

ORIGINAL ARTICLE

# The Effect of Fermented Green Fern Extract (*S. palustris*) on Tumor Necrosis Factor-Alpha and Superoxide Dismutase Levels in Malnourished Rats

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## ABSTRACT

**Background:** Malnutrition in children is still a global issue that can significantly impact the immune function and increase the susceptibility to oxidative stress. This study investigated the potential of fermented *S. palustris* on oxidative stress markers, specifically Superoxide Dismutase (SOD) and Tumor Necrosis Factor-alpha (TNF- $\alpha$ ), in malnourished rats.

**Methods:** The rats were divided into five groups of healthy controls, malnourished without intervention, and malnourished groups receiving varying doses of *S. palustris* extract (100, 300, and 500 mg/kg) for 28 days.

**Results:** A significant increase in SOD level in all intervention groups was noticed with the highest increase in 500 mg/kg group (D3) that approached the level of the healthy control group. TNF- $\alpha$  level significantly decreased in the intervention groups, with the 300 mg/kg (D2) group showing the greatest reduction.

**Conclusion:** Fermented *S. palustris* extract was shown to enhance antioxidant properties and reduce inflammation in malnourished animals that can offer a promising strategy for nutritional interventions in malnutrition-related disorders.

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## Introduction

Malnutrition remains one of the most critical health challenges worldwide, particularly affecting children in low- and middle-income countries (1). The consequences of malnutrition are severe, often leading to long-term developmental, immune, and physiological impairments. According to global reports by United Nations Children's Fund (UNICEF), World Health Organization (WHO), and the World Bank Group, approximately 45% of all child deaths under the age of five are

attributed to undernutrition, which is directly linked to protein-energy malnutrition (PEM) and micronutrient deficiencies (2, 3). PEM is characterized by deficiencies in caloric intake and essential nutrients and can significantly impact growth, immune function, and overall health. The deficiency of key micronutrients, such as vitamins and minerals, weakens the immune system, rendering malnourished individuals more susceptible to infections, particularly those affecting the gastrointestinal and respiratory

systems with reduced protein levels, rate of protein catabolism, and changes in micronutrient levels leading to oxidative stress (4-6).

One of the physiological effects of malnutrition is an increase in oxidative stress, which occurs when the body's production of reactive oxygen species (ROS) exceeds its ability to neutralize these harmful molecules. This imbalance results in cellular damages, particularly in proteins, lipids, and nucleic acids and lead to widespread tissue damages and impairments in immune function. The oxidative stress caused by malnutrition is compounded by the depletion of the body's natural antioxidant defense systems, which are vital for neutralizing ROS. Superoxide Dismutase (SOD), one of the primary antioxidant enzymes, plays a critical role in protecting cells from oxidative damages by dismutating superoxide radicals into less harmful molecules. Malnutrition is often associated with reduced activity of SOD and other antioxidants and further exacerbates the effects of oxidative damage and inflammation (7-9).

Malnutrition can increase oxidative stress in skeletal muscle because skeletal muscle is rich in mitochondria (10). Endogenous antioxidant enzymes, such as SOD play an important role in reducing oxidative stress (11). The important role of antioxidants such as SOD in preventing oxidative stress and cell damage is one of the first-line defense antioxidant enzymes (12). In addition to oxidative stress, malnutrition is also associated with increased levels of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF- $\alpha$ ) that is a multifunctional cytokine produced by various immune cells, including monocytes, macrophages, and neutrophils, and plays a central role in regulating immune responses, inflammation, and apoptosis. An elevated TNF- $\alpha$  level is commonly observed in malnourished individuals, contributing to chronic inflammation and immune dysfunction. A high level of TNF- $\alpha$  can exacerbate the inflammatory process and interfere with the body's ability to combat infections and repair tissue damages (13, 14); therefore, reducing oxidative stress and inflammation, particularly through the regulation of antioxidant enzyme levels and pro-inflammatory cytokines is crucial in addressing the health complications associated with malnutrition.

To combat the negative effects of malnutrition, alternative nutritional interventions using locally available plants and herbs are being explored. Among the promising candidates *Stenochlaena palustris* (*S. palustris*) as a type of green fern found abundantly in Indonesia has been introduced. This plant has been utilized in traditional medicine for its purported

health benefits, including anti-inflammatory, anti-aging, and antimicrobial properties. Recent studies have highlighted the antioxidant potential of *S. palustris*, especially after fermentation, which enhances the bioavailability of its active compounds, such as flavonoids, phenols, and other polyphenolic compounds (15). These compounds have been shown to exhibit potent antioxidant and anti-inflammatory activities (16), suggesting that fermented *S. palustris* may offer a promising strategy for improving nutritional status and mitigating oxidative stress and inflammation associated with malnutrition (17).

Fermentation is a well-established process that can enhance the nutritional profile of foods by increasing the bioavailability of bioactive compounds, including antioxidants. During fermentation, the enzymatic breakdown of complex compounds in plants can increase the concentration of simpler, more bioavailable antioxidants, such as phenols and flavonoids. These compounds are known for their ability to scavenge free radicals, inhibit the production of pro-inflammatory cytokines, and protect cells from oxidative damage (18). The fermentation of *S. palustris* has been shown to produce extracts with high antioxidant activity, as measured by assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), and with no table concentrations of phenolic and flavonoid compounds (17more importantly, their beneficial effect for health. Climbing swamp fern (*Sthenochlaena palustris*).

These findings suggest that fermented *S. palustris* could be a valuable functional food to improve health outcomes in malnourished populations. The potential of medicinal plants to provide antioxidant and anti-inflammatory benefits has been widely documented in scientific literature. A variety of medicinal plants, particularly those rich in phenolic and flavonoid compounds was shown to exhibit significant antioxidant and anti-inflammatory activities (18-21). This study aimed to evaluate the potential of fermented *S. palustris* extract in modulating oxidative stress markers and inflammatory cytokines, specifically SOD and TNF- $\alpha$  in a malnutrition-induced rat model. By assessing these biomarkers, the study searched to determine whether the antioxidant and anti-inflammatory properties of fermented *S. palustris* can ameliorate the physiological and immune dysfunctions associated with malnutrition.

## Materials and Methods

The fermented fern (*S. palustris*) was produced using NaCl, sucrose and LAB *L. plantarum* strain (ATCC-8014) that was fermented for 168 hours at room temperature. The resulting extract contained

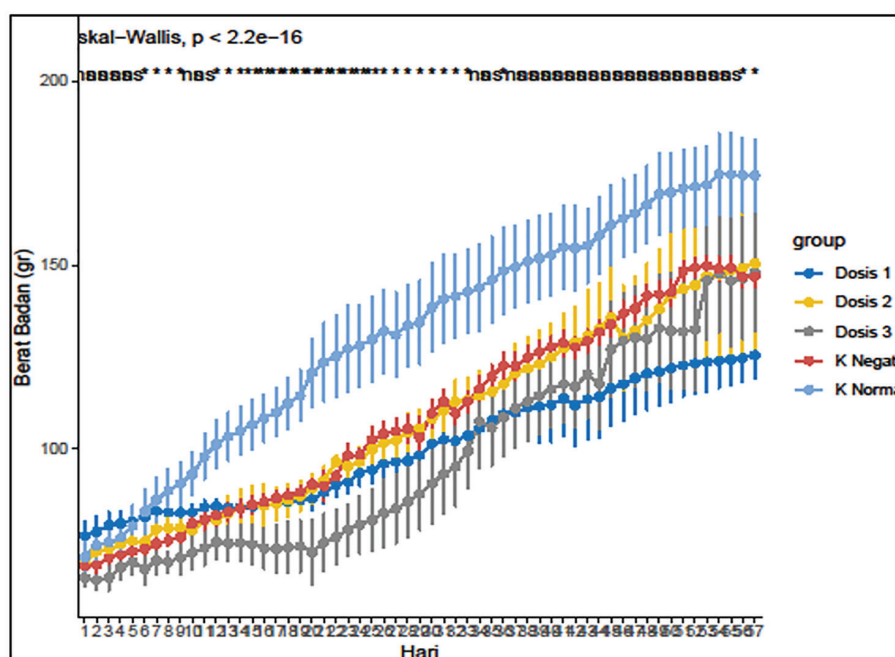
free radical inhibitory bioactivity substances (DPPH%) with  $IC_{50}$  at 315 ppm, 20% protein, 12.01 mg QE/g flavonoids and 17.31 GAE/g phenol (15). It was tested *in vivo* in rats that were modeled. Thirty Sprague-Dawley male rats were used in the study. They were 5-6 weeks old, had body weight of 80-90 grams and were obtained from iRATco Veterinary Laboratory Service. The place of research was at Perum Dramaga Cantik, Dermaga Bogor, Indonesia.

Rat rearing in the study consisted of 3 phases. The first phase (acclimatization) for 7 days aimed to ensure that all rats lived healthy and reached the supposed body weight. They were given crude protein (18%), fat (5.7%) that were according to the standard feeding formula for rodents. Drinking water was constantly available, sufficient, clean, fresh and uncontaminated. Rats placed in one cage were consisted of 6 rats, with an air handling unit facility and an air velocity of 60 Air changes per hour (ACH). Temperature and humidity were controlled with an air conditioner at a temperature range of  $22 \pm 3^\circ\text{C}$  and 55-68% humidity. Cages were lined with clean, dry, pest-free husks and irradiated with ultraviolet radiation. Light came from lamps (artificial light), with 12 hours of light and 12 hours of darkness. The cage area was  $625.5\text{ cm}^2$  with a height of 18.7 cm. The second phase was modeling for 28 days to obtain malnourished and non-malnourished rats. We conducted the modeling phase after the adaptation phase or the 8th day of maintenance (22). The rats were divided into 5 groups including group 1 with good nutritional status that was fed in the adaptation phase. The other group was malnourished that was fed with a low protein diet (8%) for 28 days. Other

treatments were the same as during the adaptation phase. The modeling was confirmed by the growth rate (Figure 1).

The third phase was intervention for 28 days enrolling 6 rats to be fed a normal diet of standard feeding as well as the adaptation phase; but they did not receive the extract. A total of 24 malnourished rats were given the extract of *S. palustris* at graded doses of 0 mg/kgBB as negative control, intervention groups with doses of 100 mg/kg (BB=D1), 300 mg/kg (BB=D2), and 500 mg/kg (BB=D3). Fermented fern extract showed free radical inhibition (DPPH%) of  $IC_{50}$  at 315 ppm, 20% protein, 12.01 mg QE/g of flavonoids and 17.31 GAE/g of phenol as described before (15). Administration of extracts was undertaken by intragastric tubing gavage method. All groups of rats were given unlimited standard feeding (*ad libitum*) and the maintenance was the same as the adaptation phase. The treatments in different groups of rats was illustrated in Table 1.

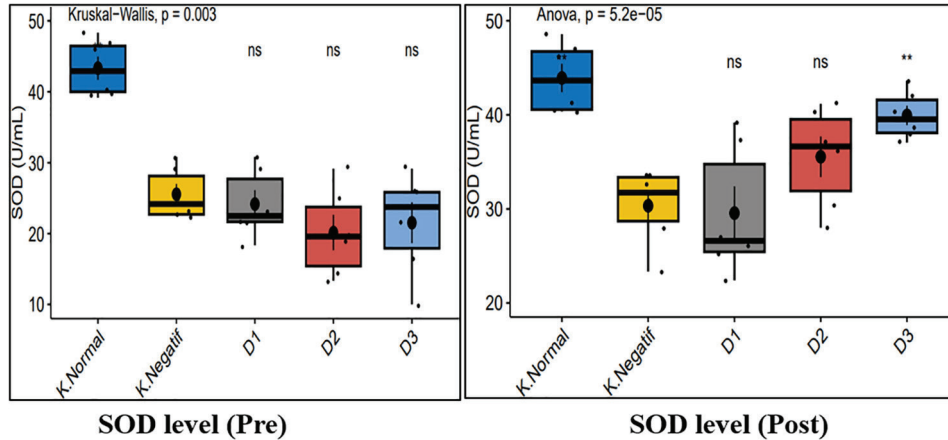
Analysis of SOD and TNF- $\alpha$  levels was carried out employing the ELISA method by measurement of samples on day 28 (end of modeling) that was referred as pre- (after 4 weeks of intervention) and post-measuring (day-56). To examine TNF- $\alpha$  level in rats, serum samples were taken through a blood collection procedure from the retroorbital vein or the heart, and then centrifuged to separate the serum. TNF- $\alpha$  level was assessed using the Enzyme-Linked Immunosorbent Assay (ELISA) method, applying an appropriate commercial kit. The ELISA procedure involved the addition of a specific antibody against TNF- $\alpha$ , followed by the addition of a substrate that produced a signal that could be measured by a spectrophotometer at the appropriate wavelength.



**Figure 1:** The growth at the time of modeling.

**Table 1:** Treatments in different groups of rats.

| Group            | Treatment                                             | Modeling/Malnutrition | Number of rats |
|------------------|-------------------------------------------------------|-----------------------|----------------|
| Control/Normal   | Healthy and normal animals were given standard feed   | No                    | 6              |
| Control/Negative | Malnourished animals without treatment                | Yes                   | 6              |
| Dose: 100 (D1)   | Fermented <i>S. palustris</i> extract (100 mg/kg, BB) | Yes                   | 6              |
| Dose: 300 (D2)   | Fermented <i>S. palustris</i> extract (300 mg/kg, BB) | Yes                   | 6              |
| Dose: 500 (D3)   | Fermented <i>S. palustris</i> extract (500 mg/kg, BB) | Yes                   | 6              |



**Figure 2:** Differences in SOD level between treatment groups (before and after treatments).

**Table 2:** Before and after treatments and the association with Delta SOD Level.

| Variable                 | K_ Normal | Malnutrition intervention Group (mg/kg/day) |       |       |       | P value     |
|--------------------------|-----------|---------------------------------------------|-------|-------|-------|-------------|
|                          |           | 0                                           | 100   | 300   | 500   |             |
| SOD_Before1 <sup>b</sup> | 43.33     | 25.55                                       | 24.17 | 20.14 | 21.53 | 0.002974*   |
| SOD_After1 <sup>a</sup>  | 43.92     | 30.33                                       | 29.55 | 35.55 | 39.94 | 0.772       |
| SOD_Delta1 <sup>b</sup>  | 0.5867    | 4.777                                       | 5.388 | 15.41 | 18.41 | 0.00005561* |
| SOD_Delta1 <sup>b</sup>  | 0.5867    | 4.777                                       | 5.388 | 15.41 | 18.41 | 0.00005561* |

<sup>a</sup>One way ANOVA test, <sup>b</sup>Kruskal Wallis test, \*Meaningful level of significance at  $\alpha=0.05$ .

The results were calculated based on a previously prepared standard curve (23).

To examine SOD level, tissue samples were homogenized in cold buffer solution. The homogenate was then centrifuged to obtain the supernatant. SOD activity was measured employing a spectrophotometric method, based on the inhibition of superoxide-induced nitrobluetetrazolium blue (NBT) formation. The reaction solution contained NBT, substrates (e.g., xanthine and xanthine oxidase), and a specific pH buffer. The decrease in absorbance at a wavelength of 560 nm was calculated to determine SOD activity. The findings were calculated in units per milligram of protein (U/mg protein) (24). Data were analyzed with R software (version 3.6.1). Before testing the difference between groups, the normality test was first carried out. Normally distributed data differed between groups with the Anova test; while those that were not normally distributed, Kruskal Willis was utilized. Test differences in normally distributed groups were evaluated by paired t-test and Wilcoxon rank test for data that were not normally distributed. The level of significance in the study was at  $p<0.05$ .

## Results

The effect of different growth modellings of rats with different feedings was exhibited Figure 1 revealing that the normal-fed rat group had a faster and more stable increase in body weight and growth than the malnourished group with a slower growth and significantly different pattern ( $p=0.000$ ). Meanwhile, feeding a hypoprotein diet (4%) for 14-16 days could cause 22% decrease in body weight confirm that a normal diet could support more optimal growth in rats. The impact of administration (before and after) of green fern (*S. palustris*) extract on SOD level was demonstrated in Figure 2 and Table 2 revealing that the K\_Normal SOD level of healthy rats was higher than that of malnourished animals. However, the administration of fermented fern extract could significantly elevate the SOD level. The largest increase was in group D3 [18.41 U/mL (delta SOD),  $p=0.000$ ].

The SOD level in D3 group was not much different from K\_Normal illustrating the positive effect of the extract to increasing SOD level in malnourished rats. Normal feeding without the extract could increase the DOD level even lower than the other 3 groups



of malnourished animals confirming the important role of adequate nutritional intake that can increase the SOD activity as an antioxidant. We found a dose dependent increasing trend that was followed by an elevation in SOD level (Table 2). Our intervention indicated to a positive effect in rising the SOD level in malnourished rats, especially in the D2 and D3 groups when compared with K\_Normal ( $p=0.0000$ ). Our intervention increased SOD level in all groups and the highest was found in group D3 ( $p=0.002$ ) (Table 3).

The measurement of TNF- $\alpha$  level was carried out similarly as measurement of SOD was conducted at the end of the 4<sup>th</sup> week of modelling and post 4<sup>th</sup> week after intervention. The TNF- $\alpha$  levels before (pre) and after the intervention (post) were presented in Figure 3. We found the extract to be effective in reducing TNF- $\alpha$  level as a pro-inflammatory

cytokine in all groups ( $p=0.002$ ); while the highest reduction was in group D2 at 15.25 pg/mL. Feeding malnourished rats with normal food could reduce TNF- $\alpha$  level even higher than the D1 and D2 groups suggesting that adequate feeding and maintaining in malnutrition can decline the TNF- $\alpha$  level as a pro-inflammatory cytokine. The identification of differences shown for each group was presented in Table 4. Furthermore, the changes in TNF- $\alpha$  level before and after interventions within groups were presented in Table 5 revealing that fern extract increased SOD level and reduced TNF- $\alpha$  level in malnourished rats, while the highest level was in group D2 ( $p=0.000$ ).

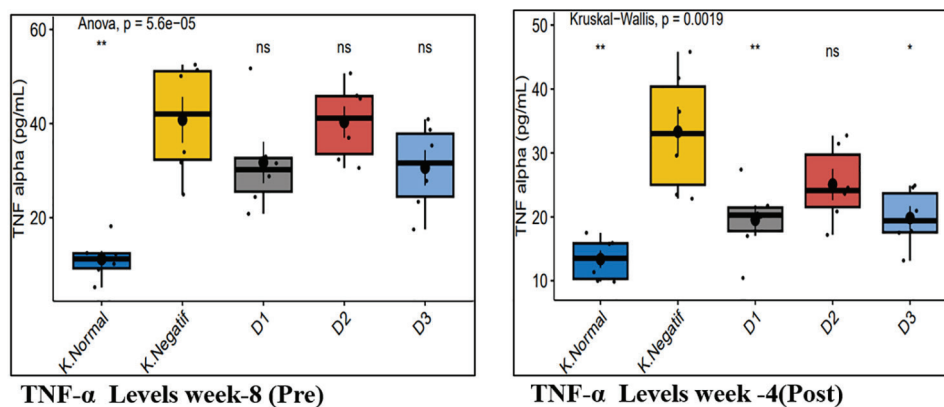
## Discussion

Phenolic compounds, such as flavonoids, phenolic acids, and tannins, are well-known for their

**Table 3:** SOD level (before and after treatments) within-group interventions.

| Measurement | K_Normal              | Malnutrition intervention groups (mg/kg/day) |                       |                        |                        |
|-------------|-----------------------|----------------------------------------------|-----------------------|------------------------|------------------------|
|             |                       | 0 (K_Negatif)                                | 100 (D1)              | 300 (D2)               | 500 (D3)               |
| Before      | 43.33                 | 25.55                                        | 24.17                 | 20.14                  | 21.53                  |
| After       | 43.92                 | 30.33                                        | 29.55                 | 35.55                  | 39.94                  |
| P value     | 0.02575 <sup>a*</sup> | 0.01917 <sup>a*</sup>                        | 0.01236 <sup>a*</sup> | 0.007256 <sup>a*</sup> | 0.002706 <sup>a*</sup> |

<sup>a</sup>Paired t-test, <sup>b</sup>Wilcoxon rank test, \*Meaningful level of significance at  $\alpha=0.05$ .



**Figure 3:** Differences in TNF- $\alpha$  level between treatment groups (Before and after interventions).

**Table 4:** TNF- $\alpha$  level before and after interventions between groups.

| Measurement              | Variable | Malnutrition intervention group (mg/kg/day) |          |          |          | P value  |
|--------------------------|----------|---------------------------------------------|----------|----------|----------|----------|
|                          |          | 0 (K_Negatif)                               | 100 (D1) | 300 (D2) | 500 (D3) |          |
| TNF_BeforeI <sup>a</sup> | 11.185   | 40.795                                      | 31.750   | 40.317   | 30.610   | 0.677    |
| TNF_AfterI <sup>b</sup>  | 13.377   | 33.323                                      | 19.533   | 25.068   | 19.820   | 0.08824* |
| TNF_Delta <sup>a</sup>   | -2.192   | 7.4717                                      | 12.217   | 15.25    | 10.79    | 0.00286* |

<sup>a</sup>One way ANOVA test, <sup>b</sup>Kruskal Wallis test, \*Meaningful level of significance at  $\alpha=0.05$ .

**Table 5:** TNF- $\alpha$  level (before and after interventions) within groups.

| Indicator     | Measurement | Control (Normal)      | Malnutrition intervention group |                       |                         |                        |
|---------------|-------------|-----------------------|---------------------------------|-----------------------|-------------------------|------------------------|
|               |             |                       | 0 mg/kg                         | 100 mg/kg             | 300 mg/kg               | 500 mg/kg              |
| TNF- $\alpha$ | Before      | 11.185                | 40.795                          | 31.750                | 40.317                  | 30.610                 |
|               | After       | 13.377                | 33.323                          | 19.533                | 25.068                  | 19.820                 |
|               | P value     | 0.03125 <sup>b*</sup> | 0.01009 <sup>a*</sup>           | 0.01004 <sup>a*</sup> | 0.0001713 <sup>a*</sup> | 0.004829 <sup>a*</sup> |

<sup>a</sup>Paired t-test, <sup>b</sup>Wilcoxon rank test, \*Meaningful level of significance at  $\alpha=0.05$ .

ability to scavenge free radicals, thus reducing oxidative stress (25). In addition, these compounds can modulate the expression of inflammatory cytokines, including TNF- $\alpha$ , by interfering with key signaling pathways that can regulate inflammation (25). For example, certain plant extracts have been shown to inhibit the activation of NF- $\kappa$ B, a transcription factor involved in the expression of pro-inflammatory cytokines (26). These properties make phenolic-rich plants potential candidates for the development of therapeutic agents to combat oxidative stress and chronic inflammation, which are prevalent in conditions such as malnutrition (27). Flavonoids, a class of polyphenolic compounds were found abundantly in many plants and were shown to possess strong anti-inflammatory effects. Flavonoids were demonstrated to modulate immune cell activity, reduce the production of pro-inflammatory cytokines such as TNF- $\alpha$ , and prevent oxidative damage to cells (24, 28).

These compounds have been linked to the reduction of chronic inflammation in various diseases, including cardiovascular diseases, diabetes, and inflammatory bowel diseases (29). Furthermore, flavonoids are known to enhance the activity of endogenous antioxidant enzymes, such as SOD and catalase, thus providing an additional layer of protection against oxidative damage (30). The presence of such compounds in fermented *S. palustris* makes it a promising candidate for alleviating the oxidative and inflammatory burdens associated with malnutrition. TNF- $\alpha$  plays a pivotal role in regulating immune responses and inflammation and is produced by various cells, including macrophages, neutrophils, and T-cells, in response to infection, injury, or stress. TNF- $\alpha$  is a key mediator of the inflammatory responses, involved in regulating the activation of immune cells, the expression of adhesion molecules, and the induction of other cytokines and chemokines (31).

However, excessive production of TNF- $\alpha$  is associated with chronic inflammation and tissue damage, which can lead to various inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and, in the case of malnutrition, impaired immune function and increased susceptibility to infections (32). In malnutrition, particularly in children, the immune system is compromised, and the production of TNF- $\alpha$  is often dysregulated. Elevated level of TNF- $\alpha$  has been found to contribute to the inflammatory responses observed in malnourished individuals that can further exacerbate the immune dysfunction and the cycle of nutritional deficiency. So reducing TNF- $\alpha$  level can be a critical therapeutic

target in improving immune function and reducing inflammation in malnourished populations (13).

A Recent study has highlighted the potential of natural products, including medicinal plants, to modulate TNF- $\alpha$  level and alleviate chronic inflammation. Fermented *S. palustris* extract, with its rich content of antioxidant and anti-inflammatory compounds, may offer an effective means of lowering TNF- $\alpha$  level, thus improving immune responses and reducing inflammation in malnourished individuals. *S. palustris* has been introduced as a plant native to Southeast Asia and is known for its rich nutritional profile, including high levels of protein, vitamins, and minerals. However, its susceptibility to spoilage due to its high moisture content requires processing techniques, such as fermentation, to enhance its shelf life and nutritional value. Fermentation not only improves the stability of the plant but also increases the bioavailability of its bioactive compounds, including polyphenols and flavonoids (33).

These compounds were shown to exhibit potent antioxidant and anti-inflammatory activities, which may help mitigate the adverse effects of oxidative stress and inflammation in malnutrition. A previous study demonstrated that fermented *S. palustris* contains significant amounts of phenolic and flavonoid compounds, which are responsible for its antioxidant properties. *S. Palustris* is widely found in Indonesia including in West Kalimantan; where the plant is obtained, thrived on peatlands, and has been used by the community as a vegetable, and in some regions as traditional medicine that can slow down aging, treat anemia and to increase breast milk supply (33). *S. palustris* is high in nutrient and water content, so it is easily rotten and needs processing technology through fermentation to be stored. It was shown that the fermented compound can scavenge free radicals, inhibit oxidative damage to DNA and lipids, and enhance the activity of antioxidant enzymes, such as SOD (15). Additionally, fermentation can increase the solubility and absorption of these bioactive compounds, making them more accessible to the body and enhancing their therapeutic effects. Therefore, the potential therapeutic benefits of fermented *S. palustris* extract in malnutrition-related oxidative stress and inflammation warrant further investigation (33).

*S. palustris* referred to as pakis hijau has garnered attention for its antioxidant properties; while its fermentation enhances the bioavailability of phytochemicals such as phenolic compounds and flavonoids which exhibit significant antioxidant activities. They act as hydrogen donors that can effectively scavenge the free radicals and thereby mitigate oxidative damage (34). They enhance

the activity of SOD and catalase too (35, 36). The induction of these enzymes can convert harmful superoxide radicals into less toxic substances and thereby reduce the inflammation and protect the cellular integrity (37). Moreover, the fermentation process of *S. palustris* can increase bioactive compounds due to the metabolic activities of microorganisms and lead to production of additional antioxidant peptides (38).

This synergistic effect can amplify the overall antioxidant capacity of the plant, contributing to a more robust defense against oxidative stress and inflammation. In terms of inflammatory biomarkers, it was shown that the consumption of antioxidant-rich foods can lead to a significant reduction in pro-inflammatory cytokines such as TNF- $\alpha$  (39). By lowering TNF- $\alpha$  level, *S. palustris* may help alleviating chronic inflammation, which is often exacerbated by oxidative stress. The relationship between antioxidants and inflammation is further supported by findings that highlight the role of Nrf2, a transcription factor that regulates the expression of antioxidant proteins and is involved in the cellular response to oxidative stress (40). Therefore, these findings are in agreement with our results revealing that fermentation of *S. palustris* not only enhances its antioxidant properties through the increased availability of phenolic compounds and flavonoids; but also plays a pivotal role in modulating inflammatory responses by reducing biomarkers such as TNF- $\alpha$  and enhancing the activity of protective enzymes like SOD. This multifaceted approach underscores the potential of *S. palustris* as a functional food with significant health benefits.

### Conclusion

Fermentation of *S. palustris* was shown to increase the content of bioactive compounds and have the potential as antioxidant and anti-inflammatory agents through inhibition of inflammatory pathways, enhancement of antioxidant enzyme activity, and modulation of macrophage polarity. *S. palustris* could contribute to lowering inflammatory biomarkers such as TNF- $\alpha$  and increasing SOD activity.

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Not available.

### Authors' Contribution

JG as first author: conducting research with the team, responsible for conducting laboratory

tests, fermenting fern leaves, providing input to laboratory technicians. TCM as second author as well as corresponding author prepared manuscript for research publication. Translated Indonesian to English and adjusted the manuscript layout to the intended journal. MM as the third author was responsible for data analysis, conducting statistical tests, and summarizing the results of the study. IS as the fourth author helped to find research materials, assisted in the analysis of fern leaf fermentation, and made research reports.

### Conflict of Interest

We state that there is no conflict of interest in the research we conducted.

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