Flavonoid-mediated immunomodulation of human macrophages involves key metabolites and metabolic pathways. Sci Rep 2019 9(1):14906.
DOI: https://doi.org/10.1038/s41598-019-51113-z
21. Wu J, Liu K, Shi X: The anti-inflammatory activity of several flavonoids isolated from *Murraya paniculata* on murine macrophage cell line and gastric epithelial cell (GES-1). Pharm Biol 2016, 54(5):868–881.
DOI: https://doi.org/10.3109/13880209.2015.1089294
22. Susilo J, Resti A: Immunomodulatory effect of ethyl acetate fraction of tempuyung leaves (*Sonchus arvensis* Linn.) to nonspecific immune responses in male mice of the BALB/C strain. Padjajaran University 2014.
23. Gao W, Wu J, Wei J, Pu L, Guo C, Yang J, Luo H: Brazilian green Propolis improves immune function in aged mice. J Clin Biochem Nutr 2014, 55(1):7–10.
DOI: https://doi.org/10.3164/jcbn.13-70
24. Mustafiah SE, Fatmawati D, Yusuf I: Phagocytic power index of peritoneal macrophages after propolis administration in mice (*Mus musculus*). Sains Medika 2011, 3(2):121–128.
DOI: https://doi.org/10.30659/sainsmed.v3i2.394
25. Sforcin JM, Bankova V: Propolis: Is there a potential for the development of new drugs? Journal Ethnopharmacol 2011, 133(2): 253–260.
DOI: https://doi.org/10.1016/j.jep.2010.10.032
26. Tripathi AK, Kohli S: Phytochemical screening and evaluation of antidiabetic activity of *Colocasia esculenta* (L) leaves on stz induced diabetic rats. Adv Pharmacol Toxicol 2013, 14 (2):1-12.
27. Kristian YY, Cahyanur R, Wulandari Y, Sinaga W, Lukito W, Prasetyawaty F, Lestari W: Correlation between branched-chain amino acids intake and total lymphocyte count in head and neck cancer patients: a cross-sectional study. BMC Nutr 2023, 9:1-8.
DOI: https://doi.org/10.1186/s40795-023-00746-5
28. Singh RB, Fedacko J, Fatima G, Magomedova A, Watanabe S, Elkilany G: Why and how the indo-mediterranean diet may be superior to other diets: the role of antioxidants in the diet. Nutrients 2022, 14(4):898.
DOI: https://doi.org/10.3390/nu14040898
29. Hassanpour SH, Doroudi A: Review of the antioxidant potential of flavonoids as a subgroup of polyphenols and partial substitute for synthetic antioxidants. Avicenna J Phytomed 2023, 13(4):354-376.
DOI: https://doi.org/10.22038/AJP.2023.21774
30. Dykes L, Rooney LW: Sorghum and millet phenol and antioxidant. J Cereal Sci 2006, 44(3):236-251.
DOI : https://doi.org/10.1016/j.jcs.2006.06.007
31. Tejasari Dr: Evaluation of ginger (*Zingiber officinale* Roscoe) bioactive compounds in increasing the ratio of T-cell surface molecules of CD3+CD4+:CD3+CD8+ in-vitro. Mal J Nutr 2007, 13(2):161-170.
32. Schoenborn JR, Wilson CB: Regulation of Interferon-γ regulation during innate and adaptive immune responses. Adv Immunol 2007, 9: 41–10.
DOI: https://doi.org/10.1016/S0065-2776(07)96002-2
33. Mertowska P, Smolak K, Mertowski S, Grywalska E: Immunomodulatory role of interferons in viral and bacterial infections. Int J Mol Sci 2023, 24(12):10115.
DOI: https://doi.org/10.3390%2Fijms241210115
34. Sashidhara KV, Rosaiah JN, Tyagi E, Shukla R, Raghubir R, Rajendran SM: Rare ripeptide and urea derivatives from roots of *Moringa oleiferaas* potential anti-inflammatory and

antinociceptiv agents. Eur J Med Chem 2009, 44(1):432-436. DOI: https://doi.org/10.1016/j.ejmech.2007.12.018

35. Dinarello CA: Proinflammatory cytokines. J Chest 2000, 118 (2):503-508. DOI: https://doi.org/10.1378/chest.118.2.503
36. Feghali CA, Wright TM: Cytokines in acute and chronic inflammation. FBL 1997, 2(4): 12–26. DOI: https://doi.org/10.2741/a171
37. Mantis N J, Nicholas R, Blaise C: Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol 2011, 4(6):603-611. DOI: https://doi.org/10.1038%2Fmi.2011.41
38. Phalipon A, Cardona A, Kraehenbuhl JP, Edelman L, Sansonetti PJ, Corthésy B: Secretory component: a new role in secretory IgA-mediated immune exclusion in vivo. Immunity 2002, 17(1):107–115. DOI: https://doi.org/10.1016/s1074-7613(02)00341-2
39. Schindler C, Levy DE, Decker T: JAK-STAT signaling: from interferons to cytokines. J Biol Chem 2007, 282(28):20059-20063. DOI: https://doi.org/10.1074/jbc.R700016200
40. Pierre JF, Heneghan AF, Feliciano RP, Shanmuganayagam D, Krueger CG, Reed JD, Kudsk KA: Cranberry proanthocyanidins improve intestinal sIgA during elemental enteral nutrition. J Parenter Enteral Nutr 2014, 38(1):107–114. DOI: https://doi.org/10.1177/0148607112473654
41. Sano Y, Gomez FE, Kang W, Lan J, Maeshima Y, Hermsen JL, Ueno C, Kudsk KA: Intestinal polymeric immunoglobulin receptor is affected by type and route of nutrition. J Parenter Enteral Nutr 2007, 31(5):351–357. DOI: https://doi.org/10.1177/0148607107031005351