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Spirulina polysaccharide as adjuvant therapy enhances the TNF-α, IFN-γ response and improve liver and spleen histopathological of mice (*Mus musculus*) infected with *Plasmodium berghei*

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Abstract

The use of adjuvant therapy can be combined with primary anti-malarial drugs to increase the efficacy and effectiveness of anti-malarial drugs and reduce more severe complications caused by their pathogenicity. This study aims to evaluate the potential of PSP (polysaccharide spirulina) as an adjuvant therapy in the response of TNF-α, IFN-γ and histopathological changes in the liver and spleen of mice infected with *Plasmodium berghei.* This experimental involving forty mices, divided into eight treatment groups: A1: Mice not infected. A2: Mice infected. B1: Mice infected and treated with artemisinin (ART) at a dose of 40 mg/kg BW. B2: Mice infected and treated with PSP at a dose of 600 mg/kg BW. B3, B4 and B5: Mice infected and treated with ART dose 40 mg/kg BW and PSP 400 mg/kg BW, 600 mg/kg BW and 800 mg/kg BW. B6: Mice not infected but treated with PSP (600 mg/kg BW). Mice were infected with 0.2 ml of 1×10^7 parasitized red blood cells (PRBCs) of *Plasmodium berghei*. Treatments were administered for 10 days. Following this, histopathological changes and immunohistochemical staining were examined to be analysed. These results showed that the uninfected groups (A1 and B6) showed significantly reduced levels of hemozoin, sinusoid congestion, Kupffer cell hyperplasia, and portal inflammation ($p < 0.05$) compared to the infected groups (A2, B1, B2, B3, B4, and B5). The spleen hemozoin and the diameter of the white pulp in group A1 were significantly different ($p < 0.05$) compared to all other groups (A2, B1, B2, B3, B4, B5, and B6). Expression of TNF-α and IFN-γ in liver and spleen cells was significantly lower ($p < 0.05$) in the negative control group (A1) compared to groups A2, B2, B3, B4, and B5.This study conlcudes that spirulina polysaccharide (PSP) as an adjuvant therapy could enhance the expression of TNF-α and IFN-γ in liver and spleen cells infected with *Plasmodium berghei*. The combination of ART and PSP at an effective dose of 400 mg/kg BW reduced hemozoin accumulation and improved liver and spleen histopathology associated with *Plasmodium berghei* infection.

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Introduction

Increased artemisinin resistance (ART) poses a significant threat to achieving malaria elimination in Asia by the 2030 target and could disrupt control efforts in other endemic regions (1). The use of adjuvant therapy as a supplementary treatment can be combined with primary antimalarial drugs to enhance their efficacy and

effectiveness, as well as to mitigate severe complications arising from the pathogenicity of malaria (2). In vivo research demonstrates that the Polysaccharide of *Spirulina platensis* (PSP), when used as an adjuvant therapy, can reduce parasitaemia levels, increase parasite growth inhibition, and lower the damage index of the liver and spleen in mice infected with *Plasmodium berghei* a model organism for malaria parasites (3). Further investigation is needed to explore the role of PSP in enhancing proinflammatory cytokines, particularly TNF-α and IFN-γ, in malaria. Such studies could provide insights into improving the efficacy and effectiveness of antimalarial drugs. Artemisinin is an antimalarial drug that effectively kills *Plasmodium* to reduce malaria morbidity and mortality rates worldwide. Its use has been shown to decrease parasitaemia levels by 51% in *Plasmodium falciparum* infections and by 28% in *Plasmodium vivax* infections (4). However, *in vitro* studies have revealed that repeated exposure to artemisinin can lead to point mutations in the *Pfatpase6* gene of the *Plasmodium falciparum* strain 2300, which are associated with artemisinin resistance (5). Similarly, *in vivo* studies have shown evidence of artemisinin resistance in mice repeatedly infected with *Plasmodium berghei* (6). Effective malaria treatment is crucial for combating this disease. Developing adjuvant therapies has become a strategic effort to reduce parasite burden and lower mortality rates by controlling the manifestations of disease pathogens (7). *Spirulina*, a species of microalgae, is frequently used in the health sector due to its various health benefits (8). Multiple studies have demonstrated that *Spirulina* possesses immunostimulatory effects both *in vitro* and *in vivo*. These effects include enhancing macrophage phagocytosis, increasing the accumulation of natural killer (NK) cells in tissues, stimulating antibody and cytokine production, activating and mobilizing T and B cells, and upregulating the expression of genes encoding IL-17, TNF-α, and IFN-γ (9).

This study aims to evaluate the potential of PSP (polysaccharide spirulina) as an adjuvant therapy in the response of TNF-α, IFN-γ and histopathological changes in the liver and spleen of mice infected with *Plasmodium berghei*.

Materials and methods

Ethical approval

This study was approved under certificate number 1/KE.020.01.2021 by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia.

Parasite, host and drug used in the research.

The parasite used in this study was *Plasmodium berghei* strain ANKA, obtained from the Institute of Tropical Disease (ITD), Airlangga University, malaria laboratory section. Male Swiss albino mice weighing 20–30 g and aged 2.5 months were used as hosts. These mice were sourced from the Veterinary Farma Surabaya (Pusvetma) Center. Artemisinin was purchased from Sigma. Rabbit IFN-γ polyclonal antibody and mouse TNF-α monoclonal antibody were procured from Santa Cruz Biotechnology. Spirulina powder was obtained from Gadjah Mada University.

In vivo antimalarial activity test in experimental animals

Forty male mice were divided into eight groups for this study. The groups were as follows A1: Mice not infected. A2: Mice infected. B1: Mice infected and treated with artemisinin (ART) at a dose of 40 mg/kg BW. B2: Mice infected and treated with PSP at a dose of 600 mg/kg BW. B3: Mice infected and treated with ART (40 mg/kg BW) and PSP (400 mg/kg BW). B4: Mice infected and treated with ART (40 mg/kg BW) and PSP (600 mg/kg BW). B5: Mice infected and treated with ART (40 mg/kg BW) and PSP (800 mg/kg BW). B6: Mice not infected but treated with PSP (600 mg/kg BW). The infection was induced by administering 0.2 mL of *P. berghei* (1×10^7) intraperitoneally. Therapy began 3 hours post-infection and continued for four days. On the seventh day post-infection, the mice were euthanized, and their organs, including the spleens and livers, were surgically removed following the necropsy procedure. The organs were then placed in a container filled with 10% formalin phosphate buffer for histopathological preparation, including Hematoxylin and Eosin (HE) staining and Immunohistochemistry (IHC) staining for further examination.

Polysaccharide *Spirulina platensis* extraction

For the extraction process, 40 g of *Spirulina platensis* powder was added to 1.6 liters of water. The pH of the mixture was adjusted to 10 by adding 1 mol/L NaOH. The solution was stirred continuously using a magnetic stirrer for 8 hours at 80°C. Afterward, the precipitate was discarded, and the resulting solution was centrifuged at 4000 rpm for 20 minutes. The supernatant was then collected and concentrated to one-fifth of its original volume. Next, 95% ethanol was added to the supernatant, and the process was repeated five times. The mixture was incubated overnight at 4°C in a refrigerator. After incubation, the solution was centrifuged at 4300 rpm for 10 minutes, and the precipitation was collected. The precipitate was washed twice with absolute acetone (using twice the volume of the precipitate) and filtered. Finally, the precipitate was frozen for 2 hours and stored for further use (10).

Organ histopathology preparation and assessment

The histopathology of the organs in this study was assessed through microscopic examination of liver and spleen samples stained with Hematoxylin and Eosin. The samples were observed using a light microscope at 400X magnification in five fields of view. Histopathological

assessment of the liver was based on changes in four histological parameters: sinusoid congestion, hemozoin deposition, portal track area inflammation, and Kupffer cell hyperplasia. Scoring for these parameters was as follows Sinusoid congestion:0: No congestion 1: 3-5 adjacent erythrocytes in the sinusoids 2: 6-8 adjacent erythrocytes in the sinusoids 3: >9 adjacent erythrocytes in the sinusoids. Hemozoin deposition: 0: No deposition 1: <10% of the field of view 2: 10-20% of the field of view 3: >20% of the field of view. Inflammation of the portal track area: 0: <5% of the portal track area 1: 5-15% of portal track area 2: 16-30% of portal track area 3: >30% of the portal track area. Kupffer cell hyperplasia: 0: <20 Kupffer cells 1: 20-35 Kupffer cells 2: 36-50 Kupffer cells 3: >50 Kupffer cells (11). Histological assessment of the spleen was performed using a light microscope to observe two parameters: the white pulp diameter at 100X magnification and hemozoin deposition at 400X magnification. Each parameter was assessed from five fields of view (12). The diameter of the white pulp was measured as the average of two perpendicular lines: the longest line of the white pulp diameter and another line perpendicular to it. The scoring for histological changes in the spleen was as follows: Hemozoin deposition: 0: No deposition 1: <10% of the field of view 2: 10-20% of the field of view 3: >20% of the field of view.

Determination of TNF-α and IFN-γ cytokine expression

The expression of cytokines in this study refers to the pro-inflammatory cytokines TNF-α and IFN-γ in liver and spleen cells, assessed using immunohistochemical staining. The staining produced yellow to brown color indicators, evaluated using the UltraVision Detection System. Immunohistochemical histopathological examination was conducted to determine the expression of TNF-α and IFN-γ in liver and spleen cells of mice (immunolabeling). Cytokine expression in each sample was assessed semi-quantitatively using the modified Remmele Immuno Reactive Score (IRS) method. Observations were made under a microscope at 400X magnification across five different fields of view. The IRS score for each sample was calculated by multiplying the score for the percentage of positive cells by the score for color intensity. The resulting IRS points ranged from 0–12 and were classified into ranges according to the IRS scoring system (0–3) (12). Scoring for TNF-α and IFN-γ Expression (Remmele Method): Percentage of Positive Cells: 0 = No positive cells 1 = <10% 2 = 10–50% 3 = 51–80% 4 = >80%. Color Reaction Intensity: 0 = No color 1 = Low 2 = Medium 3 = High. IRS Scoring Range: 0 = 0–1 point. 1 = 2–3 points 2 = 4–8 points, 3 = 9–12 points (13).

Data analysis

The data obtained from the scoring of organ histopathological changes (hemozoin deposition in the liver and spleen, sinusoid congestion, Kupffer cell hyperplasia, and portal track inflammation) and cytokine expression were analysed using the Kruskal-Wallis test, followed by the Z test for pairwise comparisons.

Results

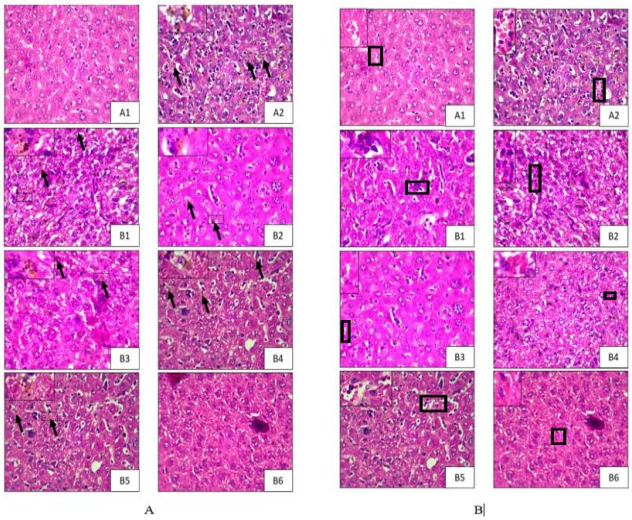
Liver histology scoring

In the liver, hemozoin levels in the uninfected groups (A1 and B6) were significantly different (P<0.05) compared to the infected groups, both treated and untreated (A2, B1, B2, B3, B4, and B5). Sinusoid congestion decreased significantly (P<0.05) in groups B1, B2, B4, and B5. Kupffer cell hyperplasia also showed a significant decrease (P<0.05) in groups B2, B4, and B5. Additionally, portal inflammation decreased significantly (P<0.05) in groups B2, B3, B4, and B5 (Table 1; Figures 1 and 2).

Table 1: Histopath means rank values and pro-inflammatory cytokine expression (TNF-α and IFN-γ) of liver mice infected with *P. berghei* and given PSP and ART using the Z test

	Hemozoin		Congestion of sinusoid		Kupffer Cell hyperplasia		Inflammatory portal track		IFN-c		TNF-a	
	Score	Mean	Score	Mean	Score	Mean	Score	Mean	IRS	Mean	IRS	Mean
A1	0	5.50^{a}	0.4	5.50^{a}	0.36	4.00^{a}	0.8	4.90^{a}	0.3	3.38^{a}	0.3	2.63^{a}
A2	2.44	35.90^{c}	1.88	36.50^{an}	2.28	35.10^{c}	2.56	35.40^{d}	2	26.00^{d}	1.85	26.38^{d}
B1	1.48	18.80^{b}	1	18.60^{bc}	1.16	16.00^{fro}	1.16	10.40^{fro}	0.85	8.75^{fro}	0.95	11.00^{ab}
B2	2.28	34.10^{c}	1.48	28.70	1.96	29.50^{c}	2.08	26.40^{cd}	1.85	24.50^{d}	1.8	24.88^{d}
B3	1.48	18.00^{b}	0.92	16.60^{ab}	1.04	14.30^{fro}	1.48	17.30^{bc}	1.65	20.38^{cd}	1.55	21.13^{cd}
B4	1.76	25.00^{bc}	1.32	25.40^{cd}	1.64	23.90^{bc}	2.4	32.20^{d}	1.55	18.50^{bc}	1.35	17.88^{bc}
B5	1.6	21.20^{b}	1.16	22.20^{bc}	2.12	32.00^{c}	2.2	27.80^{cd}	1.6	19.13^{bc}	1.4	18.88^{bc}
B6	0	5.50^{a}	0.64	10.50^{fro}	0.76	9.20^{a}	1.12	9.60^{fro}	1	11.38^{ab}	0.9	9.25^{fro}

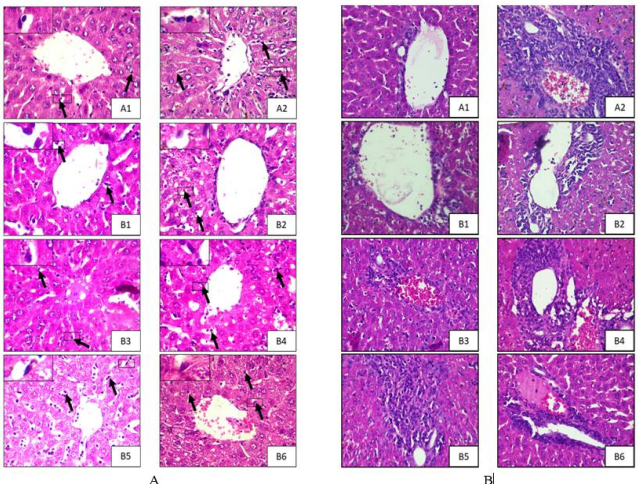
a,b Different superscripts in the same column show significant differences at P<0.05.



Figures 1: A. Microscopic images of liver hemozoin deposition; black arrow = hemozoin. B sinusoid congestion; Black Box: Congestion in sinusoids. Between groups of mice with Hematoxylin eosin stain of 400x magnification.

Spleen histology scoring using Hematoxylin Eosin (HE) staining

In the spleen, hemozoin levels increased significantly (P<0.05) when compared to groups A1, B1, B2, B3, B4, and B6. The diameter of the white pulp in group A1 was significantly different (P<0.05) from all treatment groups (A2, B1, B2, B3, B4, B5, and B6). The single ART therapy group (B1) experienced a significant decrease (P<0.05) compared to the positive control group (A2), the single PSP600 therapy group (B2), the PSP control group (B6), and all combination therapy groups (B3, B4, and B5). The ART+PSP400 combination therapy group (B3) and the PSP control group (B6) showed a significant decrease (P<0.05) compared to the positive control group (A2). These findings are detailed in table 2.



Figures 2: A. Microscopic images of Liver Kupffer cell hyperplasia. Black arrow: Kupffer cell. B. Portal inflammation. Between groups of mice with Hematoxylin eosin stain of 400x magnification.

Table 2: Histopath means rank values and pro-inflammatory cytokine expression (TNF-α and IFN-γ) of spleen mice infected with *P. berghei* and given PSP and ART using the Z test

	Hemozoin		White pulp diameter	IFN-c		TNF-a	
	Score	Mean	Mean±SD	IRS	Mean	IRS	Mean
A1	0	5.50^{a}	297.1±9.06^{a}	0.1	3.00^{a}	0.3	4.50^{a}
A2	2.52	37.40^{c}	529.62±35.72^{d}	2	25.38^{c}	1.15	18.13^{bc}
B1	1.56	20.50^{b}	354.82±14.78^{b}	1	12.75^{fro}	0.8	12.88^{ab}
B2	1.84	25.90^{bc}	525.22±51.12^{d}	2.1	26.63^{c}	1.75	25.88^{d}
B3	1.44	17.60^{fro}	437.88±31.13^{c}	1.4	17.75^{bc}	1.55	22.88^{cd}
B4	1.76	24.70^{b}	494.98±39.58^{d}	1.3	16.13^{bc}	1.3	19.63^{bc}
B5	1.84	26.90^{bc}	513.25±21.51^{d}	1.6	20.50^{bc}	1.4	19.25^{bc}
B6	0	5.50^{a}	413.56±29.01^{c}	0.75	9.88^{fro}	0.55	8.88^{fro}

a,b Different superscripts in the same column show significant differences at P<0.05.

Liver histology scoring using Immunohistochemistry (IHC) staining

The levels of IFN-γ in the liver of mice in the negative control group (A1) showed no significant difference (P>0.05) compared to the ART single therapy group (B1) and the PSP control group (B6). However, IFN-γ levels decreased significantly (P<0.05) when compared to groups A2, B2, B3, B4, and B5. Similarly, the levels of TNF-α in the liver of mice in the negative control group (A1) were not significantly different (P>0.05) from those in the ART single therapy group (B1) and the PSP control group (B6). However, TNF-α levels decreased significantly (P<0.05) when compared to groups A2, B2, B3, B4, and B5 (Figures 3).

Spleen histology scoring using Immunohistochemistry (IHC) staining

The levels of IFN-γ in the spleen of mice in the negative control group (A1) showed no significant difference (P>0.05) compared to the ART single therapy group (B1)

and the PSP control group (B6). However, IFN-γ levels decreased significantly (P<0.05) when compared to groups A2, B2, B3, B4, and B5. Similarly, the levels of TNF-α in the spleen of mice in the negative control group (A1) were not significantly different (P>0.05) from those in the ART single therapy group (B1) and the PSP control group (B6). However, TNF-α levels decreased significantly (P<0.05) when compared to groups A2, B2, B3, B4, and B5 (Figures 4).

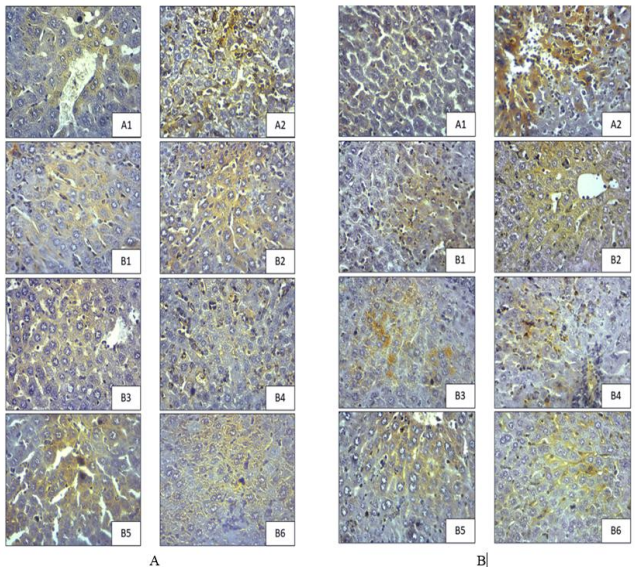
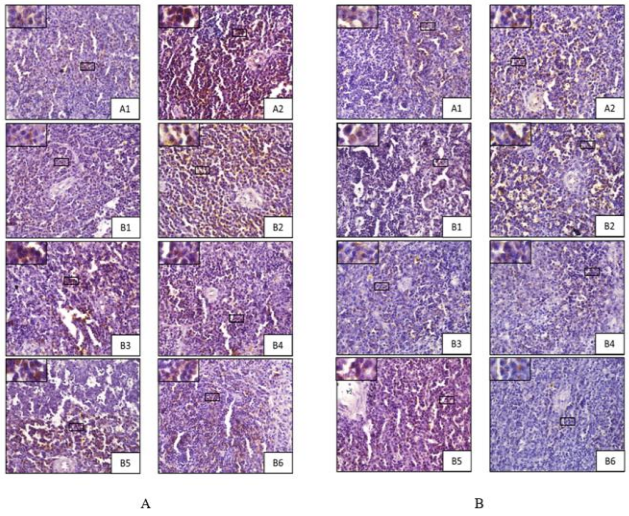


Figure 3: A. Expressing TNF-α cytokines in mice liver B. Expressing IFN-γ cytokines in liver between groups of mice with 400x magnification immunohistochemical staining.



Figures 4: A. Microscopic images of cells expressing IFN-γ cytokines. B. Cells expressing the cytokine TNF-α in the spleen. Between groups of mice with 400x magnification immunohistochemical staining.

Discussion

Polysaccharide of *Spirulina platensis* (PSP) as an adjuvant therapy in combination with artemisinin (ART), the primary antimalarial drug, on pro-inflammatory cytokine responses in mice infected with *Plasmodium berghei* at a dose of 10^7. *Spirulina platensis* is an alga with antioxidant and immunomodulatory properties that can serve as an adjuvant therapy for malaria infections. This study utilized crude polysaccharide extracts containing varying molecular weights, sulphate content, monosaccharides, and proteins (14). These crude extracts may influence the activity and mechanism of PSP in modulating the immune system. The PSP crude extract used in this study had a carbohydrate content of 13.31 mg/ml and a protein content of 0.671 mg/ml.

Additionally, the study employed ART as the primary antimalarial drug, which is the first-line treatment for malaria worldwide. The mechanism of ART involves its interaction with iron in free heme, leading to the activation of the endoperoxide bridge within the food vacuole of *Plasmodium* (15,16). The hepatic response to erythrocytic-stage *P. berghei* infection is characterized by hemozoin deposition, sequestration of infected erythrocytes, sinusoid congestion, increased hepatocyte apoptosis, activation of stellate and Kupffer cells, infiltration of inflammatory cells, increased fibrosis, and liver damage (3,17).

Hemozoin can serve as an indicator of *Plasmodium* replication in the body, as infected erythrocytes release merozoites and hemozoin into the bloodstream when ruptured schizonts break apart (18). The results of this study indicate that intraperitoneal infection with *Plasmodium berghei* at a dose of 10^7 parasites in 0.2 ml of blood led to the accumulation of hemozoin in the liver and spleen of mice in the infected group. These results indicate that after infected erythrocytes undergo lysis, hemozoin is released into the circulation and subsequently taken up by macrophages in the liver and spleen. Recent studies have shown that hemozoin accumulation in the liver of *Plasmodium berghei* infected mice correlates with the activation of Kupffer cells and increased expression of pro inflammatory cytokines such as TNF-α and IL-1β (19). The positive control group (A2) showed a significant decrease when compared to the ART therapy groups, both single (B1) and combination groups (B3, B4, and B5). The antimalarial mechanism of ART involves its interaction with iron in free heme, which activates the endoperoxide bridge within *Plasmodium* food vacuoles (15,16). Pharmacodynamic studies have revealed that the 1,2,4-trioxane compound in ART plays a crucial role in endoperoxide activity (20). Additionally, ART is thought to inhibit hemozoin biocrystallization (21). The inhibition of hemozoin leads to the accumulation of hematin (ferriprotoporphyrin IX), which disrupts parasite metabolism and is toxic to the parasite (22). In comparing the single therapy group of ART (B1) with the combination ART+PSP

groups (B3, B4, and B5), no significant differences were observed across the various doses administered. Similar findings have been reported in previous studies, where the degree of parasitaemia of *P. berghei* was not significantly different among ART+PSP combination therapy doses of 400 mg/kg BW, 600 mg/kg BW, and 800 mg/kg BW on day 7 (3). This may be attributed to the activity of PSP compounds, which can inhibit the production of reactive oxygen species (ROS), one of ART's primary mechanisms for killing *Plasmodium*. PSP is known to have strong antioxidant properties, which can neutralize reactive oxygen species (ROS). Therefore, the combination of ART with PSP may reduce the effectiveness of ART in killing the parasite, as PSP inhibits the production of ROS that is essential for ART's antiparasitic activity. This explains why there is no significant difference in the reduction of parasitemia between ART monotherapy and the ART+PSP combination therapy. Artesunate, a derivative of ART, works by producing reactive oxygen species (ROS) within parasites (23). The presence of endogenous antioxidant supplements is a known factor contributing to treatment failure when combined with artesunate (24). One of the amino acids found in *Spirulina*, cysteine, plays a key role in the synthesis of glutathione, the most abundant endogenous antioxidant in the body. However, cysteine availability for glutathione synthesis is often limited in certain cells, such as hepatocytes (25).

The insignificant results observed between the ART+PSP600 (B4) combination therapy group and the positive control group (A2) could suggest a counterproductive interaction between ART and PSP therapy. The ART+PSP800 (B5) therapy group might exhibit mechanisms that favor immune cell activation or the direct elimination of *Spirulina's* active ingredient, phycocyanin. Phycocyanin in PSP is believed to inhibit *Plasmodium* growth by disrupting hemozoin polymerization, binding to ferriprotoporphyrin-IX, a molecule harmful to *Plasmodium* metabolism (26). This mechanism may also explain why there was no significant decrease in the PSP600 (B2) single-stage group, although hemozoin deposition tended to decrease when compared to the positive control group (A2). In vitro studies have shown that PSP has potential as a primary antimalarial, with an IC50 value of 5.43 µg/ml, indicating its effectiveness in inhibiting the growth of *Plasmodium falciparum* 3D7. ART+PSP combination therapy at a dose of <400 mg/kg BW may reduce hemozoin deposition more effectively than ART (B1) single therapy. This is because the hemozoin score in the spleen for the 400 mg/kg BW dose was not significantly different from the negative control group (A1). *Plasmodium* infection can cause histological changes in the liver, one of which is sinusoid congestion (3). The control group showed some improvement in the sinusoid congestion score, though this was not significant compared to the negative control (A1). This aligns with previous research, which found that a Spirulina dose of 500 mg/kg BW caused slight congestion in liver sinusoids (27). Additionally, *Spirulina* polysaccharides have been shown to increase erythrocyte and leukocyte levels in the bone marrow, as well as raise haemoglobin levels, by stimulating erythropoietin secretion (28). The ART+PSP combination therapy group of 400 (B4) and 800 (B5) had an increase in Plasmodium followed by an increase in erythrocytes thus preventing mouse death. Plasmodium in erythrocytes uses protease enzymes to break down hemoglobin into heme and globin, where globin will become amino acids for parasite development. Hemozoin and glycosylphosphatidylinositol (GPI) attached to parasite antigens have been shown to affect erythropoiesis during Plasmodium infection. Hemozoin promotes early apoptosis of erythroid precursors (29). Plasmodium falciparum infection causes Hb degradation of about 60%-70% (20). The nature of PSP that stimulates erythropoietin secretion which further stimulates the formation of erythrocytes in the spinal cord quickly can prevent anemia which can cause hypoxia in cells in organs because this erythrocytic stage also causes severe anemia if it continues to run due to lysing erythrocytes (30). The liver of mice infected with *P. berghei* also exhibited immune cell infiltration, Kupffer cell hyperplasia (macrophages in the liver), and inflammation of portal channels, similar to the effects of *Plasmodium* infection in humans (17,11). These changes were observed in all infected groups (A2, B1, B2, B3, B4, and B5), with a significant increase compared to the uninfected groups (A1 and B6). *Spirulina's* activity as an immunostimulant occurs through the NF-kB signalling pathway via the TLR4 receptor on macrophages, such as Kupffer cells. Activated macrophages secrete TNF-α, a pro-inflammatory cytokine, and nitric oxide (NO), both of which enhance parasite phagocytosis activity (31,32). *Spirulina* is also believed to increase IL-12 secretion by macrophages, which, in turn, stimulates IFN-γ secretion from NK cells and T cells (33). This aligns with the results of the present study, which showed a tendency for increased expression of TNF-α and IFN-γ cytokines in the liver of infected mice. Kupffer cell hyperplasia in the ART single therapy group (B1) showed a significant decrease compared to the positive control group (A2). This could be due to the anti-inflammatory properties of ART, which may inhibit TNF-α. Research has shown that TNF-α levels in the ART single therapy group (B1) were lower than in both the positive control group (A2) and the single PSP therapy group (B2). In vitro studies using a murine macrophage cell line (RAW264.7) revealed that dihydroartemisinin (a derivative of ART) inhibits the secretion of TNF-α, IL-6, and NO by reducing the regulation of iNOS proteins (34).

The comparison of Kupffer cell hyperplasia in the ART+PSP400 (B3) combination therapy group showed no significant difference from the PSP control group (B6), which supports the effectiveness of the combination therapy at this dose, along with the results on hemozoin deposition

and sinusoid congestion. Kupffer cell hyperplasia can serve as an indicator of *Plasmodium* accumulation in the host's body. The PSP 400 mg/kg BW dose in group B3, when used as adjuvant therapy, may not significantly inhibit ART's mechanism for malaria elimination, likely due to the smaller dose. This finding reinforces the previous hypothesis that ART+PSP combination therapy at a dose of <400 mg/kg BW may more effectively reduce hemozoin deposition. Kupffer cells, which are macrophages in the liver, play a crucial role in preventing malaria severity and parasite release into the bloodstream (35). This correlates with the hemozoin deposition results, where a decrease in hemozoin in group B3 was accompanied by a reduction in Kupffer cell hyperplasia. This could explain the increased Kupffer cell presence observed in the PSP600 (B2) single therapy group, as well as in the ART+PSP600 (B4) and ART+PSP800 (B5) combination therapy groups.

Kupffer cell hyperplasia observed in the ART+PSP800 (B5) combination therapy and PSP600 (B2) single therapy groups, which was not statistically significant in this study, may be attributed to the mechanism by which PSP eliminates Plasmodium. Both therapeutic regimens are believed to eliminate *P. berghei* by recruiting immune cells, such as Kupffer cells and cytotoxic T lymphocytes (CTL), through the cytokines TNF-α and IFN-γ. These cytokines tend to increase in both the liver and spleen. Additionally, the phycocyanin content in PSP is thought to contribute to the destruction of hemozoin polymerization, as previously described. The negative control group (A1) did not show significant differences compared to the single therapy groups (ART (B1) and PSP control (B6)). It is well-established that ART has anti-inflammatory effects, leading to a reduction in inflammation in the ART single therapy group (B1). The lack of a significant increase in the PSP control group (B6) may be due to the immunostimulant effect of PSP, which increases the presence of inflammatory cells. This effect likely explains the increase observed in Kupffer cells. The rise in liver portal inflammation in the PSP (B6) control group is likely due to the role of IFN-γ, which enhances macrophage activity, including that of Kupffer cells, and stimulates lymphocyte proliferation in the spleen. Additionally, previous studies have shown that lymphocytes infiltrate the portal zone, which, when infected with Plasmodium, contains infiltrations of lymphocytes and plasma cells (11).

Spirulina is known to increase the secretion of IL-12 by macrophages, which plays a role in stimulating IFN-γ secretion from NK cells and T cells (33). This may explain the significant increase in portal inflammation observed in the ART+PSP600 (B4) and ART+PSP800 (B5) combination therapy groups, compared to the ART (B1) single-therapy group. The lack of a significant increase in the ART+PSP400 (B3) combination therapy group, as compared to the ART+PSP800 (B5) group, may indicate differences in the mechanism of malaria elimination between the two. The ART+PSP400 (B3) combination likely recruits immune cells, including CTLs and lymphocytes, alongside the activity of ART. Spirulina has been shown to enhance the cytolytic activity of NK and CD8+ cells, which engage in eliminating Plasmodium (33). Cytotoxic compounds, such as granzymes, detected in plasma during the erythrocytic malaria stage, suggest that parasite-infected erythrocytes are potential targets of NK cells or CD8+ T cells (CTL) in vivo (34). Lymphocyte recruitment is triggered by an increase in IL-12 secretion from PSP-activated macrophages, which, in turn, stimulates IFN-γ secretion from T cells (32). IFN-γ can activate macrophages and induce additional immune responses crucial for eliminating intracellular pathogens, such as *P. berghei* (34,35). The decrease in portal inflammation observed in the PSP600 single therapy group (B2), though not significant compared to the positive control (A2), may be attributed to the anti-inflammatory properties of PSP. The expression of pro-inflammatory cytokines, TNF-α and IFN-γ, which were also reduced (but not significantly), further supports this. The anti-inflammatory effects of Spirulina platensis, observed in rats with colitis, significantly reduced the activity of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (36).

The significant increase observed in the ART+PSP600 (B4) and ART+PSP800 (B5) combination therapy groups, compared to the ART+PSP400 (B3) combination therapy group, may be due to the pro-inflammatory cytokine activity of Spirulina, independent of TNF-α and IFN-γ. In fact, the expression of TNF-α and IFN-γ in the liver was not higher in the ART+PSP600 (B4) and ART+PSP800 (B5) combination therapy groups than in the ART+PSP400 (B3) group. Spirulina is an immunostimulant that can increase monocyte proliferation, enhance macrophage phagocytosis, cause an accumulation of NK cells in tissues, stimulate the production of antibodies and cytokines, and activate both T and B cells (37). Spirulina platensis has also been shown to significantly increase the expression of inflammatory genes such as iNOS, COX-2, TNF-α, and IL-6. Additionally, phycocyanin has been found to increase the secretion of inflammatory agents, including TNF-α, IL-1β, IL-6, and COX-2 protein expression in vitro using J774A cells (38,39).

The ART single therapy group (B1) exhibited a significant reduction in inflammation when compared to the positive control group (A2), the PSP400 single therapy group (B2), the PSP control group (B6), and the ART+PSP combination groups (B4 and B5). This reduction is attributed to the anti-inflammatory properties of ART, which inhibit IFN-γ production. This is consistent with findings that Artemether significantly suppresses T cell proliferation and IL-2 and IFN-γ production by affecting receptors on T cells, thereby inhibiting MAPK signalling pathways, including the phosphorylation of ERK1/2, Jnk, and P38 (10). The diameter of the white pulp in the PSP control group (B6) increased significantly compared to the negative control group (A1). This increase is likely due to the dietary administration of

Spirulina polysaccharide fraction, which significantly enhances IFN-γ secretion and is associated with an increase in T and NK cells in the spleen, observed on the third day after administration (40). The ART+PSP400 (B3) combination therapy group showed a significant decrease compared to the positive control group (A2), the PSP600 single therapy group (B2), and the ART+PSP combination groups (B4 and B5), but it increased insignificantly compared to the PSP control group (B6). Enlargement of the white pulp diameter in the spleen is an indication of immune system activation in response to antigens, with the proliferation of T and B lymphocytes playing a key role in defending the body from pathogens (41). This also aligns with the deposition of hemozoin, reinforcing why the combination therapy groups at the 600 and 800 doses were the most effective. The ART+PSP combination therapy groups (600 (B4) and 800 (B5)) did not show a significant decrease compared to the positive control group (A2) or the PSP600 single therapy group (B2). This could be due to the presence of high parasite levels in the mice, suggesting ongoing immune system activity in the spleen, as evidenced by the significantly higher expression of TNF-α and IFN-γ cytokines compared to the negative control group (A1). *P. berghei* can stimulate immune responses in the spleen, leading to the development of lymphocytes and reticular cells (42). Enlargement of the white pulp can occur as a result of *P. berghei* induction, further explaining why the PSP600 single therapy group (B2) did not show a significant reduction compared to the positive control group (A2) (43).

Conclusion

This study found that the response to spirulina polysaccharide (PSP) adjuvant therapy combined with artemisinin can increase levels of pro-inflammatory cytokines (TNF-α and IFN-γ) in the liver and spleen of mice (*Mus musculus*) infected with *Plasmodium berghei*. The effective dose of PSP in the ART+PSP combination therapy is 400 mg/kg BW, which can reduce hemozoin and improve the histopathology of liver and spleen tissues caused by *Plasmodium berghei* infection in this study.

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Conflict of interest

The authors declare that they have no competing interests**.**

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سبيرولينا متعدد السكاريد كعلاج مساعد لتحسين استجابة TNF-α، IFN-γ وتحسين التركيب النسجي المرضي للكبد والطحال في الفئران المخمجة بـ *Plasmodium berghei*

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الخلاصة

يمكن الجمع بين استخدام العلاج المساعد والأدوية الأولية المضادة للملاريا لزيادة تاثير وفعالية الأدوية المضادة للملاريا وتقليل المضاعفات الأكثر خطورة التي تسببها إمراضيتها. وتهدف هذه الدراسة إلى تقييم تاثير سبيرولينا متعدد السكاريد كعلاج مساعد في لتحسين استجابة TNF-α، IFN-γ وتحسين التركيب النسجي المرضي للكبد والطحال في

الفئران المخمجة بـ *Plasmodium berghei*. هذا الدراسة اجريت على أربعين من الفئران، قسمت الى ثماني مجموعات: أ-١: الفئران غير المصابة. أ-٢: الفئران المصابة. ب-١: الفئران مصابة وعولجت بالأرتيميسينين بجرعة ٤٠ ملغم / كغم من وزن الجسم. ب-٢: الفئران مصابة وعولجت بسبيرولينا متعدد السكاريد بجرعة ٦٠٠ ملغم / كغم من وزن الجسم. ب-٣ ، ب-٤ ، ب-٥: الفئران مصابة وعولجت بجرعة المعالجة المضادة للفيروسات القهقرية ٤٠ ملغم/كغم من وزن الجسم و ٤٠٠ ملغم/كغم من وزن الجسم و ٦٠٠ ملغم/كغم من وزن الجسم و ٨٠٠ ملغم/كغم من وزن الجسم. ب-٦: الفئران غير المصابة ولكن عوملت بسبيرولينا متعدد السكاريد (٦٠٠ ملغ / كغ من وزن الجسم). أصيبت الفئران بـ ٠,٢ مل من ١ x ١٠^{٧} خلايا الدم الحمراء المخمجة بـ *Plasmodium berghei*. تم إعطاء العلاجات لمدة ١٠ أيام. بعد ذلك، تم فحص التغيرات النسيجية المرضية والمقاطع المصبوغة بالصبغات الكيميائية المناعية. أظهرت النتائج أن المجموعات غير المصابة (أ-١ و ب-٦) انخفاضا كبيرا في مستويات الهيموزوين، واحتقان الجيوب الأنفية، وتضخم خلايا كوبفر، والتهاب البوابة (إ < ٠,٠٥) مقارنة بالمجموعات المصابة (أ-٢، ب-١، ب-٢، ب-٣، ب-٤ و ب-٥). كان هيموزوين الطحال وقطر اللب الأبيض في المجموعة أ-١ مختلف بشكل معنوي (أ< ٠,٠٥) مقارنة بكل المجموعات الأخرى (أ-٢، ب-١، ب-٢، ب-٣، ب-٤، ب-٥ و ب-٦). التعبير لكل من TNF-α و IFN-γ في خلايا الكبد والطحال كان منخفض معنويا" (إ < ٠,٠٥) في مجموعة السيطرة السلبية أ-١ مقارنة مع المجموعات أ-٢، ب-٢، ب-٣، ب-٤ و ب-٥. أستنتج من هذه الدراسة أن السبيرولينا متعدد السكاريد يمكن أن يكون علاج مساعد يعزز التعبير لكل من TNF-α و IFN-γ في خلايا الكبد والطحال المخمجة بـ *Plasmodium berghei*. كما أن الجمع بين العلاج بالأرتيميسينين و سبيرولينا متعدد السكاريد بجرعة فعالة ٤٠٠ ملغم / كغم من وزن الجسم يقلل من تراكم الهيموزوين ويحسن التركيب النسجي للكبد والطحال المرتبطة بالمخمج بـ *Plasmodium berghei*.