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The effect of ethanol extract of pasak bumi (*Eurycoma longifolia* Jack.) on neurogenesis and neuroinflammation of rat post protein malnutrition

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Abstract. Protein malnutrition may affect changes in morphology, neurochemistry, neurogenesis and immune system in the brain. Pasak bumi is often used as an aphrodisiac which is almost the same as Ginseng. The neurogenesis development can be stimulated by ginseng extract intervention. This study aimed to prove the effect of pasak bumi on neurogenesis and neuroinflammation in post-protein malnutrition rats. Experimental research design, rats were divided into 6 groups: KN=normal rats+standard feed, P1=malnutrition rats+aquadest, P2=malnutrition rats + 70% ethanol extract of pasak bumi (EPB) 7.5 mg/kg BW, P3=malnutrition rats + EPB 15 mg/kg BW, P4=malnutrition rats + EPB 22.5 mg/kg BW, P5=malnutrition rats + EPB 30 mg/kg BW. EPB administration for 5 weeks. Parameters examined were levels of BDNF, IL6, TNFα, and serotonin by ELISA method. Statistical analysis using ANOVA and Kruskal Wallis test with 95% confidence level. The results of the study: the mean BDNF level in the P3 group was significantly highest (p=0.047). However, there was no significant difference between groups in IL6, TNFα, and serotonin. Conclusion: The 70% ethanol extract of pasak bumi did not affect neuroinflammation and brain serotonin levels in post-malnutrition rats, but increased BDNF levels in post-malnourished rats at a dose of 22.5 mg/kg BW.

Keywords: protein malnutrition, neuroinflammation, neurogenesis, Pasak bumi

1. Introduction

Malnutrition is a serious problem in Indonesia. The results of the 2018 Basic Health Research in South Kalimantan reported that the prevalence of malnutrition was 28%, stunting was 40%, and thin and very thin 12%. South Kalimantan is ranked 5th out of 18 provinces that have children under five who are underweight above the national average. Children with malnutrition tend to lose intelligence by 10-15 points [1]. Brain cells need some protein, minerals, vitamins, and essential fatty acids for brain development. Observations on neurogenesis due to protein malnutrition show decreased prenatal neurogenesis in dentate granule cells in the formation hippocampus [2]. A study found that protein restriction in the early period of life may lead to neural progenitors decrease in the hippocampus. This condition may influence a decrease in object recognition [3]. In neurophysiology, changes occur in metabolism, signal distribution [4], and oxidative stress [5]. Protein malnutrition also causes changes in the immune system. In malnutrition, anti-inflammatory biomarkers are decreased and pro-inflammatory biomarkers are increased [6]. Research on changes in the brain's immune system in conditions of malnutrition has not been done much. However, it is suspected that changes in the immune system in the brain will also affect cell development and brain function.

Malnutrition could be solved by providing appropriate and balanced food. South Kalimantan has abundant food resources to solve the malnutrition problem. Some studies showed that seluang fish from South Kalimantan can increase IGF-1, bone growth, protein [7], improve oxidative stress condition in the brain [8], and improve memory [9] in malnourished rats. In addition to the provision of foods that contain high protein and essential fatty acids, several studies prove that the process of neurogenesis can be enhanced by ginseng extract treatment [10]. Triawanti et al proves that pasak bumi (Eurycoma longifolia. Jack) which is widely found in South Kalimantan can reduce brain oxidative stress in protein-deficient rats. [5]. Pasak bumi has several active compounds among others 14,15 beta-dihydroxyklaine-anone; 9-methoxy-canthin-6-one, -carboline-1-propionic acid, and 7-methoxy-β-carboline-1-propionic acid, eurycomaside, canthin-6-one alkaloids, -carboline alkaloids, tirucallane-type triterpenes, squalene derivatives, biphenylneolignans [11]. Some of the content of pasak bumi belonging to flavonoid compounds has the potential as antioxidants and alkaloid compounds as anti-inflammatory. Previous studies have shown that pasak bumi can inhibit the expression of IL6, NFκB and iNOS which are involved in lipopolysaccharide (LPS)-induced inflammatory processes. [12]. The administration of pasak bumi was expected to improve neurogenesis and inflammation due to malnutrition.

This study aimed to determine neuroinflammation characterized by levels of IL-6 and TNFα, neurogenesis characterized by levels of BDNF, and neurotransmitters characterized by levels of serotonin after malnutrition and after70% ethanol extract of pasak bumi root treatment.

2. Material and Method

This study was an experimental study with posttest-only control group design. We conducted a study with approval from the ethics committee of the Faculty of Medicine, Universitas Lambung Mangkurat, Banjarmasin, Indonesia (No. 298/KEPK-FK UNLAM/EC/2019).

2.1. Materials

Pasak bumi root from Martapura South Borneo, low protein feed (AIN-76A with composition casein 60 g/kg, DL-methionine 0.9 g/kg, sucrose 609.1 g/kg, cornstarch 183 g/kg, corn oil 50 g/kg, cellulose 50 g/kg, mineral mix#200000 35 g/kg, vitamin mix #300050 10 g/kg, choline butartrate 2 g/kg), standard feed (BR2 with composition crude protein 19%, crude fat 4%, crude fiber 4,5%, calcium 0.9-1.1%, phosphor 0.7%, coccidiostat and antibiotic), 70% ethanol, 90% ethanol, *Rattus norvegicus* rats, PBS pH 7.4, aquadest, IL-6 ELISA kit (BT Lab Cat. No E0135Ra), TNFα ELISA kit BT Lab Cat. No E0764Ra), BDNF ELISA kit (BT Lab Cat. No E0476Ra), serotonin ELISA kit (BT Lab Cat. No E0866Ra), ELISA reader Human®.

2.2. Preparation of experimental animal malnutrition

From the day rats were born, they were made malnourished by feeding the mother with low protein feed for 4 weeks during breastfeeding. After weaning the 4 weeks old male rat continued with low protein feed (AIN-76A) for 4 weeks. Rats were considered malnourished when plasma protein levels <4 g/dL. Protein content was measured by the Biuret method. A total of 5 rats that had been fed AIN-76 for 4 weeks were sacrificed and their blood was taken and plasma protein levels were measured.

2.3. Preparation 70% ethanol extract pasak bumi roots (EPB)with maceration method

The roots of the pasak bumi are shaved and then dried without being exposed to direct sunlight. Then blend until it becomes a powder. A total of 200 grams of pasak bumi root powder was soaked in 1.5 L of 70% ethanol at room temperature for 5 days and then filtered. Pasak bumi root powder dregs were re-macerated in 500 mL 70% ethanol at room temperature for 2 days and then filtered. The entire filtrate was collected and concentrated with a rotary evaporator at a temperature of 50°C to obtain a thick extract that still contains a small amount of solvent. Further evaporation using an oven at 40°C to obtain a thick extract.

2.4. Examination of TNFα, IL6, serotonin, and BDNF levels

After administration of extract ethanol 70% pasak bumi (EPB) for 5 weeks, rats were sacrificed for brain collection and measurement of levels of TNFα, IL6, serotonin, and BDNF by ELISA method. The ELISA inspection procedure follows the manual provided by the manufacturer.

2.5. Data analysis

The data were analyzed using the Shapiro Wilk normality test, followed by the Anova test if distribution was normal and the Kruskal Wallis test if it was not normally distributed. The level of confidence was 95%.

3. Result

3.1. Neuro-inflammation

After administration of EPB for 5 weeks, the brain TNFα and IL-6 levels were examined to determine the inflammatory process that occurred during the malnutrition and recovery phases. The levels of IL-6 and TNFα are presented in figures 1 and 2.

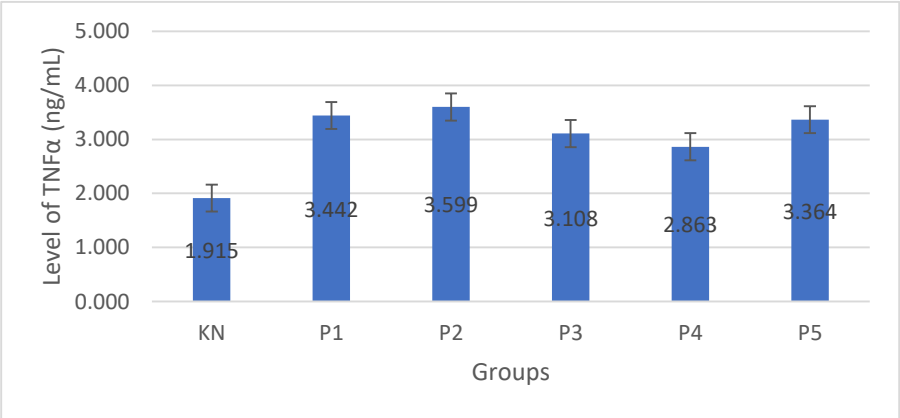

Figure 1. Mean levels of brain TNFα after administration of pasak bumi (*Eurycoma longifolia* Jack.) 70% ethanol extract. (KN= normal + placebo; P1= malnutrition + placebo; P2= malnutrition + EPB 7.5 mg/kgBW; P3 = malnutrition + EPB 15 mg/kgBW; P4= malnutrition + EPB 22.5 mg/kgBW; P5= malnutrition + EPB 30 mg/kgBW; p=0.175. EPB: 70% ethanol extract pasak bumi).

Figure 1 showed TNFα levels in the normal group were lower than in other groups. Meanwhile, in the malnourished group, higher TNFα levels were seen. After the statistical test, the data obtained were normally distributed and then continued with the ANOVA test, the p-value = 0.175. This means that there is no significant difference in TNFα levels in all treatment groups. However, there was a tendency for the average TNFα level to be lower in the normal group than in the treatment group.

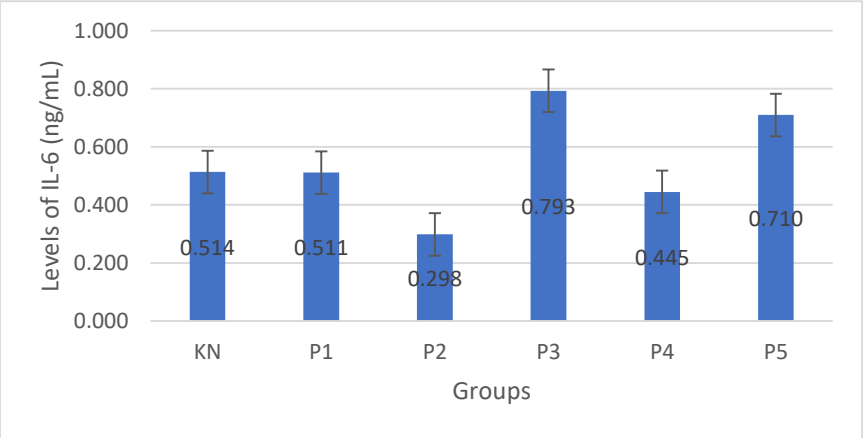

Figure 2. Mean levels of brain IL-6 after administration of pasak bumi (*Eurycoma longifolia* Jack.) 70% ethanol extract. (KN= normal + placebo; P1= malnutrition + placebo; P2= malnutrition + EPB 7.5 mg/kgBW; P3 = malnutrition + EPB 15 mg/kgBW; P4= malnutrition + EPB 22.5 mg/kgBW; P5= malnutrition + EPB 30 mg/kgBW; p=0.071; EPB : 70% ethanol extract pasak bumi).

In this study, apart from TNFα, IL-6 was also examined as a proinflammatory cytokine. Figure 2 showed the levels of IL-6 in the normal control group and the malnourished rats that were given a placebo had almost the same mean levels of IL-6, namely 0.514, while the P3 group had the highest levels. Based on the results of the statistical test, the data was not normal, so it was continued with the Kruskal Wallis test with a 95% confidence level. The results of the Kruskal Wallis test showed that there was no significant difference in all treatment groups (p=0.071).

3.2. Neurogenesis dan Neurotransmitter

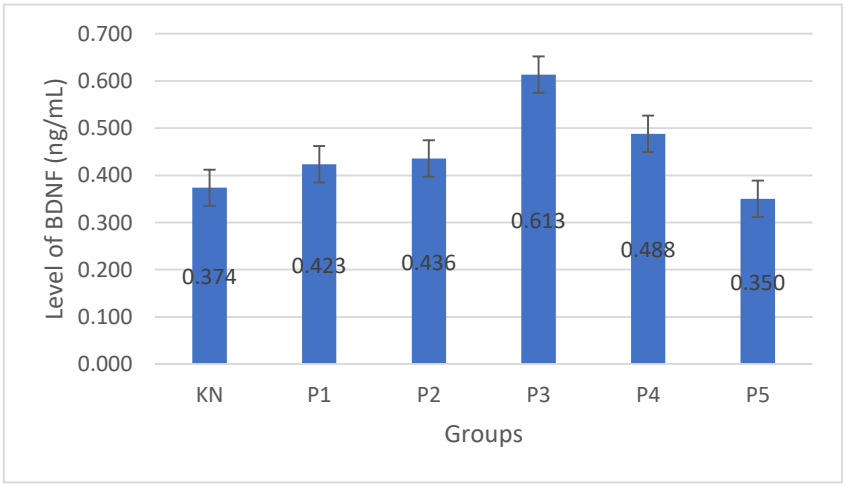

Figure 3. Mean levels of brain BDNF after administration of pasak bumi (*Eurycoma longifolia* Jack.) 70% ethanol extract. (KN= normal + placebo; P1= malnutrition + placebo; P2= malnutrition + EPB 7.5 mg/kgBW; P3 = malnutrition + EPB 15 mg/kgBW; P4= malnutrition + EPB 22.5 mg/kgBW; P5= malnutrition + EPB 30 mg/kgBW; p=0.047; EPB : 70% ethanol extract pasak bumi).

Figure 3 showed the highest mean BDNF levels in the P3 group compared to other groups. The results of statistical tests obtained data that were not normally distributed, so it was continued with the Kruskal Wallis test with a 95% confidence level. Kruskal Wallis test results obtained p-value = 0.047, which means there is a significant difference in the treatment group. Mann Whitney further test showed that there was a significant difference between the normal group (KN) and the P3 treatment (malnutrition

group that was given EPB 15 mg/kg BW). Meanwhile, there were no differences between the other groups.

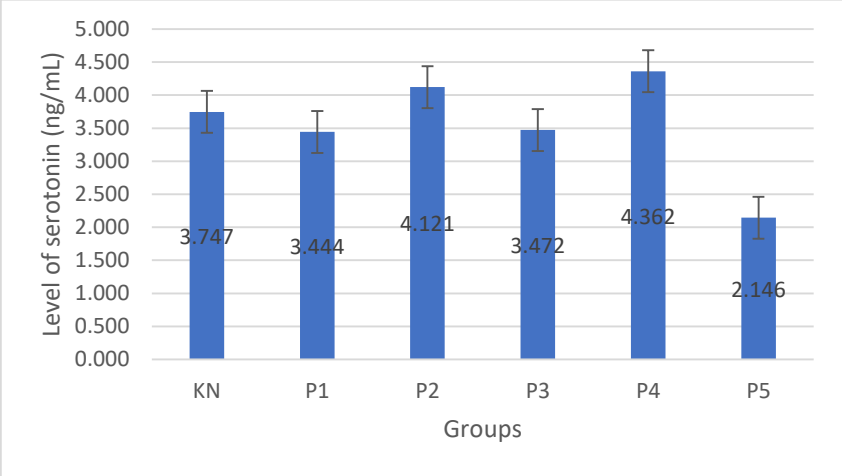

Figure 4. Mean levels of brain serotonin after administration of *Eurycoma longifolia* Jack. 70% ethanol extract. (KN= normal + placebo; P1= malnutrition + placebo; P2= malnutrition + EPB 7.5 mg/kgBW; P3 = malnutrition + EPB 15 mg/kgBW; P4= malnutrition + EPB 22.5 mg/kgBW; P5= malnutrition + EPB 30 mg/kgBW; p=0.329; EPB: 70% ethanol extract pasak bumi).

In this study, the measured neurotransmitter is serotonin levels (figure 4). The results of the normality test showed that the data was not normal, so it was continued with the Kruskal Wallis nonparametric test. Based on the Kruskal Wallis test, p-value = 0.329, which means that there is no significant difference in serotonin levels between groups with a 95% confidence level.

4. Discussion

In this study, measurements of TNFα and IL-6 as pro-inflammatory markers were carried out. Figure 1 showed a tendency to increase TNFα levels in the malnourished group compared to normal controls. The process of developing the immune system occurs in the first 1000 days of life. If a specific nutrient deficiency occurs, it will affect T cell metabolism. Malnutrition also alters mediators of energy homeostasis including glucocorticoid hormones that regulate inflammation and trigger thymus and neurocognitive development, which is disrupted in malnourished children [13]. In malnutrition due to eating disorders, there are changes in inflammatory biomarkers, such as increasing IL-1, IL-6, TNF-alpha and decreasing CD4, CD8, C3, and lymphocytes [6]. In addition, infants who have been malnourished since the time of conception show impaired immune development and thymus atrophy. Compared with mice that were adequately nourished, mice that experienced protein-energy malnutrition during lactation had a smaller thymus and a greater thymic pro-inflammatory response to leptin as a result of higher leptin receptor expression [14]. Protein deficiency increases the inflammatory mediator TNF-α, which is important for activating immune mechanisms. The abnormal increase in its expression can lead to extensive tissue damage caused by leukocytes activating chronic inflammation. In mice fed a low-protein diet, the expression of TNFα and IL-6 in the rat kidney was significantly higher than normal. Increased TNFα and IL-6 can cause cell death [15]. If this happens to brain cells, it will certainly have an impact on decreasing brain function, including intelligence. In malnutrition, there is also an increase in ROS which of course will exacerbate this condition [16].

Although statistically not significant, the group given EPB 22.5 mg/kg BW showed lower TNFα levels than the other malnourished groups given EPB at different doses. However, this still cannot prove that pasak bumi can reduce the inflammatory process during periods of protein malnutrition.

Figure 2 shows that the levels of IL-6 in the control and malnutrition groups were not significantly different, although the malnourished group who was given EPB 7.5 mg/kg BW looked lower than the other groups. Interleukin 6 is generally produced by macrophages in response to specific microbial

molecules. IL-6 can cross the brain barrier. Animal studies have shown that IL-6 in the central nervous system mediates suppression of food intake and body weight through stimulation of the Glucagon Like Peptide-1 (GLP1) receptor [16].

In several studies, it was known that pasak bumi has the potential as an anti-inflammatory. Research by Ruan et al [12] 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-B (NF-κB). The pasak bumi significantly inhibited the expression of IL-6, NF-κB, and iNOS in the lipopolysaccharide (LPS)-induced NF-κB signaling pathway. Meanwhile, the research of Hien et al [17] proved that the alkaloid extract of pasak bumi root showed anti-inflammatory activity through suppression of proinflammatory mediators namely NO, iNOS, and COX2, so protected mice from death in the LPS-induced septic shock model. Research by Emelda [18] also showed the same thing, namely the anti-inflammatory potential of 70% ethanol extract of pasak bumi root in lymphocyte cells taken from mouse spleen through the COX-2 suppression mechanism. However, there have been no studies showing the anti-inflammatory effects of pasak bumi root in malnourished rat brain cells.

In addition to the inflammatory parameters in this study, the process of neurogenesis was also investigated. Protein malnutrition can affect morphological, neurochemical, and neurophysiological changes in hippocampus formation. In the prenatal period, protein malnutrition causes a deficit in the total surface and volume plexus of the mossy fiber layer of the hippocampus [19]. Studies on granule cells in the dentate gyrus and CA3 pyramidal cells of the hippocampus showed that protein malnutrition affected all of these cells, with a dominant effect on the dentate gyrus [20]. Observations on neurogenesis due to protein malnutrition showed decreased prenatal neurogenesis in dentate granule cells in the hippocampus formation [21]. Another study found that protein restriction early in life led to a decrease in neural progenitors in the hippocampus. This leads to decreased recognition of objects as adults. In neurophysiology, changes occur in metabolism and signaling [3].

In the present study the levels of brain-derived neurotrophic factor (BDNF) were examined which is a neurotrophin that is mostly expressed in the CNS both by neurons and by astrocytes. BDNF has an important role in the function of neurogenesis, the formation of neuroplasticity, and the survival of neurons [22, 23]. The results showed that the malnourished group had higher BDNF levels than the normal group (figure 3). The group that was given EPB 15 mg/kgBW showed the highest levels of BDNF significantly compared to the normal group. However, there was no significant difference between the other treatment groups. This shows that the effect of administration of 70% ethanol extract of pasak bumi is not dose dependent, where a high dose of 30 mg/kgBW actually shows the lowest level of BDNF. This is known as the biphasic dose-response effect, i.e. the effect is not directly proportional to the dose but forms a biphasic curve where at higher doses the effect will appear to decrease. It has two phases: the positive and the downfall to negative phase.

The BDNF secreted by neuronal cells will bind to the TrkB receptor causing phosphorylation of Glycogen Synthase Kinase-3β Serine 9 (GSK-3β Ser9) thus triggering Long Term Potentiation (LTP) [24]. This endogenous BDNF will trigger gene expression and protein synthesis that underlie LTP and memory formation, whereas exogenous BDNF, one of which is by astrocytes, will trigger LTP at many synapses of other brain structures [25]. In malnutrition, theoretically, BDNF levels should be lower than normal. However, in this study, it was the opposite. This is thought to be due to the brain tissue mechanism to repair, namely by increasing the secretion of BDNF by astrocyte cells. In the study of Sinha et al. [26], the expression of GFAP in the malnourished group was significantly higher than the normal group. GFAP expression indicates the number of astrocytes expressed. This means that there is an increase in the number of astrocytes and morphological changes in rats experiencing protein malnutrition. In addition to astrogliosis, astrocytes also experience enlargement and thickening of the cell body. In the current study, administration of EPB 15 mg/kgBW caused higher BDNF levels than other groups. It is suspected that EPB 15 mg/kgBW triggers an increase in the number of astrocytes that secrete BDNF as a compensatory mechanism in malnutrition conditions so that BDNF levels become

higher. High levels of BDNF are needed to increase LTP so that memory becomes stronger. How the pasak bumi mechanism triggers an increase in BDNF levels still needs further research.

This study also examined serotonin, a neurotransmitter that modulates various neuropsychological processes and nerve activity and is the target of many psychiatric and neurological drugs. Figure 4 showed that serotonin levels between the normal and malnourished groups who were given a placebo or EPB were not significantly different. This is different from the study of Manuel-Apolimar et al, [27] it was reported that prenatally undernutrition rats showed higher serotonin expression in the hypothalamus than normal rats. Another study conducted by Mokler et al, [28] reported that adult rats with prenatal undernutrition had significantly higher serotonin levels in the right ventral medial prefrontal cortex than normal rats. Serotonin is associated with negative regulation of food intake, indicating hyperphagia related to fetal programming. Previous studies have shown that compounds that are similar to or can increase serotonin activity result in hypophagia and weight loss, and inhibition of neuronal neuropeptide Y (NPY) activity. In contrast, drugs that inhibit serotonin release stimulate eating and NPY [27]. Increased serotonin in prenatal protein malnourished rats was associated with increased impulsivity, especially in the cortico-striatal circuits including the prefrontal cortex. In addition, cognitive rigidity has also been reported in animal models of malnutrition [28].

In this study, measurements of TNFα, Il-6, BDNF, and serotonin levels came from all over the brain, so it could not explain in more detail each part of the brain, such as the hypothalamus, or other parts. In addition, the number and morphology of astrocyte cells were not measured so that they could not explain the mechanism underlying the increase in BDNF levels when given EPB 15 mg/kgBW.

5. Conclusion

Based on the results of the research that has been done, it can be concluded that the 70% ethanol extract of pasak bumi had no effect on neuroinflammation and brain serotonin levels in post-malnutrition rats, but increased BDNF levels in post-malnourished rats at a dose of 15 mg/kg BW. Further research should be conducted to explain the mechanism of pasak bumi to increase BDNF in malnutrition.

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