



Research Article

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Immunomodulatory and Erythropoietic Effects of Fermented Leaf Extract of Fern (*Stenochlaena palustris*): Dose-Dependent Recovery of Blood Disorders in a Malnourished Rat Model



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Abstract

This study investigates the immunomodulatory and erythropoietic potential of fermented fiddlehead fern (*Stenochlaena palustris*) leaf extract of Fermented Fiddlehead Fern Leaf Extract (FFFE) in a malnourished rat model. Protein-energy malnutrition disrupts hematological homeostasis, and FFFE, fermented with *Lactobacillus plantarum* to enhance bioactivity, was evaluated as a nutraceutical intervention. Using a post-test-only control group design, malnourished rats received FFFE at 100, 300, or 500 mg/kg BW/day for 30 days. Hematological analysis revealed a dose-dependent recovery of blood parameters. The highest dose (500 mg/kg BW) produced the most potent erythropoietic effect, significantly elevating red blood cell count, hemoglobin, and hematocrit levels. Immunomodulatory effects were dose-specific: the 100 mg/kg BW dose preferentially stimulated lymphocyte proliferation (adaptive immunity), whereas the 300 mg/kg BW dose was more effective at increasing neutrophil count (innate immunity). Platelet count also improved significantly at the highest dose. The findings demonstrate that FFFE effectively restores hematological and immune parameters disrupted by malnutrition. Fermentation is identified as a critical step for enhancing its efficacy, positioning FFFE as a promising, sustainable nutraceutical candidate for addressing malnutrition-related dysfunctions.

Keywords: Malnutrition; Fermented Fern Extract; Hematology; Immunomodulator; Local Food

Introduction

Malnutrition remains a complex and multi-dimensional global health issue, adversely affecting the quality of human resources, cognitive development, and economic productivity of a nation [1]. Haematologically, protein and energy deficiency directly disrupts blood system homeostasis. Impaired synthesis of proteins, haemoglobin, and blood cell growth factors triggers anaemia, which is clinically characterised by decreased erythrocyte, Haemoglobin (Hb), and Haematocrit (HCT) values [2]. Immune system dysfunction associated with malnutrition is also reflected in significant alterations in the leukocyte profile, including lymphopenia and dysregulation of neutrophil responses, which increase susceptibility to infection [3].

Conventional nutritional interventions to address malnutrition often focus on single micronutrient supplementation or food fortification. Although these strategies have proven effective in some contexts, their implementation faces obstacles in the form of high costs, sustainability challenges, and limited accessibility, particularly in remote and low-income communities [4]. Therefore, exploring and utilising local food sources that are nutrient-rich, easily accessible, and low-cost is essential in formulating innovative and contextual strategies to address malnutrition.

In Indonesia, fern leaves (*S. palustris*) are one of the abundant local food sources that have not been optimally utilised. This

plant is known to have promising nutritional content, including protein, essential minerals such as iron and zinc, and various bioactive compounds such as flavonoids and phenols that have the potential to support cell regeneration and haematopoietic function [5]. The iron content in fern leaves is an important precursor for haemoglobin synthesis, zinc plays a role in immune cell proliferation, and bioactive compounds can act as antioxidants that protect blood cells from oxidative damage [6].

However, the nutritional potential of fern leaves is hindered by the presence of anti-nutritional compounds, particularly tannins and phytates. These compounds have the ability to bind minerals (such as iron and zinc) and proteins in the digestive tract, thereby reducing bioavailability and nutritional value that can be absorbed by the body [7]. To overcome this obstacle, a food technology approach through fermentation offers a promising solution. The fermentation process, utilising probiotic microorganisms such as *Lactobacillus plantarum*, has been proven to significantly reduce anti-nutritional compound levels. These microbes produce phytase and tannase enzymes that degrade phytate and tannin, releasing bound minerals and proteins [8]. Furthermore, fermentation has also been shown to improve protein quality through proteolysis, producing bioactive peptides, and increasing the content of B-complex vitamins (such as folate and B12) that are essential for blood cell synthesis and nervous system function [7-9]. Thus, the transformation of fern leaves through fermentation is expected to produce food products with superior nutritional value and bioavailability compared to their raw materials.

Several previous studies have indicated the benefits of various plant extracts in improving haematological profiles. For example, supplementation with moringa leaf extract (*Moringa oleifera*) has been reported to increase haemoglobin levels in anaemic rats [10]. Specifically for fern leaves, a preliminary study by [11] showed that administration of non-fermented fern leaf ethanol extract can increase haemoglobin levels in white mice [11]. However, the literature examining the effects of fern leaves, particularly in fermented form, on complete blood parameters-which include not only the erythrocyte system but also leukocytes and thrombocytes-remains very limited. This critical knowledge gap lies in the lack of strong empirical evidence regarding the efficacy or benefits of fermented fern leaf extract in comprehensively restoring haematological disorders in malnutrition models.

Based on the above description, this study was designed to test the hypothesis that fermentation with *Lactobacillus plantarum* can increase the nutritional value and bioavailability of active compounds in fern leaves, so that the administration of the extract can act as an effective nutritional intervention in improving haematological parameters in malnourished rats. This study critically analyses the impact of the intervention not only on the erythrocyte system (including RBC, Hb, HCT, MCH, MCHC) but also on the immune system through comprehensive differential

leukocyte analysis and platelet parameters. The findings of this study are expected to provide a scientific basis for the innovative, sustainable, and low-cost utilisation of local food resources as part of a strategic solution to address malnutrition and its haematological effects.

Materials and Methods

Study Design and Test Animals

This study employed a pure experimental design with a **post-test-only control group** and was arranged in a **randomised group design** to minimise confounding variables. A total of 25 male Sprague-Dawley rats, aged 5-6 weeks with an initial body weight of 80-90 g, were used in this study. The sample size was determined using Federer's formula for animal studies [12], which considers the number of treatments (t) and replications (r). With 5 treatment groups, the calculation $(t-1)(r-1) \geq 15$ resulted in a minimum of 5 mice per group ($r=5$), for a total of 25 mice.

The mice were randomly allocated into five groups ($n=5$ each):

K_Positive (Normal Control): Healthy mice fed a standard normal diet.

K_Negative (Negative Control): Malnourished mice fed a low-protein diet.

D1 (Dose 1): Malnourished mice fed standard feed + 100 mg/kg BW of fermented fern leaf extract.

D2 (Dose 2): Malnourished rats fed standard feed + fermented fern leaf extract 300 mg/kg BW.

D3 (Dose 3): Malnourished rats fed standard diet + fermented fern leaf extract 500 mg/kg BW.

The selected dose range (100–500 mg/kg BW) was based on preliminary studies and previous research using plant extracts for haematological improvement in mouse models, which generally used a similar range to establish a dose-response relationship [10-11].

Preparation of Fermented Fern Leaf Extract

The intervention material was prepared using an optimised method based on *Response Surface Methodology* (RSM) [14]. Fresh fern leaves (*S. palustris*) were harvested in the morning (06.00-08.00), selected, washed with running water, and cut into uniform pieces (3-5 cm). *Lactobacillus plantarum* inoculum was prepared in MRS Broth and incubated for 48 hours, reaching a density of approximately 2.67×10^9 CFU/mL, referring to our previous studies.

The fermentation process was carried out using **100 grams** of prepared fern leaves mixed with salt (NaCl) and sucrose at the optimal concentration determined by RSM (e.g., 2.5% salt and 3% sucrose, w/w). A total of 5% (v/b) *L. plantarum* inoculum was added, equivalent to a final concentration of $\sim 1 \times 10^7$ CFU/g.

Fermentation was carried out in closed plastic containers at room temperature for 72 hours. After fermentation, the solid biomass was separated, dried in an oven at 60°C for 72 hours, ground, and sieved to obtain a fine powder (100 mesh).

Bioactive compounds were extracted from the fermentation powder using maceration with 96% ethanol (1:50, w/v) for 3×24 hours on an orbital shaker (200 rpm). The supernatant was collected after centrifugation (3000 rpm, 3 minutes), concentrated using a rotary evaporator at 60°C, and the residual solvent was evaporated in a water bath to obtain a dry extract. The total flavonoid content of the optimised extract was determined using the aluminium chloride colorimetric method and expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g) [14].

Malnutrition Modelling and Intervention Phase

Mice were housed individually in metabolic cages under controlled conditions: 12-hour light/dark cycle, temperature 22–25°C, and relative humidity 55 ± 10%. Cage bedding was changed every 3 days. Body weight and feed intake were monitored every 48 hours [12].

Malnutrition induction and intervention consist of three phases:

Adaptation Phase (7 days): All mice were fed standard normal feed for acclimatisation and to achieve stable body weight.

Malnutrition Induction Phase (30 days): Twenty rats were fed a low-protein diet to induce malnutrition, while five rats (K_Positive) continued to receive standard normal feed. The feed composition is detailed in (Table 1). Malnutrition was confirmed when the body weight of mice in the low-protein group was less than 20% of the average body weight of the normal control group (K_Positive) [8].

Table 1: Experimental Feed Composition (g/kg feed).

Ingredient	Normal Feed	Low-Protein Feed
Casein (>85% protein)	200	40
Sucrose	100	100
Fibre	10	10
Corn oil	80	80
Mineral Mix ^{a^}	40	40
Vitamin mixture ^{a^}	10	10
L-Methionine	1.5	1.5
Choline Bitartrate	2.5	2.5
Corn Flour (Kanji)	556.5	716.5
Total	1000	1000

^{a^}Mineral and vitamin mixture formulated according to AIN-93 standards.*

Intervention phase (30 days): The twenty malnourished rats were redistributed into the K_Negative, D1, D2, and D3 groups (n=5 each). The K_Positive group continued to receive standard feed. The K_Negative group received standard feed without extract. The D1, D2, and D3 groups received standard feed supplemented with fermented fern leaf extract at doses of 100, 300, and 500 mg/kg BW/day, respectively, via oral gavage.

Haematological Analysis

At the end of the intervention period, blood samples (approximately 1 mL) were taken from the retro-orbital plexus of the rats under light anaesthesia. Blood was collected in EDTA tubes for complete blood count analysis. Haematological parameters, including total Red Blood Cell (RBC) count, Haemoglobin (Hb), Haematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Red Cell Distribution Width (RDW), Total White Blood Cell Count (WBC), leukocyte differential count (neutrophils, lymphocytes, monocytes, eosinophils, basophils), and Platelet Count (PLT), were analysed using an automated veterinary haematology analyser (BC2800Vet, Mindray) [15]. Serum was isolated by separating a portion of the blood using centrifugation at 2,500 rpm for 10 minutes for potential future biochemical analysis.

Data Analysis

Data were analysed using R software version 3.6. The normality of data distribution was tested using the Shapiro-Wilk test, and the homogeneity of variance was tested using the Levene test. For data that met the assumptions of normality and homogeneity of variance, a one-way Analysis of Variance (ANOVA) was performed, followed by a post-hoc test (e.g., Tukey's HSD) for multiple comparisons. For data that did not meet these assumptions, the non-parametric Kruskal-Wallis test was used, followed by Dunn's post-hoc test. P-values less than 0.05 were considered statistically significant.

Ethical Approval

The experimental protocol involving animals was reviewed and approved by the Ethics Committee of the iRATco Veterinary Laboratory Service (Ethics Number: 4.2.019-08/KEHI/VIII/2024), located at Perumahan Dramaga Cantik, Dramaga, Bogor, Indonesia. All procedures were strictly carried out in accordance with the principles of care and use of laboratory animals.

Results

Leukocyte Profile

The analysis of the leukocyte profile showed significant variations in both the total leukocyte count and its differential cells among the groups after the intervention.

The malnourished group without intervention (K_Negative) exhibited the highest absolute total leukocyte count in the post-test phase (31.17×10^9 g/L). All groups receiving FFFE (D1, D2, D3) demonstrated a greater increase in the delta of total leukocytes (ranging from $+12.63$ to $+14.32 \times 10^9$ g/L) compared to the K_Negative group ($+5.01 \times 10^9$ g/L), with the highest increase observed in the D2 group ($+14.32 \times 10^9$ g/L) (Table 2).

A distinct pattern was observed in the lymphocyte count.

The D1 group (100 mg/kg BW) produced the highest increase in lymphocytes ($\Delta +13.42 \times 10^9$ g/L) compared to other groups. For neutrophil counts, the K_Negative and D1 groups showed a decrease after the intervention (negative delta), while the D2 and D3 groups showed an increase (positive delta). The highest increase in neutrophils was observed in the D2 group ($\Delta +1.08 \times 10^9$ g/L) (Table 2). No statistically significant differences were found in the counts of monocytes, eosinophils, and basophils between the groups ($p > 0.05$).

Table 2: Leukocyte Profile of Mice Before (Pre) and After (Post) Intervention.

Group	Parameter								
	Leukocytes (10^9 g/L)			Neutrophils (10^9 g/L)			Lymphocytes (10^9 g/L)		
	Pre	Post	Δ	Pre	Post	Δ	Pre	Post	Δ
K_Positive	16.68	17.81	1.13	3.58	1.6	-1.98	11.25	13.95	2.71
K_Negative	26.16	31.17	5.01	4.22	2.87	-1.36	18.76	23.83	5.07
D1	13.85	26.48	12.63	3.45	2.04	-1.41	7.92	21.34	13.42
D2	15.61	29.93	14.32	2.83	3.9	1.08	11.2	22.25	11.06
D3	19.1	32.52	13.42	3.2	3.99	0.79	13.53	24.98	11.45
<i>P-value</i>	0.387	0.003*	0.071	0.878	0.030*	0.002*	0.159	0.005*	0.133

*Note: Δ = Delta (Change); * = Significant ($p < 0.05$)*

Erythrocyte and Platelet Profiles

The erythrocyte profile analysis indicated that FFFE administration, particularly at the highest dose, had a potent erythropoietic effect.

Group D3 (500 mg/kg BW) showed the most significant improvement in primary erythrocyte parameters. The increase in Red Blood Cell (RBC) count ($\Delta +1.61 \times 10^{12}$ /L) and hemoglobin (Hb) level ($\Delta +2.33$ g/dL) in group D3 was markedly higher

than in all other groups. The final hemoglobin level in group D3 (14.75 g/dL) nearly reached that of the normal control group, K_Positive (15.00 g/dL) (Table 3). The erythrocyte indices (MCV, MCH, MCHC) were relatively stable and did not show significant differences between the intervention groups.

For Platelet Count (PLT), group D3 showed the highest increase ($\Delta +293.50 \times 10^9$ /L) compared to all other groups (Table 3).

Table 3: Red Blood Cell and Platelet Profile of Mice Before (Pre) and After (Post) Intervention.

Group	Parameter								
	Red Blood Cells (10^{12} /L)			Haemoglobin (g/dL)			Platelets (PLT) (10^9 /L)		
	Pre	Post	Δ	Pre	Post	Δ	Pre	Post	Δ
K_Positive	8.3	9.14	0.84	14.8	15	0.2	910.33	800.17	-110.17
K_Negative	5.4	5.68	0.28	10.28	11.43	1.15	765	791	26
D1	6.57	7.39	0.82	12.46	12.62	0.16	522.8	678	155.2
D2	6.94	7.57	0.63	12.68	13.2	0.53	1003.5	752.5	-251
D3	6.52	8.12	1.61	12.43	14.75	2.33	839.5	1,133.00	293.5
<i>P-value</i>	0.007*	0.001*	0.799	0.063	0.056	0.834	0.05	0.065	0.215

*Note: Δ = Delta (Change); * = Significant ($p < 0.05$)*

Discussion

This study demonstrates that Fermented Fern Leaf Extract (FFFE) effectively restores hematological parameters in a malnourished rat model, with the effects being both multifaceted and dose-dependent.

The observed highest increase in total leukocytes in the K_Negative group likely reflects a systemic stress response or subclinical infection due to immunosuppression, a common condition in malnutrition [3]. The more relevant finding is the substantial increase in the delta of total leukocytes in all FFFE-treated groups, suggesting an active immunostimulatory role

of the extract. The unique, dose-specific effects on lymphocyte and neutrophil populations are particularly noteworthy. The preferential stimulation of lymphocytes at the low dose (100 mg/kg BW) indicates a potent effect on the adaptive immune system. This can be attributed to the improved bioavailability of zinc, a critical cofactor for lymphocyte proliferation and DNA synthesis, which is released from phytate complexes during fermentation [6-11]. Conversely, the effectiveness of the medium dose (300 mg/kg BW) in increasing neutrophil counts points towards an enhancement of innate immunity. The distinct response patterns strongly suggest that FFFE acts as an immunomodulator, fine-tuning the immune response based on the dosage, rather than providing a non-specific boost.

The most potent erythropoietic effect was achieved with the highest dose of FFFE (500 mg/kg BW), as evidenced by the dramatic increases in RBC, Hb, and HCT. This finding aligns with a previous study using non-fermented fern leaf extract [11], but the superior results in our study underscore the critical role of fermentation. The enhancement is likely multi-mechanistic. First, fermentation with *L. plantarum* reduces anti-nutritional factors like tannins and phytates, thereby increasing the bioavailability of iron, an essential component of hemoglobin [5-16]. Second, the fermentation process can enhance the content of B-complex vitamins, such as folate and B12, which are crucial for erythrocyte maturation [17]. Third, the increase in bioactive compounds like flavonoids post-fermentation may protect erythrocytes from oxidative damage, which is often elevated in malnutrition. This is consistent with our previous findings where the same fermented extract increased SOD levels and decreased TNF- α , indicating reduced oxidative stress and inflammation [18]. The stability of erythrocyte indices (MCV, MCH, MCHC) across groups indicates that FFFE supports the production of new, normocytic and normochromic red blood cells rather than altering the characteristics of existing cells.

The significant increase in platelet count at the highest dose further supports the role of FFFE in promoting overall bone marrow health and hematopoiesis. Adequate availability of proteins and micronutrients is a prerequisite for the differentiation of hematopoietic stem cells into various lineages, including megakaryocytes, the precursors of platelets [15].

In conclusion, the efficacy of FFFE is intrinsically linked to the fermentation process. By degrading anti-nutrients, fermentation unlocks the full nutritional potential of fern leaves, enhancing the bioavailability of critical minerals and potentially generating novel bioactive peptides. This addresses the primary limitation of using unprocessed local food sources [19-20]. The dual hematopoietic and immunomodulatory functions of FFFE position it as a promising, sustainable nutraceutical candidate for addressing the

complex dysfunctions associated with malnutrition.

Conclusion

Based on the research results, it can be concluded that Fermented Fern Leaf Extract (EDPF) effectively and in a dose-dependent manner restores haematological disorders in malnourished mouse models, with high doses (500 mg/kg BW) showing the strongest erythropoietic potential to overcome anaemia and low-medium doses (100 and 300 mg/kg BW) act as immunomodulators that selectively stimulate adaptive and innate immunity. This efficacy is strongly suspected to be triggered by the fermentation process with *Lactobacillus plantarum*, which increases the bioavailability of nutrients and bioactive compounds by reducing anti-nutrient levels. This study not only provides empirical evidence for the innovative use of local food sources as a sustainable strategy to combat the systemic effects of malnutrition, but also introduces a new dimension of EDPF as a nutraceutical candidate with dual haematopoietic and immunomodulatory functions. To validate these findings and develop their application, further research such as long-term toxicity testing, identification of specific bioactive compounds, exploration of molecular mechanisms, and clinical trials in at-risk populations are highly recommended.

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