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Effect of pasak bumi (*Eurycoma longifolia Jack*), DHA, and seluang fish (*Rasbora* spp.) on neuroinflammation and neurotransmitter alterations in malnourished rats

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ABSTRACT

Background: Malnutrition has detrimental effects on brain development, leading to neuroinflammatory and neurotransmitter disorders. A nutrient-rich diet is advocated to mitigate these effects. In South Kalimantan, natural resources such as pasak bumi (*Eurycoma longifolia* Jack.) and seluang fish (*Rasbora* spp.) are recognized for their potential antioxidant and anti-inflammatory properties.

Objective: This study aimed to evaluate the effects of pasak bumi in comparison with seluang fish and DHA on neuroinflammation and neurotransmitter imbalances in malnourished rats.

Methods: The study involved dividing malnourished rats into six experimental groups: (1) untreated control, (2) treated with pasak bumi extract, (3) treated with DHA, (4) treated with a combination of DHA and pasak bumi extract, (5) treated with seluang fish, (6) treated with a combination of seluang fish and pasak bumi extract; and a control group of normal rats receiving standard feed and placebo. The primary outcomes measured were levels of IL-6, TNF-α, and serotonin.

Results: The study revealed that malnutrition in rats significantly elevated IL-6 and TNF-α levels. Treatments involving pasak bumi extract, both alone and in combination with DHA or seluang fish, reduced IL-6 levels. Similarly, combination of pasak bumi extract and DHA or seluang fish lowered TNF-α levels than single treatment of pasak bumi extract ($p > 0.05$). All treatments did not reduce the serotonin level.

Conclusion: The findings of this study underscore the potent anti-inflammatory capabilities of pasak bumi extract, particularly when combined with DHA or seluang fish, in mitigating the inflammatory response in malnourished rats.

Keywords: malnutrition, pasak bumi, seluang fish, neuroinflammation, neurotransmitter

Introduction

Malnutrition has long been a significant public health concern in Indonesia. The latest data from the *Studi Status Gizi Indonesia* (SSGI) in 2021 indicates a decrease in stunting prevalence from 27.7% to 24.4% compared to the previous year. However, this persistent issue continues to affect various regions unevenly. Notably, South Kalimantan ranks sixth nationally in stunting among children under five and third in the underweight category, which is particularly alarming [1].

Protein-deficient malnutrition can disrupt the synthesis of enzymes that function as antioxidants, reducing their numbers [2,3]. This results in an imbalance between antioxidants and oxidative stress in the brain, causing excess free radicals [4]. These free radicals can damage cellular components, including proteins, DNA, membrane phospholipids, and enzymes [5,6]. Furthermore, malnutrition disrupts the immune system's balance, reducing anti-inflammatory biomarkers and increasing pro-inflammatory ones [7,8]. This harmful alteration in the immune system is suggested to contribute to neuroinflammation in the brain, potentially impairing neuronal development and affecting a child's intelligence over time [9,10].

One approach to overcome malnutrition is providing highly nutritious, particularly high-protein foods, to protein-malnourished children [11,12]. A previous study demonstrated that the administration of *seluang* fish, high in protein and commonly consumed in South Kalimantan, increased IGF-1 levels, benefiting bone growth and haemoglobin levels in an animal model [13]. This intervention also alleviated oxidative stress in the brain [14] and improved memory in malnourished rats [15]. Another local nutrient source, pasak bumi (*Eurycoma longifolia* Jack), is believed to hold the potential for addressing brain damage caused by malnutrition-induced inflammation [8-17]. The active compounds in *E. longifolia*, such as quassinoids, biphenylneolignan, tirucallane-type triterpenes, β-carboline alkaloids, canthin-6-one alkaloids, and squalene derivatives, have demonstrated antioxidant and anti-inflammatory properties in previous studies [18,19].

This study employed an experimental rat model (*Rattus norvegicus*) with protein deficiency. First, we focused on determining the effective dose of pasak bumi extract to mitigate the adverse effects of malnutrition [19]. The results indicated that rats given a 15 mg/kg BW dose of pasak bumi extract showed improvements in oxidative stress and higher retention of spatial memory compared to the control group and other doses [19]. Second, we compared this effective dose with the administration of seluang fish, DHA, and the combination of each with pasak bumi extract in overcoming neuroinflammation and neurotransmitter disorders in the brains of malnourished rats. We assessed neuroinflammation through IL-6 and TNF-α levels and evaluated neurotransmitter function using serotonin levels [20,21].

Methods

Ethical approval

This study received ethical approval from the Health Research Ethics Commission, Faculty of Medicine, Lambung Mangkurat University (approval No. 061/KEPK-FK UNLAM/EC/II/2020).

Feed composition

In this study, we used two distinct types of feed: a low-protein feed and a standard feed. The low-protein feed is comprised of the AIN-76A purified rodent diet [Dyets Inc., USA], which includes cornstarch (183 g/kg), corn oil (50 g/kg), cellulose (50 g/kg), DL-methionine (0.9 g/kg), sucrose (609.1 g/kg), casein (60 g/kg), a mineral mix#200000 (35 g/kg), a vitamin mix #300050 (10 g/kg), and choline bitartrate (2 g/kg). Conversely, the standard feed contains 20–22% protein, 5–7% fat, 5–7% cinder, 3–5% fiber, 9–11% calcium, 0.6–0.8% phosphorus, and an energy content of 2900–3100 kcal.

Preparation of experimental animal malnutrition model

Newborn rats were breastfed by mothers on a low-protein diet (AIN76A, 6% protein) for four weeks, then continued on the same diet post-weaning for another four weeks. Rats with serum protein levels below 4.7 g/dL were classified as malnourished. Serum protein was measured from 1 mL of blood obtained via tail vein puncture and centrifugation [22].

Treatments

Rats were divided into seven groups (five rats per group):

- Normal control: healthy rats on standard feed and placebo
- Untreated control: malnourished rats on standard feed and placebo
- P1: malnourished rats on standard feed and pasak bumi ethanol extract (15 mg/kg BW)
- P2: malnourished rats on standard feed and DHA (1 mg/kg BW)
- P3: malnourished rats on standard feed and combination DHA (1 mg/kg BW) and pasak bumi extract (15 mg/kg BW)

- P4: malnourished rats on seluang fish
- P5: malnourished rats on combonation seluang fish and pasak bumi extract (15 mg/kg BW).

The treatment duration was five weeks, based on prior studies identifying the optimal antioxidant activity dose [19].

Examination of TNF-α, IL-6, and serotonin levels

At the 5-week mark, rats were euthanized using anaesthetic drug and cardiac puncture, followed by brain extraction for TNF-α, IL-6, and serotonin level measurement via the ELISA method [23]. The procedure was conducted according to the manufacturer's guidelines (BioTechnology Laboratory®).

Data analysis

Data were analyzed using SPSS software. The Shapiro-Wilk test checked data normality. For normally distributed data, an ANOVA test with a 95% confidence level and LSD posthoc test were used. For non-normally distributed data, the non-parametric Kruskal–Wallis test followed by the Mann–Whitney test was applied, maintaining a 95% confidence level.

Results

Effect treatments to IL-6 level

The study demonstrates that malnourished rats, serving as the untreated control group, exhibited elevated IL-6 levels in the brain compared to a normal group (Figure 1). This indicates that malnutrition may increase the inflammatory response. When malnourished rats were treated with pasak bumi extract, their IL-6 levels were lower than those in the untreated group, suggesting the extract's potential to mitigate IL-6 levels. Conversely, treatment with DHA alone did not significantly alter IL-6 levels compared to the untreated control. However, a combined treatment of pasak bumi extract and DHA significantly reduced IL-6 levels, surpassing DHA treatment alone.

Similarly, administering seluang fish alone did not impact IL-6 levels, maintaining them on par

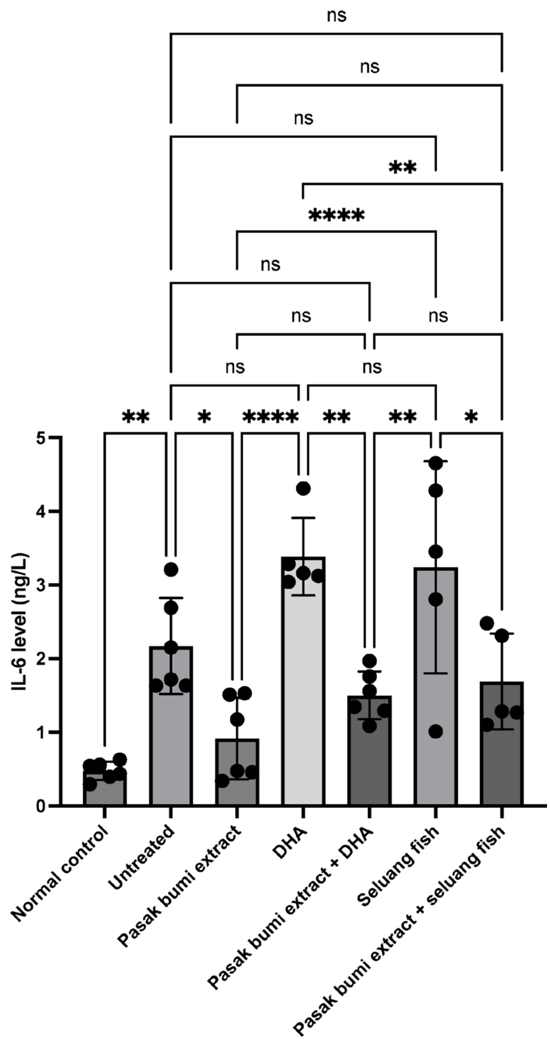

Figure 1. IL-6 levels of malnourished rat brain after intervention. Normal control: healthy rats on standard feed and placebo; Untreated control: malnourished rats on standard feed and placebo; P1: malnourished rats on standard feed and pasak bumi extract (15 mg/kg BW); P2: malnourished rats on standard feed and DHA (1 mg/kg BW); P3: malnourished rats on standard feed, pasak bumi extract (15 mg/kg BW) and DHA (1 mg/kg BW); P4: malnourished rats on seluang fish; P5: malnourished rats on pasak bumi extract (15 mg/kg BW) and seluang fish

with the untreated group. Notably, a combined treatment involving pasak bumi extract and seluang fish effectively reduced IL-6 levels. These findings underscore the crucial role of pasak bumi extract in diminishing IL-6 levels in malnourished rats.

Effect treatments to TNF-alpha levels

Our findings indicate that TNF- α levels were elevated in the malnourished, untreated group compared to the normal group (Figure 2). Notably, malnourished rats treated with a combination

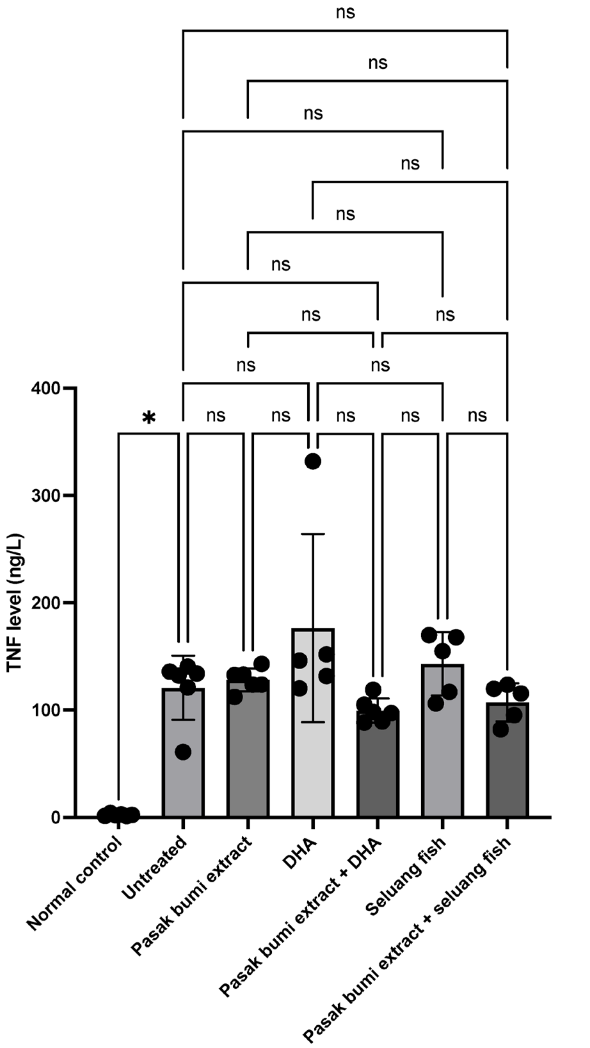

Figure 2. TNF-α levels of malnourished rat brain after intervention Normal control: healthy rats on standard feed and placebo; Untreated control: malnourished rats on standard feed and placebo; P1: malnourished rats on standard feed and pasak bumi extract (15 mg/kg BW); P2: malnourished rats on standard feed and DHA (1 mg/kg BW); P3: malnourished rats on standard feed, pasak bumi extract (15 mg/kg BW), and DHA (1 mg/kg BW); P4: malnourished rats on seluang fish; P5: malnourished rats on pasak bumi extract (15 mg/kg BW) and seluang fish

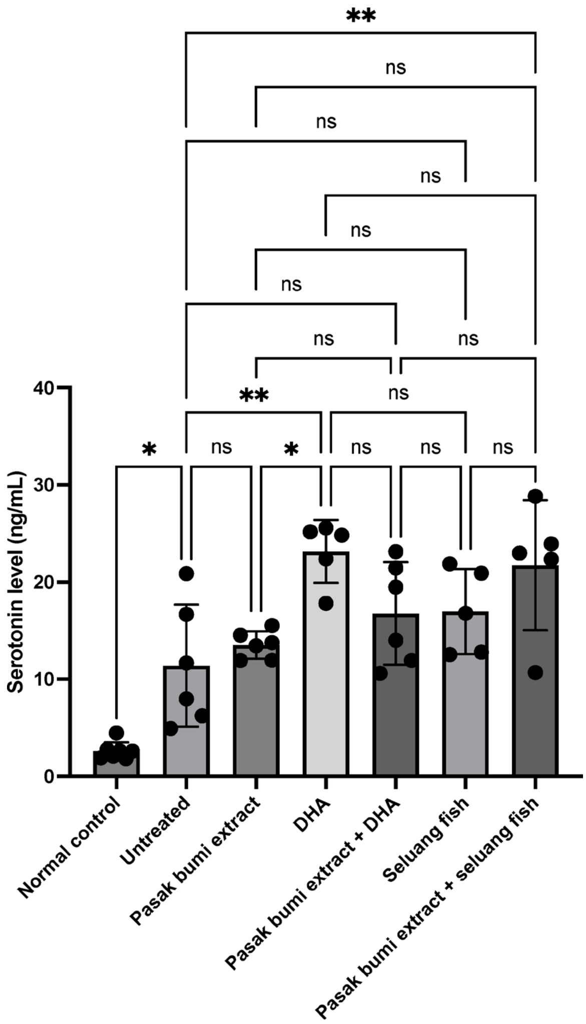

Figure 3. Level of brain serotonin in malnourished rats after intervention Normal control: healthy rats on standard feed and placebo; Untreated control: malnourished rats on standard feed and placebo; P1: malnourished rats on standard feed and pasak bumi extract (15 mg/kg BW); P2: malnourished rats on standard feed and DHA (1 mg/kg BW); P3: malnourished rats on standard feed, pasak bumi extract (15 mg/kg BW), and DHA (1 mg/kg BW); P4: malnourished rats on seluang fish; P5: malnourished rats on pasak bumi extract (15 mg/kg BW) and seluang fish

of pasak bumi extract and DHA exhibited lower TNF-α levels than those in the untreated group. Furthermore, this combination was more effective in reducing TNF-α levels in malnourished rats than treatments involving only pasak bumi extract, DHA, or seluang fish.

Effect treatments to serotonin level

Regarding serotonin levels, the data revealed that the average brain serotonin in malnourished rats exceeded that of normal rats (Figure 3). Interventions involving pasak bumi extract, DHA, and seluang fish in malnourished rats resulted in higher serotonin levels compared to the normal control group. Among the intervention groups, treatment with pasak bumi extract alone resulted in the lowest brain serotonin levels.

Discussion

In this study, the effects of seluang fish and DHA on neuroinflammation and neurotransmitter levels in malnourished rats were examined, both as a single treatment and in combination with pasak bumi extract. The findings revealed that malnourished rats exhibited significantly elevated levels of IL-6 and TNF-α (Figures 1 and 2) compared to the normal group. This aligns with prior research indicating that protein deficiency can increase inflammatory mediators IL-6 and TNF-α levels. Notably, administering pasak bumi extract led to the most substantial reduction in IL-6 levels among all intervention groups. When pasak bumi extract was combined with DHA, IL-6 levels did not differ significantly from those observed with the administration of pasak bumi extract alone. A similar trend was observed with the combination of pasak bumi extract and seluang fish, where IL-6 levels were not significantly different from those in rats treated solely with pasak bumi extract. Furthermore, both the combination of pasak bumi extract with DHA and the combination with seluang fish resulted in lower TNF-α levels compared to their respective treatments alone. These results suggest that pasak bumi extract possesses considerable anti-inflammatory potential.

Pasak bumi extract demonstrates significant anti-inflammatory effects, as evidenced by various studies, through mechanisms such as (i) inhibiting the release of inducible nitric oxide synthase (iNOS), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and interleukin-6 (IL-6), which are critical inflammation-related proteins, within the lipopolysaccharide (LPS)-induced NF-κB signaling pathway [24]; and (ii) suppressing the activity of nitric oxide (NO), iNOS, and cyclooxygenase-2 (COX2), while also protecting against death in LPS-induced septic shock models in mice [25,26]. Adding docosahexaenoic acid (DHA) and fish to the treatment regimen enhances the anti-inflammatory potential of the intervention.

DHA, an omega-3 fatty acid, serves as an anti-inflammatory mediator [27,28], impacting inflammation through various mechanisms related to the composition of cell membranes [29]. Alterations in membrane composition can influence fluidity and cell signaling, thereby affecting gene expression and the production of lipid mediators. Inflammation-involved cells typically contain high levels of arachidonic acid, an omega-6 fatty acid [30]. However, oral supplementation with EPA (eicosapentaenoic acid) and DHA can modify the levels of arachidonic acid, EPA, and DHA. Eicosanoids derived from arachidonic acid, including prostaglandins, thromboxanes, and leukotrienes, play roles in inflammation regulation through processes such as vasoconstriction and enhancing immune responses, often promoting pro-inflammatory mediators [30].

In contrast, EPA produces eicosanoids with anti-inflammatory properties, distinct from those derived from arachidonic acid [32]. Both EPA and DHA inhibit additional inflammatory mechanisms, such as neutrophile chemotaxis [33], the production of eicosanoids (like prostaglandins and leukotrienes) from arachidonic acid [34], and the production of cytokines and T-cell reactivity [35]. These effects are mediated through various mechanisms, including activating cell surface molecules (GPR120) and intracellular receptors (PPAR-gamma) that regulate inflammatory cell signaling and gene expression patterns [32,36].

Previous study has also demonstrated that EPA and DHA can inhibit IL-6 and IL-8 production stimulated by endotoxins in human endothelial cell cultures and reduce TNF-alpha production in monocyte cultures induced by endotoxins [32]. Furthermore, EPA reduces the activation of NF-κB triggered by endotoxins in monocytes, correlated with decreased phosphorylation of IκB [38]. The omega-3 fatty acids from marine fish have been studied for their role in reducing inflammation by inhibiting the transcription factor NF-κB, through mechanisms including reducing eicosanoid mediator production from arachidonic acid, increasing production of anti-inflammatory eicosanoids from EPA, enhancing the production of anti-inflammatory and resolvin molecules from EPA and DHA, decreasing leukocyte chemotaxis, suppressing the expression of leukocyte and endothelial adhesion molecules to reduce adhesive interactions, and inhibiting the production of pro-inflammatory cytokines and proteins by affecting the NF-κB signaling pathway [27].

This study also used seluang fish to treat brain disorders due to malnutrition, one of which was neuroinflammation. Besides containing DHA, seluang fish contains complete amino acids [39]. The group with the combination of pasak bumi extract and seluang fish showed lower levels of IL-6 and TNF-α compared to those given pasak bumi extract or seluang fish alone, and similar to being given a combination of DHA and pasak bumi extract (Figures 2 and 3). Amino acids found in seluang fish include essential and non-essential amino acids. Several studies have proven the role of amino acids in the inflammatory process [40,41,42]. In inflammatory bowel disease (IBD), the anti-inflammatory activity of the following amino acids has been demonstrated: tryptophan, glycine, methionine, cysteine, and arginine [43]. Tryptophan inhibits the activation of proinflammatory cytokines (TNF-α, IL-6, IFN-γ, IL-12p40, IL-1β, and ICAM-1) [44], glutamine reduces the expression of CCR9, LFA-1, and PSGL-1 produced by Th cells [45], glutamate inhibits T cell response and inflammation [46], cysteine inhibits the expression of local inflammatory mediators (TNF-α, IL-6, IL-12p40, and IL-1β) [47], histidine reduces the production of TNF-α, IL-6, and also inhibits NF-κB signaling [48], arginine reduces proinflammatory cytokines and chemokine expression [49], glycine prevents the increase of IL-1β and TNF-α [48], taurine inhibits NF-κB activity [50].

Branched-chain amino acids (BCCAs) such as leucine also promote anti-inflammatory effects [51,52,53]. BCAAs modulate inflammation by synthesizing glutamine, which occurs via BCAA transaminases and produces glutamate from α-ketoglutarate [54]. Glutamate is then converted to glutamine using glutamine synthetase [55]. Glutamine affects the release of TNF-α, IL-2, IL-6, IL-10, and IFN-γ [56]. Studies have also shown that glutamine plays a crucial role in the NF-κB signal transduction pathway, contributing to the reduction of local inflammation [57,58]. Glutamine is also involved in the inflammatory pathway in ulcerative colitis [59,60]. Glutamine inhibits this process by increasing the heat shock protein activity, which can reduce the expression of the transcription factor NF-κB and stimulate genes associated with the inflammatory response [61]. Administration of short-term glutamine supplementation proved to have a significant decrease in NF-κB [62, 63]. Glutamine can also reduce inflammation by inhibiting the activation of STAT protein and normalizing the production of nitric oxide [63].

Figure 3 illustrates that serotonin levels in the malnourished group were notably higher than those in the normal group, aligning with existing research indicating an increase in serotonin levels under malnutrition conditions [64,65]. This rise is attributed to the impact of undernutrition, occurring during pre- and post-natal stages, on the serotonin neurotransmission system. Such nutritional deficits activate the brain's serotonergic biosynthetic system and metabolism, consequently elevating the free fraction of L-Tryptophan (FFT), a precursor to serotonin [66,67]. Additionally, protein deficiency alters the plasma albumin's capacity to bind L-Trp, further increasing FFT levels in the plasma. This elevated FFT can cross the blood-brain barrier, which is absorbed by brainstem serotonergic neurons, triggering serotonin synthesis [68]. The unusually high serotonin levels in malnutrition may also result from brain developmental reprogramming due to nutritional deficiencies [69].

Abnormally low serotonin levels can impair learning abilities and memory [70], whereas excessively high levels are similarly detrimental [71]. Elevated serotonin has been linked to the dysregulation of food intake, indicating hyperphagia as a result of fetal programming [72]. Furthermore, high serotonin levels are associated with decreased appetite, increased impulsivity, and diminished reference memory [73]. This study found that all malnourished groups—whether treated with pasak bumi extract, DHA, seluang fish individually, or in combination—exhibited elevated serotonin levels compared to the normal group. Notably, the group treated with pasak bumi extract alone displayed the lowest serotonin levels among the intervention groups, suggesting that pasak bumi extract may prevent serotonin levels from rising excessively.

Deficiency in omega-3 fatty acids leads to increased basal levels and diminished stimulated levels of serotonin [73]. In contrast, serotonin release can diminish under pharmacological stimulation. The restoration of serotonin release impaired by dietary deficiency is achievable through an adequate omega-3 diet during critical neurodevelopmental stages, including at birth, between 7-14 days postnatal, or during lactation [74]. Administering an adequate diet during lactation can replenish the fatty acid composition in the brain and facilitate serotonin release [74]. However, when such a diet is introduced post-weaning, it fails to restore serotonin levels despite normalizing DHA levels within the hippocampal membrane. This finding clarifies why, in the present study, diets rich in DHA and seluang fish did not bring serotonin levels back to normal level, given that the interventions were implemented after the weaning phase. Consequently, it underscores the importance of ensuring an adequate intake of nutrients during the first 1000 days of life to support optimal neurodevelopment and prevent long-term deficits in serotonin levels.

Conclusion

Protein deficiency-induced malnutrition adversely impacts the brain, escalating the neuroinflammatory response and triggering excessive serotonin production. The study reveals that combining pasak bumi extract with DHA, as well as pasak bumi extract with seluang fish, effectively reduces levels of IL-6 and TNF-α in the brains of malnourished rats. Furthermore, pasak bumi extract alone demonstrates a unique ability to diminish serotonin levels in the brains of these rats compared to other interventions. Consequently, the synergistic effects of pasak bumi extract with either DHA or seluang fish highlight their potential as new therapeutic agents for mitigating malnutrition.

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Author contributions

Conceptualization, T; Methodology, T, D.D.S; Investigation, D.I.A, M.S.N; Writing – Original Draft, D.I.A., T; Writing – Review & Editing, T, M.S.N; Funding Acquisition, T; Resources, D.D.S; Supervision, T, M.S.N.

Declaration of interest

None.

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