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Effects of The Combination of Seluang Fish (*Rasbora* spp.) and Pasak Bumi (*Eurycoma Longifolia* Jack) on Systemic Inflammation and Neurotransmitter in Stunting Model Rat (*Rattus Novergicus*)

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Abstract

Background: Stunting causes growth and development issues which are characterized by a systemic inflammatory response and neurotransmitter disorder. Pasak Bumi (*Eurycoma longifolia* Jack) and seluang fish are abundant in South Kalimantan, and they are the potential to be adjunctive therapy for stunting management.

Objective: To analyze the effect of Pasak Bumi extract (EPB) and seluang fish administration in overcoming systemic inflammation and neurotransmitter disorders in the stunting rats model.

Methods: We used six groups of stunting rats: positive control (KP) group: placebo and standard diet, P1 group: 70% EPB 15 mg/kg body weight (BW) and standard diet; P2 group: DHA 1 mg/kg BW and standard diet; P3 group: EPB 15 mg/kg BW, DHA 1 mg/kg BW and standard diet; P4 group: seluang fish; P5 group: EPB 15 mg/kg BW and seluang fish for five weeks; We measured levels of IL-6, TNF- α , and serotonin. The data were analyzed by the ANOVA test with a 95% confidence interval.

Results: The levels of TNF- α ($p = <0.001$) and serotonin ($p < 0.001$) were reduced significantly in the treatment groups.

Conclusion: The administration of PBE and seluang fish was able to inhibit systemic inflammation and neurotransmitter dysregulation.

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INTRODUCTION

The first 1,000 days of life are critical for long-term immune system development.^{1,2} This period determines variations in the phenotype and function of the immune system in individuals, which is also associated with various factors such as infection, maternal health, vaccination, environmental pollution, and nutritional status.³ A disharmonizing and destructive interaction of these factors could interfere with immune system regulation. Malnutrition is known to be responsible for the incidence of immunodeficiency in children in low and middle-income countries.⁴ One model of malnutrition is stunting, which is characterized by low stature (height-or length-for-age Z score (LAZ) < -2).

Stunting occurs due to chronic malnutrition or growth failure in the past since early life and is used as a long-term indicator of malnutrition in children. Children with stunting have a high number of symptomatic and asymptomatic pathogens and have a greater risk of mortality than children of their normal age.⁵ Studies show that stunting causes immune system dysregulation characterized by a systemic inflammatory response,^{6,7,8} namely an increase in IL-1, IL-6, and TNF- α accompanied by a decrease in CD4 and CD8 lymphocytes.^{9,10} This was positively related to circulating levels of pathogen-associated molecular patterns (PAMPs) and anti-pathogenic antibodies,¹ but negatively affected linear growth factors resulting in stunted bone growth.^{11,12}

Stunting was also associated with neurotransmitter disorders, especially serotonin. Several previous studies, both on experimental animals

and humans, have shown an increase in serotonin levels in the brain in malnourished conditions.^{13,14,15} The relationship between serotonin and malnutrition is not clearly understood, but recent findings regarding the role of peripheral serotonin in the inflammatory response and the immune system may provide a broader picture. Serotonin is thought to be able to modulate the peripheral immune system by activating the work of T lymphocytes and macrophages through 5-HT receptors.¹⁶ Serotonin imbalance is associated with impulsivity and reduced long-term memory.¹⁷

If neglected, stunting conditions could lead to long-term complications that jeopardize the children's future. Therefore, it encourages research on modified therapy to overcome the negative effects of stunting by utilizing local materials. Pasak Bumi (*Eurycoma longifolia* Jack) is a perennial plant that grows endemic to Indonesia, especially Kalimantan. Pasak Bumi has many active compounds, such as flavonoids, antioxidants, and alkaloids which function as anti-inflammatory agents.¹⁸ Recent studies showed that Pasak Bumi **COULD** reduce the expression of IL6, NFkB, and iNOS. These markers are involved in lipopolysaccharide (LPS)-induced inflammatory activities.¹⁹

Seluang fish (*Rasbora* spp.) is a highly nutritious food from South Kalimantan. It has a protein content of 40% w/w and DHA of 1.04% w/w fatty acids per 100 g of seluang fish. DHA has a role as an anti-inflammatory mediator.^{20,21} This study used an experimental rat model (*Rattus Norvegicus*) with protein deficiency to mimic the stunting condition. This study aimed to analyze the effect of Pasak Bumi (EPB) extract and seluang fish on systemic inflammation and neurotransmitters in stunting rats.

The parameters used to assess systemic inflammation are serum levels of IL-6 and TNF- α ,²² and serum levels of serotonin as a neurotransmitter in the blood.²³

METHODS

This study had ethical approval No.154/KEPK-FK ULM/EC/VI/2022 from the Ethics Committee, Faculty of Medicine, Lambung Mangkurat University.

Materials

We prepared six groups of *Rattus norvegicus* rats. Pasak Bumiroots, seluang fish, pand ure DHA were served as treatments. We served low protein diet with a mixture of AIN-76A, DL-methionine 0.9 g/kg, sucrose 609.1 g/kg, purified rodent diet [Dyets Inc., USA] casein 60 g/kg, vitamin mix #300050 10 g/kg, cornstarch 183 g/kg, corn oil 50 g/kg, cellulose 50 g/kg, mineral mix#200000 35 g/kg, and choline bitartrate 2 g/kg. The standard diet was made consisted of protein 20–22%, fat 5–7%, fiber 3–5%, cinder 5–7%, calcium 9–11%, phosphorus 0.6–0.8%, and energy 2900–3100 kcal. We also provide aquadest, 70% ethanol, and 90and % ethanol. We used a TNF- α ELISA kit, IL-6 ELISA kit, and serotonin ELISA kit for measurements.

Procedures

Preparation of experimental animal model

We gave the breastfeeding rat's mother the low-protein diet for four weeks to induce stunting rat pups until the pups were weaned. After weaning, we continued the administration of a low-protein diet for the next four weeks. We withdrew the 1 mL of blood from the tail vein. The blood was centrifuged, and the serum protein level was measured. The

pups were classified as undernourished if the serum protein level was below 4.7 g/dL.²⁴

Experimental procedures

We used six groups of stunting rats, consisting of five rats.

- Positive control (KP) group: placebo and standard diet;
- P1 group: 70% EPB 15 mg/kg body weight (BW) and standard diet;
- P2 group: DHA 1 mg/kg BW and standard diet;
- P3 group: EPB 15 mg/kg BW, DHA 1 mg/kg BW, and standard diet;
- P4 group: seluang fish;
- P5 group: EPB 15 mg/kg BW and seluang fish for 5 weeks;
- Negative control group (KN): healthy rats given a placebo and standard diet for 5 weeks.

The use of EPB at a dose of 15 mg/kg BW was based on the results of the previous studies, which showed the best effect compared to a dose of 7.5 mg/kg BW.²⁵

Extraction of the rat's blood sample by cardiac puncture

After the intervention period was over, the rats were sacrificed for further surgery on the thorax to collect blood from the heart. Blood sampling for measurement of inflammatory parameters (IL-6 and TNF- α) and neurotransmitter parameters (serotonin) by ELISA method.

Examination of TNF- α and IL-6

The ELISA method refers to the TNF- α rate and the IL-6 ELISA Kit manual (Novateinbio, USA). A total of 100 L of standard solution, blank solution, and sample were dissolved by

dilution and placed in a tube to be incubated for 3 hours at room temperature. Then washed with phosphate-buffered saline (PBS) 4 times. The conjugate was filled in the tube with as much as 200 L. The suspension was washed **FOUR** times, and then 200 L of Subtract solution was added and incubated for 30 minutes at room temperature.

Examination of serotonin

Following the ELISA Kit manual (Novateinbio, USA), a total of 100 L of standard solution, blank solution, and the sample was dissolved by dilution and placed in a tube to be incubated for 3 hours at room temperature. Then washed with PBS 4 times. The conjugate was filled in the tube with as much as 200 L. The suspension was washed four times, and then 200 L of Subtract solution was added and incubated for 30 minutes at room temperature.

Data Analysis

IBM SPSS Statistic⁴ was running to analyze the data result. These data were tested for normality and homogeneity. If the data distribution is normal, it is followed by an analysis of the Anova test with a 95% confidence level and the Tuckey HSD further test. However, if the data distribution is not normal, a non-parametric Kruskal Wallis test is carried out, followed by Mann-Whitney with a 95% confidence degree.

RESULTS

IL-6 Activity

The average level of IL-6 activity are shown in Figure 1. below. From the table, it was found that the malnourished group (KP) had a higher level of IL-6 activity than the normal rat group (KN). Among all groups given the seluang fish

intervention, EPB and DHA showed that the group given EPB 15mg/kg BW (P1) had the lowest level of IL-6 activity.

The Shapiro-Wilk normality test showed that the data were normally distributed ($p > 0.05$) so that further analysis could be carried out using the ANOVA test, which yielded a p-value of 0.639, which means that there was no significant difference in IL-6 activity between each group.

TNF- α Activity

The average level of TNF- α activity, as seen in Figure 2. show a higher level of TNF- α activity in the malnourished rat group (KP) than in the normal rat group (KN) and the treated group except in the P5 group. Among all groups given the Seluang Fish intervention, EPB and DHA showed that the group given EPB 15mg/kg BW (P1) had the lowest level of TNF- α activity.

³ The normality test shows that the data are not normally distributed. It continued with the Kruskal Wallis test and obtained $p = 0.000$, indicating a significant difference in TNF- α activity in each group. The Mann-Whitney post hoc test found that the groups with significant differences were between KN and KP, KN with P5, P1 with KP, and P1 with P5.

Serotonin Activity

The average serotonin activity are shown in Figure 3. Serotonin activity in the KP group was higher when compared to normal rats (KN) and the P1 treatment group. Among all groups given the Ikan Seluang intervention, EPB, and DHA showed that the group given EPB 15mg/kg BW (P1) had the lowest level of serotonin activity.

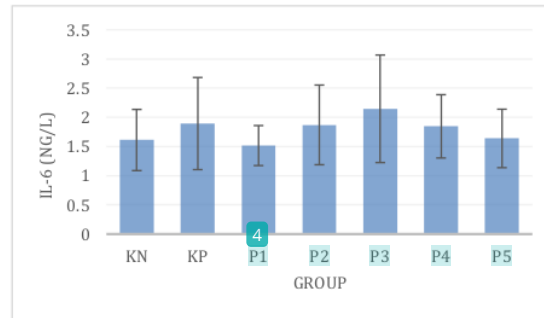


Figure 1. Average ± SD of activity of IL-6 in the serum of malnourished rats after intervention (p=0.639).

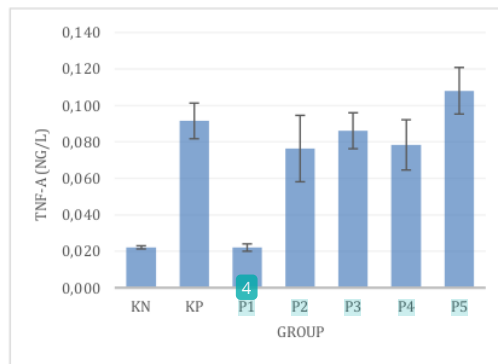


Figure 2. Average ± SD activity of TNF-α in the serum of malnourished rats after intervention (p<0.001).

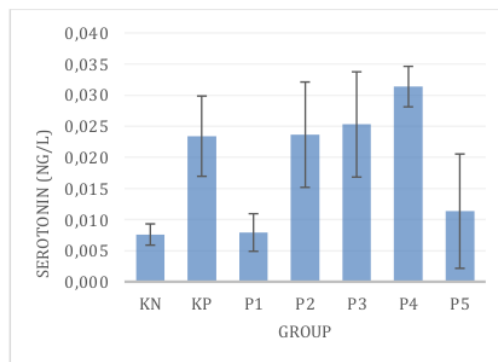


Figure 3. Average ± SD activity serotonin activity in the serum of malnourished rats after intervention (p=0.001).

The normality test results give the results of data that are not normally distributed. Kruskal Wallis test results obtained p = 0.000, indicating a significant difference in the serotonin activity of each group. From the post hoc test, it

was found that the groups with significant differences were between KN and P3, KN and P4, P1 and P4, and P5 and P4.

DISCUSSION

The relationship of inflammation in stunting to bone growth

Inflammation in stunting occurs due to disturbances in the body's immune system by micronutrient deficiency, resulting in increased susceptibility to infection.^{10,26} Malnutrition and infection trap the immune system in a vicious cycle. In the event of a severe or prolonged infection, the immune system is mobilized to protect the body at the expense of high nutritional costs. These costs are due to the effects of pathogens on host tissues and the inflammatory response to infection leading to decreased food intake, malabsorption of nutrients, loss of nutrients, increased metabolism of nutrients, and alterations in nutrient transport and storage.²⁷ Moreover, inadequate nutritional intake in cases of malnutrition causes the body's defense system to weaken, thereby increasing the frequency and severity of infections.²

When the infection is caused by a pathogenic bacteria that infiltrates gastrointestinal mucosa, not being destroyed, the inflammatory response will enter a chronic phase characterized by excessive production of enzymes, chemokines, and proinflammatory cytokines.^{12,28} Cytokines are signaling molecules that mediate intercellular and intracellular communication, in this case, triggering an inflammatory response.^{29,30} The most commonly dysregulated cytokines in inflammatory diseases are Interleukin-6 (IL-6) and Tumor Necrosis Factor-alpha (TNF- α).^{11,31,32} Systemic inflammation is known to be related to the inhibition of the bone growth mechanism, which underlies short stature.

Bone growth is the result of the process of endochondral ossification.¹¹ This process begins with a differentiation cascade initiated by the expansion of the stem cell clone as proliferative chondrocytes, which is followed by cell hypertrophy, cartilage matrix secretion, and apoptosis which releases angiogenic factors that stimulate vascular invasion and migration of osteoblasts and osteoclasts, leading to cartilage remodeling, calcified and trabecular bone formation.¹² This process is tightly regulated by systemic endocrine and local anabolic paracrine/apocrine influences and several micronutrients such as zinc and leptin.^{33,34}

Endocrine components related to anabolic processes in the pre-growth plate include growth hormone/insulin-like growth factor 1 (GH/IGF-1), thyroid hormones, androgens, estrogens, vitamin D, glucocorticoids, and leptin.^{11,12,35} GH produced in the pituitary gland after being stimulated by the GHRH hormone has a direct effect that stimulates the proliferation of chondrocyte cells in the epithelial plate, as well as an indirect effect mediated by IGF-1 in the form of cell hypertrophy. Postnatally, insulin, GH, and IGF may have overlapping functions.³⁶ Possible crosstalk between IGF-1 and insulin at the receptor level and identical post-receptor signaling involving the Ras/mitogen-activated protein kinase pathway signaling cell proliferation and PKB/Akt kinase and the mammalian target pathway of rapamycin complex 1 (mTORC1) which stimulates cell growth.³⁷ Despite the differences in GH receptors, some GH receptors Janus activated kinase-2 (JAK2) initiate signals similar to post-insulin/IGF-1 receptor signaling.³⁸ Due to the GH receptors found on chondrocytes from all growth plate zones, the action of GH is more complex than simply activating the expression

of IGF-1, bone morphogenetic proteins, and other genes in resting zone chondrocytes via the JAK/STAT pathway.³⁹ Similar to GH, IGF-1 also plays a role in stimulating chondrocyte proliferation in the resting zone and subsequent chondrocyte hypertrophy. A distinguishing feature of IGF-1's action is its regulation via IGF-binding proteins (IGFBP) 1-6.¹²

Figure 1 shows that the levels of IL-6 in the control group and the malnutrition group were not significantly different. However, the levels of IL-6 in the malnourished group (KP) were higher than in the normal group (KN). This may indicate that there is an increase in IL-6 levels in malnutrition. In one study, mice over-expressing IL-6 showed an impaired growth phenotype with a reduction in the size of 50-70% compared to their other siblings. This is associated with decreased levels of IGF-1 and IGFBP3 with the normal distribution of GH cells in the pituitary and GH production.¹¹ Several studies have shown that IL-6 acts systemically to inhibit growth by disrupting the balance of activity of the GH/IGF-1 axis, although recent studies have also suggested that IL-6 has a direct inhibitory effect on chondrocyte cells in the growth plate.⁴⁰ The group given EPB 15 mg/kg BW (P1) showed the lowest effect on IL-6 levels among all intervention groups, but it is still not statistically proven that Pasak Bumi affects IL-6 levels.

Meanwhile, Figure 2 shows that the levels of TNF- α were significantly different between the control and malnourished groups. High concentrations of TNF- α , as found in the KP group, can inhibit growth in a dose-dependent manner by decreasing the rate of proliferation and hypertrophy of chondrocyte cells while increasing apoptosis.^{11,12} TNF- α which can be

produced endogenously along the growth plate, inhibits chondrocyte proliferation, especially in combination with IL-1 β .¹²

TNF- α and IL1b have been shown to inhibit IRS1, Akt, and MAPK phosphorylation and induce IGF1 resistance in several different cell types.⁴¹ In addition, TNF- α was found to be able to inhibit steroid hormone production by acting directly on gonadal cells or inhibiting gonadotropin-releasing hormone (GnRH) secretion.⁴² TNF- α also works synergistically with IL-6 to interfere with IGF-1 production on the GH/IGF-1 axis [11]. Giving EPB 15 mg/Kg BW (P1) decreased TNF- α levels significantly, with the lowest level compared to other intervention groups.

There has been no research on the effect of giving Pasak Bumi on systemic inflammation in stunting rats. Several previous studies have shown that the compounds contained in Pasak Bumi have the potential as anti-inflammatory agents.¹⁵ The active compounds include quassinoids, canthin-6-one alkaloids, -carboline alkaloids, tirucallane-type triterpenes, piscidinol A, scopoletin, squalene derivatives, and biphenylneolignans.¹⁸ The content of quassinoid (Eurycomanone and Eurycomalactone) and canthin-6-one alkaloid was able to significantly inhibit nitric oxide (NO) production in LPS-induced RAW 264.7 macrophage cell culture.⁴³ Ruan et al. proved that the content of piscidinol A, 24-epi-piscidinol A, bourjotinolone A, and scopoletin can inhibit the release of inflammation-related proteins, namely inducible nitric oxide synthase (iNOS), IL-6 and nuclear factor kappa-light-chain, -enhancer (NF- κ B).¹⁹ Flavonoid compounds can reduce the expression of proinflammatory cytokines such as IL-

6, IL-8, TNF- α IL-1 β , and monocyte chemoattractant protein-1 (MCP-1) in RAW macrophage cells and inhibit NF- κ B-induced transcriptional pathways.²⁶ Another study also proved that the alkaloid extract of Pasak Bumi roots exhibited anti-inflammatory activity through suppression of proinflammatory mediators NO, iNOS, and COX2 and protected mice from death in an LPS-induced septic shock model.⁴⁴

Neurotransmitter disturbance in stunting

Neurotransmitters such as serotonin (5-HT) are endogenous chemicals that allow neurons to communicate with each other throughout the body. They enable the brain to provide a variety of functions through the process of chemical synaptic transmission.⁴⁵ Disruption of the neurotransmitter serotonin occurs when levels are too low or too high. Low serotonin levels below normal affect learning and memory abilities.¹⁵ Meanwhile, an excessive increase in serotonin is associated with negative regulation of food intake, indicating hyperphagia associated with fetal programming.⁴⁶ High serotonin is also associated with behavioral disorders in the form of increased impulsivity.⁴⁷ The results of the data analysis showed that there were significant differences in serum serotonin levels between the normal group and the malnourished group. Serotonin levels in the malnourished group were higher than normal, and research by Manuel-Apolinar et al. showed that under conditions of malnutrition, there was an increase in serotonin levels.⁴⁶ The mechanism by which serotonin synthesis is increased in malnutrition has not been fully elucidated but may be related to stress in early life which can increase the expression of 5-HT_{2A} receptors.^{48,49} Another mechanism, namely

malnutrition in the pre-, peri-, and post-natal periods can cause an increase in the free fraction of L-Tryptophan (FFT) in plasma which will later be used in the synthesis of serotonin.⁵⁰ The increase in FFT is also followed by an increase in L-tryptophan in the brain which indicates excessive activation of Tryptophan-5-hydroxylases (TPH).⁵¹

Only a small fraction of serotonin circulates in the brain. As much as 95% of the serotonin in the body is synthesized by cells in the intestinal mucosa called enterochromaffin (EC) cells through the amino acid L-tryptophan, which comes from food to be circulated in the bloodstream by platelets.^{16,52} Rossman et al. in Herr et al. showed that peripheral serotonin has an immunomodulatory role on circulating immune cells, as evidenced by the presence of 5-HT receptors on T cells, macrophages, dendritic cells, and mast cells.⁵² The immunomodulatory role of serotonin is known to be immunostimulating and immunosuppressive. This is thought to be because the role of serotonin depends on the location of activated receptors in these subsets of cells.¹⁶ For example, serotonin can function in the innate and adaptive immune systems by stimulating monocytes and lymphocytes to secrete cytokines. In addition, specific activation of the 5HT-2A receptor subtype is superpotent and capable of inhibiting TNF- α -induced inflammation.^{52,53} This may explain the increase in peripheral serotonin levels in the blood that is linear with the systemic inflammatory conditions in stunting. However, it is necessary to further investigate whether the increase in serotonin induces the release of proinflammatory cytokines or the increase in serotonin as a compensatory mechanism to decrease the inflammatory process.

Seluang fish administration to the rat group (P4) showed the highest serotonin levels, even exceeding the control group (KP). This is because omega-3 fatty acids such as DHA and EPA contained in seluang fish are known to increase the release of serotonin and its receptor activity [54]. Serotonin secretion is inhibited by the E2 series of prostaglandins produced from arachidonic acid and omega-6 fatty acids produced from linoleic acid in animals. EPA inhibits the formation of the E2 series of prostaglandins which further inhibits the formation of arachidonic acid in individuals.^{55,56} DHA modulates serotonin receptor function by increasing cell membrane flow.⁵⁴ Serotonin receptors are a type of protein-coupled G receptor that cross cell membranes SEVEN times and are strongly influenced by membrane lipid composition. When flow across the membrane is reduced, serotonin binding to its receptors decreases significantly because serotonin receptors have lower accessibility.⁵⁷

4 The administration of 15 mg/kg BW of EPB (P1) and the combination of 15 mg/kg BW of EPB plus seluang fish (P5), on the other hand, were significantly able to reduce serotonin levels close to the normal group. There are no studies that explain the direct effect of the active compounds in Pasak Bumi that they can reduce serotonin levels. However, with the view that there is a relationship between the work of the immune system and serotonin, it can be suspected that the combination of Pasak Bumi extract and seluang fish reduces systemic inflammation (as evidenced by the decrease in TNF- α levels) so that serotonin levels in plasma also decrease due to its unnecessary role. It is supported by a study that shows the combination of flavonoid compounds and EPA and DHA significantly increases the anti-

inflammatory effect.⁵⁸ EPA and DHA themselves have anti-inflammatory effects through the inhibition of cytokines, prostaglandins, and the transcription factor NF-B.^{20,21} However, the mechanism of how the administration of seluang fish alone is still not able to bring serotonin to normal levels through the pathway of decreasing systemic inflammation still needs to be investigated further.

CONCLUSION

This study proved that the administration of EPB 15 mg/kg BW was able to inhibit systemic inflammation in stunting rats by reducing serum levels of TNF- α . Meanwhile, the combination of 15 mg/kg BW of EPB and seluang fish can overcome neurotransmitter disorders by regulating serotonin levels in a stunting rat model.

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