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The supplementation of pasak bumi (*Eurycoma longifolia* Jack.) in undernourished rats to increase spatial memory through antioxidant mechanism

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SUMMARY

Undernourished interferes with the synthesis of antioxidants enzymes, causing oxidative stress in the brain. They will damage the cellular components of the brain and result in decreased intelligence. Pasak bumi (*Eurycoma longifolia*. Jack) is an endemic plant in Kalimantan that has potential as an antioxidant so it is thought to increase intelligence after undernourished through antioxidant mechanisms. This study aims to prove that the pasak bumi root can improve the spatial memory of *Rattus norvegicus* after undernourished through antioxidant mechanisms. The study design was post test only with control design. Four treatment groups: normal (KN), P1 = undernourished rat + placebo, P2 = undernourished rat + 70% ethanol extract pasak bumi (EPB) 7.5 mg/kgBW, P3 = undernourished rat + EPB 15 mg/kgBW. The brain oxidative stress parameters examined were SOD activity, H_2O_2 level, catalase activity and MDA level. Spatial memory of rats was measured by the Morris Water Maze method. Data analysis used Anova and Kruskal Wallis test with a confidence level of 95%. The results showed that the group given EPB 15 mg/kgBW had higher SOD and catalase activity and lower H_2O_2 and MDA levels than the other groups. Spatial memory of mice given EPB 15 mg/kgBW tended to be better than other groups even though not statistically significantly different. Conclusion: the administration of EPB 15 mg/kgBW in undernourished rats was able to improve the brain

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oxidative stress and tended to improve the spatial memory of rats compared to the EPB dose of 7.5 mg/kgBW.

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1. Introduction

The prevalence of malnutrition of children under five in South Kalimantan in 2018 includes 28% malnutrition, 40% stunting, 12% thin and very thin. This figure is above the national average for malnutrition of 17.7%, stunting 30.8% and thin 10.2% [1]. Children with poor nutritional status and short or very short risk of losing intelligence by 10–15 points [2]. Protein, minerals, vitamins and essential fatty acids are needed in the development of brain cells. The results of studies using omega 3 fatty acid diets in rats can improve learning and spatial memory [3].

Undernourished due to protein deficiency also interferes with the synthesis of enzymes that act as antioxidants, causing deficiency of antioxidants and oxidative stress in the brain [4]. Excessive levels of free radicals will damage cellular components such as proteins, DNA, phospholipid membranes, and enzymes [5]. This has an impact on brain damage resulting in decreased intelligence.

Undernourished is overcome by providing high nutritious food. South Kalimantan has a variety of potential food sources to overcome the problem of undernourished. Previous studies have shown that giving of wild fish from South Kalimantan can increase IGF-1, bone growth, Hb levels, protein [6], overcome oxidative stress in the brain [7], and improve memory [8] in mice that were malnourished.

The results of previous studies prove that the process of neurogenesis can be improved by administering ginseng extract [9]. In South Kalimantan, there are plants with the same potential as ginseng, the pasak bumi (*Eurycoma longifolia* Jack.), both of which are often used as powerful

aphrodisiacs. The active chemical compound contained by the pasak bumi are 14.15 beta-dihydroxyklaine-anone; 9-methoxy-canthin-6-one, β-carboline-1-propionic acid, and 7-methoxy-β-carboline-1-propionoc acid, eurycomaoside, canthin-6-one alkaloids, β-carboline alkaloids, tirucallane-type triterpenes, squalene derivates, biphenylneolignans [10]. Based on this, it should be suspected if the pasak bumi also has the same potential as ginseng in repairing the brain in malnutrition.

This study used experimental animals *Rattus norvegicus* which were made to be protein deficient by feeding low protein (AIN 76A). One parameter that can be measured to describe mouse intelligence is spatial memory. The research aims to prove that the pasak bumi (*E. longifolia* Jack.) root, was able to improve the spatial memory of *R. norvegicus* after undernourished through antioxidant mechanisms.

2. Methods

This study had been received approval from the ethics committee of the Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia (Number 298/KEPK-FK UNLAM/EC/VII/2019). This study was an experimental study with posttest-only with control group design. The *R. norvegicus* as sample were female, 3 months old, and the number were 30 rats. Female Rattus novergicus were mated by the male ones, and then they were separated. The rat pup's that are born are selected male and then included in the group. The groups consisted of 6 rat pup's.

2.1. Material

Pasak bumi (*E. longifolia*. Jack) root, low protein feed (AIN-76A, Purified rodent diet [Dyets Inc, USA] – 6% Casein), standard feed, aquadest, 70% ethanol, 90% ethanol, *R. norvegicus*, $FeCl_2$ 2 mM, o-phenanthroline, H_2O_2 40 mM, PBS pH 7.4, aquadest, HCl 2, 5 M, 2% glucose, NaCl 0.9%, Na_2CO_3, standard MDA, TCA, Thiobarbituric acid, HCl 1 N, acetic acid, $K_2Cr_2O_7$, Morris water maze.

2.2. Procedures

2.2.1. Preparation of experimental animals malnutrition

Rat pup's were made undernourished from the day they were born by feeding the rat's mother that were breastfeeding with low protein feed (AIN-76A) for 4 weeks, after weaning the rat pup's continued with low protein feed (AIN-76A) for 4 weeks. Totally, rat pup's received low-protein feed for 8 weeks (56 days). Composition of AIN-76A were 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 bitartrate 2 g/kg. Rats are considered undernourished when plasma protein levels <4,7 g/dL [6]. *R. norvegicus*'s normal protein level is 4,7–5,2 g/dL [7].

2.2.2. Administration of the ethanol extract 70% Pasak bumi root (EPB)

Once the rats were undernourished, they were divided into 3 groups, namely positive control (P1): undernourished rats + placebo + standard feed; (P2): undernourished rats + 70% ethanol extract pasak bumi root (EPB) 7.5 mg/kg + standard feed; (P3): undernourished rats + 70% ethanol extract pasak bumi root (EPB) 15 mg/kg + standard feed for 5 weeks; plus 1 negative control group (KN) that is healthy rats given placebo and standard feed for 5 weeks. Standard feed consisted of protein 20–22%, fat 5–7%, fiber 3–5%, cinder 5–7%, Calcium 9–11%, Phosphor 0.6–0.8%, energy 2,900–3,100 kcal. Ethanol extract 70% pasak bumi root and placebo was given by oral giving once a day. The body weight measurements of rat pup's were 8 weeks old and after 13 weeks old.

2.2.3. Spatial memory measurement

Spatial memory of the rats was evaluated using the Morris water maze (MWM) task, on days 1–25 after last sonde. The maze involved a circular tank (diameter, 200 cm; height, 60 cm) filled with water (24 °C ± 26 °C) to a depth of 30 cm. A hidden circular black platform (diameter, 10 cm) was submerged 2 cm below the water surface and placed at the same location in the northeast quadrant throughout the training period. In addition, colored posters were pasted on the wall to aid the rats in learning the

platform location. The water pool was divided by two hypothetical lines into four imaginary quadrant zones (North, South, East, and West) of an equal surface area, representing four starting points for the test. All rats were subjected to two trials per day for eighth consecutive days. On day 1–8, each rat was lowered into the pool facing the wall and allowed 90 s to find the platform. If the rat failed to find the platform within this time period, it was gently guided to it and allowed a 30-second rest on the platform before being taken out from the maze. The constant daily sequence of starting points for all test trials was randomly selected until the completion of four starting points per rat for each day and the sequence was not repeated on the next test. On day 9, each rat was subjected to a single 90-second trial without the platform, and the crossing times to the platform zone and the time spent in the target quadrant were recorded [11].

2.3. SOD, peroxide (H_2O_2), catalase and MDA levels assay from brain homogenate

The brain was pounded with mortar at room temperature and added with 1 mL of PBS pH 7.4 until it became liquid. Then taken 5 mL and centrifuged at 8000 rpm for 20 min. The supernatant was then taken for measurement of H_2O_2 [12], SOD [13], katalase [14], and MDA by *thiobarbituric acid* (TBA) method.

2.3.1. Measurement of brain SOD levels

Incubation was performed on 3 mL of a solution containing 0.05 M Na_2CO_3, 0.1 M EDTA pH 10.2. Furthermore, the solution was added 100 μL brain homogenate and 100 μL adrenaline with (3.10^{-4}) BM 189 M. Initial absorption measurements (A_0) was performed with a spectrophotometer at 480 nm wavelength. After that, the sample was incubated for 5 min at 30 °C and got the absorbance (A_1).

2.3.2. Measurement of brain H_2O_2 levels

Measurement of H_2O_2 was using a spectrophotometer. At first, making a standard curve. A total of 20 μmol H_2O_2 was added with 2 mL of dichromate:glacial acetic acid (1:3) mixture. Then the mixture was heated in boiling water for 10 min. Then the cooled mixture was measured for absorbance at a wavelength of 570 nm. The same procedure was done for 40, 60, 80, 100, 120, 140, 160 and 180 μmol H_2O_2. A graph was made between the absorbance on the Y-axis with levels of H_2O_2 on the X-axis to obtain a linear equation.

Preparation of test solution was made with a total of 1 mL of brain homogenate was added 5 mL of PBS pH 7.4. A mixture of 1 mL was taken and added to 2 mL of dichromate:acetate (1:3) mixture and then wrapped in aluminum foil for 30 min. The mixed solution was heated using a water bath for 10 min at 100 °C. The solution was cooled to room temperature. The solution was then transferred into the cuvette and measured its absorbance using UV-VIS at a wavelength of 570 nm.

2.3.3. Measurement of brain MDA levels

From the last procedure, 200 μL supernatant was taken for measurement of MDA levels. The first thing to do was making MDA standard curve. As many as 0.05 μM MDA standard added 1 mL of distilled water, then placed in Eppendorf tube. Thereafter, 100 μL of 100% TCA, 100 μL sodium thiobarbituric 1%, and 250 μL HCl 1 N were added respectively. Then heated at 100 °C for 20 min, and centrifuged 3500 rpm for 10 min. Subsequently, 450 μL supernatant was taken and the distilled water added to 3500 μL. Then read with the spectrophotometer with a maximum wavelength of 540 nm. The same thing was done to 0.025, 0.0125, 0.00625, 0.003125 and 1.56×10^{-5} μM MDA. Then making graphs for the relationship between absorbance on the Y-axis and MDA levels on the X-axis to obtain a linear equation.

2.4. Data analysis

Data were tested for normality and homogeneity. If normal, proceed with the Anova test analysis with a 95% confidence level and a tuckey HSD test. If it is not normal then a Kruskal Wallis non-parametric test is followed by Mann Whitney with a 95% confidence level.

3. Result

3.1. Oxidative stress

After administration of low protein feeding (AIN-76A) for 8 weeks (56 days) post natal it is known that the mean rats protein level was 1.9 g/dL below the normal level which is 4.7 g/dL [7]. The average body weight after 8 weeks of low protein feeding (AIN-76A) were P1 = 118.3 ± 9.83 g; P2 = 74.28 ± 11.33 g; P3 = 76.67 ± 5.16 g, respectively. After 5 weeks of placebo, EPB administration and standard feeding, the rats experienced an increase in body weight. There are P1 = 173.33 ± 44.62 g; P2 = 167.14 ± 45.46 g; P3 = 185 ± 45.37 g, respectively. While in the control group (no undernourished), the mean body weight of 15 weeks post natal was 181.42 ± 19.51 g. Levels of SOD, H_2O_2, catalase, and MDA of rat brain after administration of 70% ethanol extract pasak bumi root (EPB) are presented in Figs. 1–4.

Brain SOD enzyme activity is presented in Fig. 1. In the undernourished group who were given of the EPB 15 mg/kgBW (P3), SOD activity was higher than the P1 and P2 groups. Because the data are not normal then the Kruskal Wallis test is used which shows that there are significant differences between groups (p = 0.000). Further tests using Mann–Whitney showed that there were differences between each treatment group.

Figure 2 shows that peroxide levels in the group of undernourished rats given placebo (P1) were higher than other groups. Kruskal wallis statistical test results showed that there were significant differences in peroxide levels between groups (p = 0.000). Mann–Whitney further tests showed there were differences between each group. This proved that in the condition of undernourished there is an increase in the peroxide level and after giving the EPB was seen decreasing in peroxide. The administration of EPB 15 mg/kgBW dose showed a greater decrease than the 7.5 mg/kgBW dose.

Brain catalase activity in undernourished rats given a placebo appeared to be very low compared to other groups (Fig. 3). EPB can increase catalase activity, at a dose of 15 mg/kgBW the increase was higher than the dose of 7.5 mg/kgBW. Anova test proved there are significant differences between

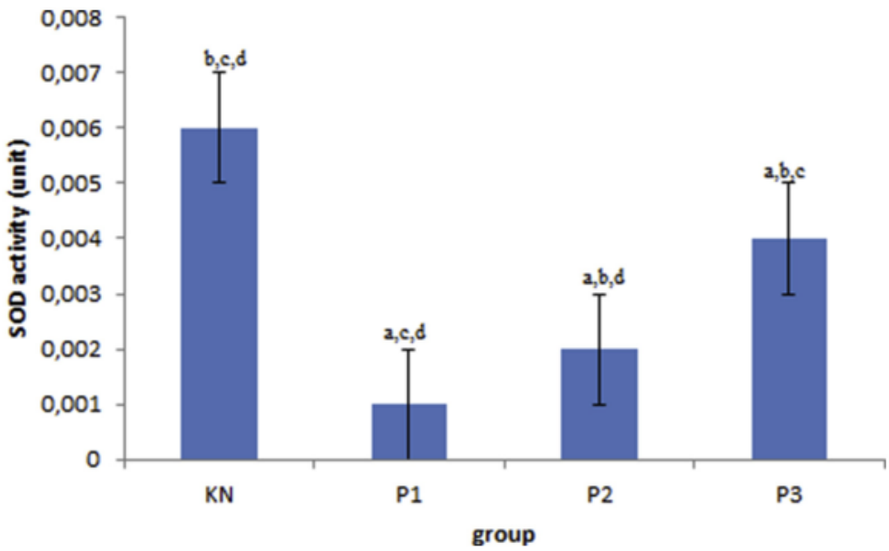

Fig. 1. Superoxide dismutase (SOD) activity of *Rattus norvegicus* brain after administration of *Eurycoma longifolia* Jack. extract ethanol 70%. Note: KN = normal + placebo; P1 = undernourished + placebo; P2 = undernourished + EPB 7,5 mg/kgBW; P3 = undernourished + EPB 15 mg/kg BW; p = 0.000. n = 6, [a]: there are significant differences compared with KN group (p < 0.05); [b]: there are significant differences compared with P1 group (p < 0.05); [c]: there are significant differences compared with P2 group (p < 0.05); [d]: there are significant differences compared with P3 group (p < 0.05).

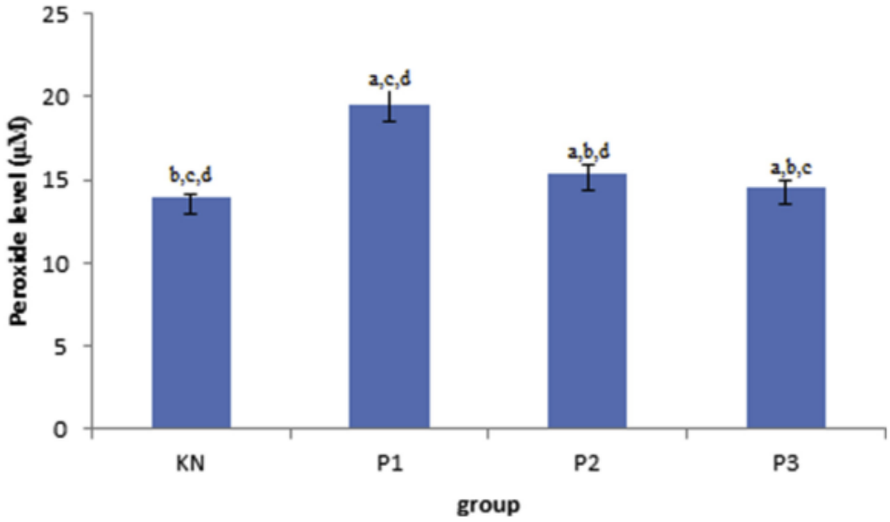

Fig. 2. Peroxide level of *Rattus norvegicus* brain after administration of *Eurycoma longifolia* Jack. extract ethanol 70%. Note: KN = normal + placebo; P1 = undernourished + placebo; P2 = undernourished + EPB 7,5 mg/kgBW; P3 = undernourished + EPB 15 mg/kgBW; p = 0.000. n = 6, [a]: there are significant differences compared with KN group ($p < 0.05$); [b]: there are significant differences compared with P1 group ($p < 0.05$); [c]: there are significant differences compared with P2 group ($p < 0.05$); [d]: there are significant differences compared with P3 group ($p < 0.05$).

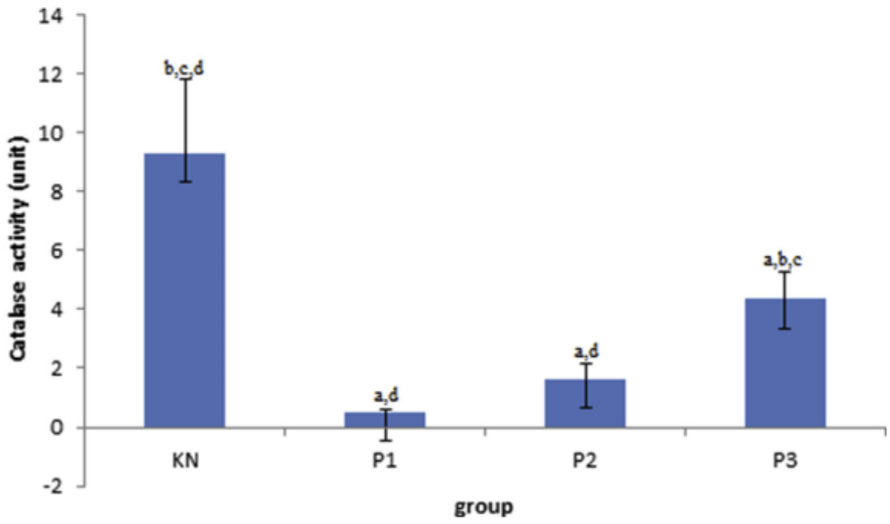

Fig. 3. Catalase activity of *Rattus norvegicus* brain after administration of *Eurycoma longifolia* Jack. extract ethanol 70%. Note: KN = normal + placebo; P1 = undernourished + placebo; P2 = undernourished + EPB 7,5 mg/kgBW; P3 = undernourished + EPB 15 mg/kgBW; p = 0.000. n = 6, [a]: there are significant differences compared with KN group ($p < 0.05$); [b]: there are significant differences compared with P1 group ($p < 0.05$); [c]: there are significant differences compared with P2 group ($p < 0.05$); [d]: there are significant differences compared with P3 group ($p < 0.05$).

groups ($p = 0.000$). The LSD difference test showed that there were differences between KN and P1, P2 and P3, P1 with P3 and P2 with P3, while there were no differences between P1 and P2 ($p = 0.169$).

Malondialdehyde (MDA) is a product of the lipid peroxidation reaction. Figure 4 shows that MDA levels in the undernourished group given placebo (P1) were higher than in the normal group. Administration of EPB 7.5 mg/kgBW can significantly reduce MDA levels. At a dose of 15 mg/kgBW MDA

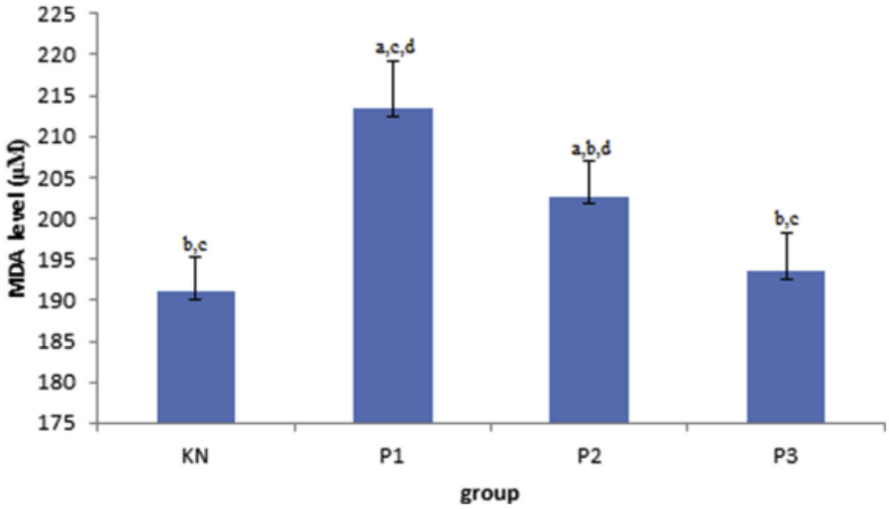

Fig. 4. MDA level of *Rattus norvegicus* brain after administration of *Eurycoma longifolia* Jack. extract ethanol 70%. Note: KN = normal + placebo; P1 = undernourished + placebo; P2 = undernourished + EPB 7,5 mg/kgBW; P3 = undernourished + EPB 15 mg/kgBW; p = 0.000. n = 6, [a]: there are significant differences compared with KN group ($p < 0.05$); [b]: there are significant differences compared with P1 group ($p < 0.05$); [c]: there are significant differences compared with P2 group ($p < 0.05$); [d]: there are significant differences compared with P3 group ($p < 0.05$).

levels appear to be lower than a dose of 7.5 mg/kgBW. The Kruskal wallis test proved a significant difference between the groups ($p = 0.000$). Mann–Whitney different test showed that there were differences between the KN and P1 and P2 groups, the P1 and P2 and P3 groups, the P2 and P3 groups, whereas in the KN group there was no difference with P3 ($p = 0.557$). This means that MDA levels in undernourished rats given EPB at a dose of 15 mg/kgBW were almost the same as MDA levels in normal rats.

3.2. Spatial memory

Spatial memory measurements were carried out using the Morris Water Maze method, the results was presented in Fig. 5.

The results of the test probes in Fig. 5 show that the length of time in the D quadrant spent by the undernourished group given EPB 15 mg/kgBW is a maximum of 37.2 s compared to other groups. The longer the rat in the quadrant, the better its spatial memory. This shows that the spatial memory of rats in this group has a better tendency than other groups, although the ANOVA statistical test showed no significant difference between the treatment groups ($p = 0.524$).

4. Discussion

Protein undernourished gets special attention because protein is one of the important substances that contain amino acids to synthesize structural and functional proteins (enzymes, neuropeptides, and neurotransmitters). Protein deficiency in early life can reduce enzyme activity, resulting in the disruption and synthesis of protein structures. This results in the incorporation of lipids with cell membranes including disrupted neuron cells. An imperfectly formed neuron membrane can disrupt neuronal circuits and cause a decrease in the quality of learning [15,16].

The central nervous system is very vulnerable to damage due to oxidative stress due to its high metabolic activity, the brain requires large amounts of molecular oxygen, which is then followed by the formation of high levels of free radicals. The brain also contains polyunsaturated fatty acids that are very easily oxidized [17]. In addition, the total antioxidant capacity of the central nervous system is

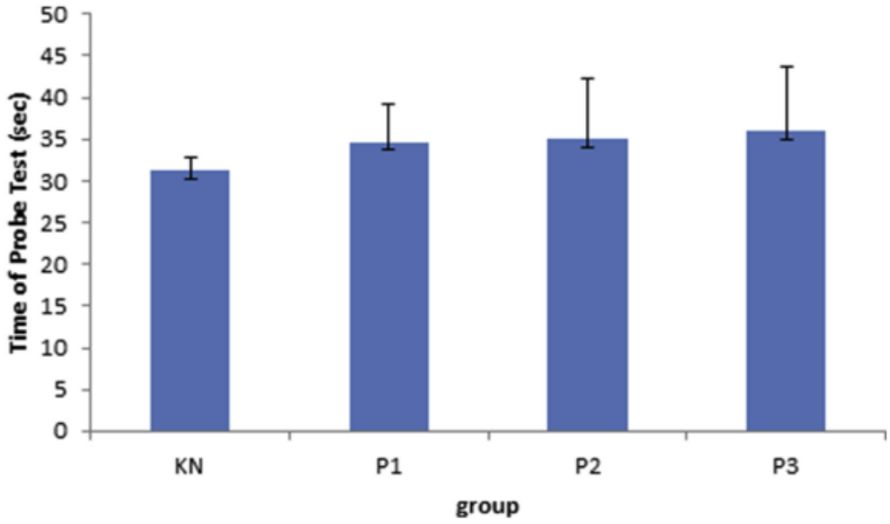

Fig. 5. Spatial memory of *Rattus norvegicus* brain after administration of *Eurycoma longifolia* Jack. extract ethanol 70%. Note: KN = normal + placebo; P1 = undernourished + placebo; P2 = undernourished + EPB 7,5 mg/kgBW; P3 = undernourished + EPB 15 mg/kgBW; p = 0.524. n = 6.

relatively small [5]. Increased oxidative stress may also be the result of adverse effects calorie deficiency and micronutrient intake [18].

In this study it was proven that the *R. norvegicus* undernourished and only given a placebo showed an increase in brain oxidative stress where the enzymatic antioxidant activity was lower than that of oxidants. SOD and catalase activity was lower than in the normal group (Figs. 1 and 3), while peroxide and MDA levels were higher than in normal rats (Figs. 2 and 4). Under normal circumstances, the brain is protected from damage by free radicals by a balance between peroxidants and antioxidant mechanisms, including antioxidant enzymes and chemical free radical scavengers. This balance appears to be impaired in protein energy malnutrition (MEP). Many clinical and pathological manifestations of MEP are thought to result from an imbalance between free radical defense and free radical production. Pathological features in protein deficiency include shrinking brain size and increased lipid peroxidation in the brain [19].

SOD is an antioxidant that is widely available in the brain and plays an important role in preventing brain damage due to oxidative damage [20]. In protein malnutrition there is a decrease in SOD levels [21]. The findings in this study indicate that the 70% ethanol extract pasak bumi (*E. longifolia* Jack.) root increasing SOD levels and reducing MDA levels in rats brain. This is thought to be the presence of bioactives contained in the pasak bumi, including phenols, alkaloids, flavonoids and glycosids [22].

Protein restriction early in life causes a decrease in neural progenitors in the hippocampus. This results in decreased recognition of objects as adults [23]. Decreased recognition of this object will cause a decrease in spatial memory. In the study of Wang and Xu [24], it was found that mice with protein malnutrition since the womb had lighter brain weight compared to controls, the total protein level in the hippocampus is significantly lower, the hippocampal BDNF content was lower, the MWM test showed learning ability and memory is interrupted too. These results indicate that protein malnutrition affects spatial navigation of experimental animals, which is caused by low BDNF concentrations in the hippocampus.

In protein malnutrition, there is also a decrease in volume in the dentinal gyrus and also the CA1 hippocampus. The decrease occurred up to 66%. The presence of lesions in the gyrus dentatus and CA1 hippocampus triggers a decline in task acquisition with short-term memory delays and impaired working memory performance. Working memory is the ability to store and manipulate mnemonic information to guide the journey (map) of his behavior including spatial memory [25].

In this study, the group of rats were given EPB 15 mg/kgBW showed a tendency to have better spatial memory compared to the control and undernourished group were given placebo, although not

statistically significantly different. Previous studies have shown pasak bumi also improves cognition in normal mice 4 weeks old [26]. In this study the tendency to increase spatial memory was thought to be through antioxidant mechanisms. This was evident from the reduction in oxidative stress in the brain which is characterized by an increase in the activity of SOD enzymes, catalase and a decrease in peroxide and MDA. Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS), consisting of superoxide, hydrogen peroxide, and hydroxyl radicals, with inadequate oxidative defenses, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) [19].

Excessive formation of ROS causes neuronal cell damage and induces death through the apoptotic and necrotic pathways. Previous research has shown that there is a link between oxidative stress and mitochondrial dysfunction and the development of cell death in various neurological disorders. Mitochondrial dysfunction includes bioenergetic failure and increased cytosolic calcium, oxidative stress, mitochondrial permeability transition pore opening, and release of key proteins into cells triggering cell death. Oxidative stress enzymes induce various cellular problems that can trigger mitochondrial dysfunction and accumulation of ROS/RNS not only contribute to macromolecular lesions such as lipids, proteins and DNA but also affect bioenergetics, glutamate excitotoxicity, and DNA, by inducing apoptotic signals [27].

Increases in free radicals or reactive oxygen species must be balanced with increased enzymatic antioxidant synthesis. However, this is often not enough to overcome only with endogenous antioxidants, so exogenous antioxidants are needed. The inadequate defense of endogenous antioxidants in the brain can be overcome by administering exogenous antioxidants, so that the balance of reactive oxygen species and antioxidants can be formed again [4,5].

The 70% ethanol extract pasak bumi root was used in this research contains active compound 8.73% saponin, 14.47% alkaloid, 21.5 mg/mL flavonoid, 42.28 mg/mL steroid, 244.3 mg/mL terpenoid and 2.33 tannin mg/mL. Flavonoid compounds have been known to have strong antioxidant potential. Flavonoid compounds in some plants have also been shown to be potential antioxidants by suppressing the formation of free radicals by inhibiting enzymes or by metal ionic chelating involved in the production of free radicals and through reducing free radicals [10].

In this study, although the tendency for spatial memory in the group given EPB was 15 mg/kgBW higher than the other groups, it was not statistically significantly different. This can be caused by a lack of dose EPB. If the dose is increased to 1.5 or 2 times a significant difference may be seen. Besides that, there are other factors that can affect spatial memory, namely the reaction of astrogliosis (reactive astrocytes). In the protein undernutrition an increase in glial fibrillary acidic protein (GFAB) is a marker of astrocytes reactivity [28]. If astrocytes reactivity increases, the docosahexaenoic acid (DHA) and arachidonic acid (AA) production by astrocytes will also increase [29]. The DHA and AA play a role in increasing spatial memory. This is thought to cause spatial memory in undernourished rats that are no different from normal rats due to the effect of DHA and AA production by astrocytes.

The brain structure that plays a role in spatial memory is the hippocampus. In this study whole brain was used to measure oxidative stress. This is a limitation of research because it cannot describe specifically what part of the hippocampus that experiences the highest oxidative stress compared to other parts of the brain. However, it can be concluded that in general undernourished causes an increase in oxidative stress 2 the brain and in this study can be reduced by giving 70% ethanol extract pasak bumi root starting at a dose of 7.5 mg/kgBW.

5. Conclusion

According the data, we concluded that the administration of 70% ethanol extract pasak bumi root (EPB) 15 mg/kgBW in the undernourished rats was able to improve the oxidative stress condition of the brain and tended but still could not to improve the spatial memory of rats compared to the EPB dose of 7.5 mg/kgBW.

Ethics approval and consent to participate

This research had been received approval from the ethics committee No. 298/KEPK-FK UNLAM/EC/VII/2019 of the Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia.

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Authorship

Triawanti formulated the research questions, designed the study, nutritional and biochemical consultant, and carried it out. Meitria Syahadatina Noor and Didik Dwi Sanyoto contributed in experimental laboratory procedure and statistical analysis and manuscript. All authors contributed in writing the article and approved it before submission.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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