Research Article

The Physiological and Immunological Effects of Aqueous Extract of *Spirulina platensis* in Albino Male Rats Treated with Methotrexate

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Abstract | *Spirulina platensis* is a promising nutritional supplement recognized for its beneficial bioactive components that contribute to overall human health. Recently, interest has emerged in enhancing the immune system through the use of natural products to prevent disease. The present study aimed to evaluate the effects of *S. platensis* supplementation on multiple biomarkers in methotrexate-induced immunocompromised rats, including immune markers (TNF-α, IL-1, and IL-6), oxidative stress parameters (MDA, SOD, and CAT), and physiological markers such as liver function enzymes. Thirty male albino rats, aged 2–3 months and weighing 160–175 g, were used in this experiment. The rats were divided into three groups: a negative control group treated with normal saline, a positive control group treated with methotrexate (50 mg/kg), and a treatment group receiving methotrexate (50 mg/kg) along with an aqueous extract of *S. platensis* (250 mg/kg) for 25 days. The results showed that methotrexate treatment significantly disrupted immune markers, oxidative status, and liver function, indicating immunodeficiency and hepatic impairment. In contrast, *S. platensis* supplementation significantly restored catalase and superoxide dismutase levels and improved liver function by significantly reducing AST, ALT, and ALP levels ($P < 0.05$). Additionally, a significant reduction in pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) was observed ($P < 0.05$). These findings suggest that *Spirulina platensis* enhances immune responses by reducing inflammation, restoring immune homeostasis, and improving oxidative status, thereby decreasing free-radical production and associated liver and organ damage.

Keywords | *Spirulina platensis*, Natural antioxidant, Methotrexate-toxicity, Phytochemical compound, Immune-stimulation

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INTRODUCTION

Spirulina is a blue-green microalga that has attracted considerable attention in recent years due to its nutritional value and health-promoting potential. This photosynthetic microorganism has been extensively studied, with researchers exploring its diverse applications in the fields of food, feed, and biotechnology (Yarnold *et al.*, 2019; Pereira *et al.*, 2019). *Spirulina* grows naturally in warm-climate regions and has long been used as a dietary supplement for both humans and animals. In addition to its commercial importance, *Spirulina* exhibits significant pharmaceutical potential owing to its rich chemical composition, including high-quality proteins, vitamins,

essential amino acids, dietary minerals, essential fatty acids, and antioxidant compounds. These characteristics make *Spirulina* a sustainable and nutritious food source capable of meeting the demands of an expanding global population (Asghari *et al*., 2016; Singh *et al*., 2005; Patras *et al*., 2019). Furthermore, *Spirulina* has various industrial applications, including wastewater treatment, energy generation, and the extraction of valuable bioactive compounds. Its ability to thrive under extreme environmental conditions and to be cultivated sustainably makes it an attractive alternative to traditional agricultural resources (Mariana *et al*., 2020).

In the medical field, *Spirulina* has demonstrated multiple therapeutic benefits, including antidiabetic, cardioprotective, hepatoprotective, antiviral, antibacterial, and anticancer effects. It has also been reported to repair DNA damage, modulate immune responses, induce cell cycle arrest and apoptosis, suppress lipid peroxidation, scavenge free radicals through antioxidant activity, and protect against metal toxicity (Spínola *et al*., 2024; Lympaki *et al*., 2022; Zhang *et al*., 2021). Recently, oxidative stress has gained significant attention in scientific research due to its involvement in numerous diseases. Disruption of oxidative enzyme balance leads to excessive production of reactive oxygen and nitrogen species (ROS and RNS), which are highly reactive and can damage vital macromolecules such as DNA, RNA, lipids, and proteins. *Spirulina* is considered a potent antioxidant owing to its bioactive compounds, including flavonoids, β-carotene, phycocyanin, iron, and vitamins, which contribute to the reduction of inflammation and oxidative damage. Previous studies have demonstrated that *Spirulina* enhances both innate and adaptive immune responses by modulating gut microbiota and strengthening intestinal immune barriers, highlighting its potential role in autoimmune disease management (Fadhil and Aadim, 2024; Gentscheva *et al*., 2023; Salahuddin *et al*., 2025).

Methotrexate (MTX), a synthetic folic acid derivative, is widely used as a therapeutic agent for various conditions ranging from oncological disorders to autoimmune diseases. As a disease-modifying antirheumatic drug, MTX is highly effective in delaying the progression of rheumatoid arthritis, a condition characterized by chronic inflammation and joint damage (Castro *et al*., 2013; Hernandez-Baldizon, 2012). However, MTX is associated with several adverse effects on multiple organs, leading to severe toxicity (Zachariae *et al*., 1990). Therefore, optimal MTX use requires careful consideration of its pharmacological properties, dosage regimens, routes of administration, and concurrent therapies to ensure patient safety and therapeutic efficacy. MTX toxicity can impair hematopoietic function, resulting in complications such as myelosuppression, and can induce hepatotoxicity, which may progress to fibrosis or cirrhosis if not properly monitored (Hamed *et al*., 2022). Additionally, MTX acts as an immunosuppressive agent by inhibiting T- and B-cell activation (Chu *et al*., 2010). This property underlies its use in the present study to induce immunosuppression in rats, thereby enabling the evaluation of *Spirulina* microalgae for its potential immune-stimulating effects and overall health-restorative capacity. Therefore, this study aimed to evaluate the immunomodulatory and protective effects of *Spirulina platensis* supplementation in MTX-induced immunocompromised rats.

MATERIALS AND METHODS

Aqueous extract preparation for antioxidant experiment

One hundred grams of dried *S. platensis* powder were suspended in 1 L of distilled water and shaken in a shaker incubator at 25 °C for 48 h. The mixture was then centrifuged at 5000 rpm for 10 min, followed by filtration using Whatman filter paper No. 1. The filtrate was evaporated at 40 °C and 60 rpm using a rotary evaporator. The resulting concentrate was converted into a paste, transferred to Petri dishes, and dried in an oven at 40 °C until completely dried (Zhao *et al*., 2022).

In vitro antioxidants activity of *S. platensis*

The antioxidant capacity of *S. platensis* was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Different concentrations of the extract (0.25, 50, 75, 125, 500, and 1000) were prepared and mixed with DPPH solution, which exhibits a dark violet color due to the presence of an unpaired electron. The ability of the extract to scavenge free radicals, resulting in a pale-yellow color, was measured using a spectrophotometer at a wavelength of 517 nm (Kumar *et al*., 2008).

Animal treatment

In this experiment, thirty male albino rats of comparable age and weight were randomly divided into three groups, with ten rats in each group. The first group served as the normal control and received normal saline. The second group was treated with MTX (50 mg/kg), while the third group received MTX (50 mg/kg) in combination with an aqueous extract of *S. platensis* (250 mg/kg). All treatments were administered for a period of 25 days. The animals were maintained under standard laboratory conditions, including a controlled temperature of 20–26 °C, a 12 h light/12 h dark cycle, free access to standard pellet diet, and filtered tap water. These conditions were maintained to minimize stress and ensure reliable experimental outcomes. At the end of the experimental period, 5 mL of blood was collected via cardiac puncture under anesthesia for subsequent biochemical and immunological analyses.

Measurement of TNF-α, IL-1, and IL-6

Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentrations of TNF-α, IL-1, and IL-6 according to the manufacturer's instructions (CUSABIO, USA). Commercial ELISA kits with catalog numbers CSB-E11987r (TNF-α), CSB-E10397r (IL-1), and CSB-E04640r (IL-6) were used. Monoclonal antibodies were pre-coated onto 96-well plates specific for each cytokine. Standard solutions of known concentrations were added to generate standard curves, and serum samples were added in triplicate. Plates were incubated at room temperature as specified in the kit protocols. Subsequently, biotin-labeled antibodies, conjugate solutions, and stop solution were added sequentially, with washing steps performed three times between each step. Absorbance was measured at 450 nm using a microplate reader, and cytokine concentrations were calculated from the standard curves using linear regression analysis.

Measurement of superoxide dismutase (SOD) and catalase (CAT)

The activities of superoxide dismutase (SOD) and catalase (CAT) were measured using a quantitative sandwich immunoassay, following the manufacturer's instructions (MyBiosource, USA). A total of 100 µL of each sample and standard was added to the pre-coated wells, and the assay was performed using the same sequential steps as described for the cytokine measurements (TNF-α, IL-1, IL-6). Absorbance was measured according to the kit protocol, and enzyme activities were calculated based on the standard curves.

Measurement of malondialdehyde (MDA)

Malondialdehyde (MDA) levels in serum were assessed using a lipid peroxidation MDA assay kit (BioVision, USA). The assay is based on the reaction between MDA and thiobarbituric acid (TBA), producing an MDA–TBA_2 adduct that can be detected spectrophotometrically at 532 nm. In brief, serum samples were mixed with the supplied lysis buffer and treated with TBA for 60 min at 95 °C. After incubation, samples were centrifuged to remove precipitates and then cooled on ice. MDA concentrations were calculated from a standard curve prepared according to the kit instructions.

Liver function enzymes

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were determined colorimetrically using commercial kits (GenWay Biotech, Inc.) according to the manufacturer's instructions. ALT and AST activities were measured at 505 nm based on the color development resulting from enzymatic conversion of their specific substrates. ALP activity was determined at 405 nm, relying on the hydrolysis of p-nitrophenyl phosphate, which produces a yellow-colored product proportional to enzyme activity.

Statistical analysis

Differences between experimental groups were analyzed using the Statistical Analysis System (SAS, 2018). Least Significant Difference (LSD) tests were applied to determine significant differences between means. Data are presented as mean ± standard deviation (SD), and differences were considered statistically significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Clinical and preclinical studies in recent years have increasingly focused on the potential health benefits of natural, non-chemical substances with minimal side effects, such as cyanobacteria particularly *Spirulina*. *Spirulina* has been shown to significantly improve metabolic parameters that are often impaired by various diseases and conditions (Dąbrowska *et al*., 2024). The present study aimed to investigate the effects of an aqueous extract of *Spirulina* on selected physiological biomarkers.

Table 1: *In vitro* antioxidant activity of aqueous *Spirulina* extract assessed by DPPH free radical scavenging assay.

Spirulina extract concentration (µg/mL)	Absorbance at 517 nm (Mean ± SE)	Antioxidant activity (%)
0	0.43±0. 01	38.85
25	0.16±0. 02	49.04
50	0.12±0.002	63.43
75	0.08±0.004	73.97
125	0.079±0.001	80.53
250	0.05±0.005	84.08
500	0.031±0.002	89.33
1000	0.027±0.02	93.80

Table 1 shows that antioxidant activity increased proportionally with the concentration of *Spirulina* extract, with 1000 µg/mL exhibiting the highest activity (93.80%) compared to 25 µg/mL, which showed 49.04% activity. These results highlight the role of *Spirulina* as a potent antioxidant, likely due to its bioactive components (Seyidoğlu *et al*., 2021). Similar findings were reported by Seyidoğlu *et al*. (2021), who observed a positive correlation between *Spirulina* extract concentration and antioxidant activity.

Data presented in Table 2 revealed a significant increase in MDA levels in the MTX-treated group (4.85 ± 0.02 µmol/L) compared with the control group (1.67 ± 0.19 µmol/L). Treatment with *Spirulina* significantly reduced MDA levels to 2.54 ± 0.45 µmol/L, mitigating the

oxidative stress induced by MTX. Similarly, SOD activity was significantly elevated in MTX-treated rats (3.85 ± 0.76 IU/L) compared to controls (1.46 ± 0.06 IU/L). Administration of *Spirulina* restored SOD levels close to normal (1.80 ± 0.04 IU/L).

Table 2: Effects of aqueous *Spirulina* extract on SOD, CAT, and MDA levels in methotrexate-treated albino rats.

Groups	SOD (IU/L)	CAT (IU/L)	MDA (µmol/L)
Control	1.46 ± 0.06	1.81 ± 0.02	1.67 ± 0.19
Methotrexate 50 mg/kg	3.85±0.76	4.97±0.03	4.85±0.02
Methotrexate 50 mg/kg + *Spirulina* 250 mg/kg	1.80±0.04	2.32±0.05	2.54±0.45
LSD	0.97**	1.02**	0.85**

Values are presented as mean ± SD. **Significant difference between groups at P < 0.05.

Assessment of catalase (CAT) activity showed a significant increase in the MTX group (4.97 ± 0.03 IU/L) relative to controls (1.81 ± 0.02 IU/L), whereas *Spirulina* treatment reduced CAT activity to near-normal levels (2.32 ± 0.05 IU/L). These findings indicate that aqueous *Spirulina* extract exerts a potent antioxidant effect by modulating oxidative stress markers in MTX-treated rats.

The reduction in MDA, SOD, and CAT levels after *Spirulina* administration is likely due to its rich composition of bioactive compounds, including β-carotene, astaxanthin, phycocyanin, tocopherol, selenium, and phenolic acids (Salah *et al*., 2025). These compounds scavenge reactive oxygen species (ROS) generated by MTX, thereby decreasing lipid peroxidation and normalizing antioxidant enzyme activities. Elevated SOD and CAT levels are typically associated with oxidative stress and ROS overproduction, which target polyunsaturated fatty acids in cell membranes. By reducing these enzyme activities toward normal levels, *Spirulina* helps protect overall cellular health (Sreedhar *et al*., 2013; Pak *et al*., 2012; Khawaja *et al*., 2024). These results are consistent with previous findings by Park *et al*. (2018), who reported that feeding *S. platensis* increased antioxidant defenses in serum due to its bioactive chemical components.

Statistical analysis showed a significant reduction in the total white blood cell (WBC) count in the MTX-treated group (3.43 ± 0.06 ×10^3/µL) compared to the control group (6.65 ± 1.96 ×10^3/µL). Treatment with *Spirulina* extract significantly restored WBC levels to 5.23 ± 1.67 ×10^3/µL, indicating an improvement in immune function and a potential suppression of MTX-induced inflammatory processes (Table 3).

Table 3: Effects of aqueous *Spirulina* extract on total and differential white blood cell counts in methotrexate-treated albino rats.

Groups	WBC (×10^3/µL)	Neutrophils (%)	Lymphocytes (%)
Control	6.65±1.96	60.32 ± 1.05	50.06±5.78
methotrexate50 mg/kg	3.43±0.06	43.65±4.65	24.95 ± 1.17
Methotrexate50 mg/kg+ *Spirulina* 250 mg/kg	5.23±1.67	54.47±6.89	38.33±4.32
LSD	1.65**	7.85**	9.65**

Values are presented as mean ± SD. **Significant difference between groups at P < 0.05.

Analysis of differential counts revealed that MTX reduced neutrophil percentages to 43.65 ± 4.65%, compared to 60.32 ± 1.05% in controls. Administration of *Spirulina* increased neutrophils to 54.47 ± 6.89%. Similarly, lymphocyte percentages were significantly decreased by MTX (24.95 ± 1.17%) relative to controls (50.06 ± 5.78%), while *Spirulina* supplementation elevated lymphocyte levels to 38.33 ± 4.32% (Table 3).

These results indicate that MTX-induced leukopenia adversely affects the immune response, likely due to its cytotoxic effects on bone marrow and the gastrointestinal tract, which may result in diarrhea and blood loss. Supplementation with *Spirulina* partially restored WBC counts and supported neutrophil and lymphocyte maintenance, enhancing overall immune function. This protective effect may be attributed to bioactive compounds such as C-phycocyanin and carotenoids, which are known to improve hematopoiesis and immunity (Park *et al*., 2018; Ge *et al*., 2019; Patel *et al*., 2014; Youssef *et al*., 2023).

Table 4: Effects of aqueous *Spirulina* extract on ALT, AST, and ALP levels in methotrexate-treated albino rats.

Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Control	25.54±2.23	23.33±0.56	80.54±2.85
Methotrexate 50 mg/kg	34.60±3.74	40.43±4.90	79.65±1.89
Methotrexate 50 mg/kg + *Spirulina* 250 mg/kg	27.32±2.43	29.67±3.67	82.56±3.30
LSD	4.32**	7.85**	4.30 ^{NS}

Values are presented as mean ± SD. **Significant difference between groups at P < 0.05. ^{NS} not significant

Data in Table 4 showed that alanine transaminase (ALT) was significantly elevated in MTX-treated rats (34.60 ± 3.74 IU/L) compared to the control group (25.54 ± 2.23 IU/L). Treatment with *Spirulina* slightly increased ALT relative to controls but markedly attenuated the MTX-induced elevation to 27.32 ± 2.43 IU/L. A similar trend

was observed for aspartate transaminase (AST), which increased to 40.43 ± 4.90 IU/L following MTX treatment and was reduced to 29.67 ± 3.67 IU/L after *Spirulina* supplementation. Alkaline phosphatase (ALP) showed a non-significant change among groups (Table 4).

Methotrexate is a well-known hepatotoxic agent that limits its clinical use, with mechanisms including oxidative stress, induction of hepatic apoptosis, and disruption of anti-inflammatory mediators (Nasirian *et al*., 2017). Measurement of liver function enzymes in this study confirmed the hepatotoxic effects of MTX, as indicated by the significant elevations in ALT and AST, likely due to increased ROS production. Conversely, administration of *Spirulina* significantly reduced these enzyme levels, highlighting its hepatoprotective potential. These findings are consistent with Khafaga and El-Sayed (2018), who reported that *Spirulina* supplementation protected liver function in MTX-treated rats.

Table 5: Effects of aqueous *Spirulina* extract on IL-1, IL-6, TNF-α, and CRP levels in methotrexate-treated albino rats.

Groups	IL-1 (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)	CRP (mg/L)
Control	5.75±1.09	4.96±0.78	300.98±5.75	3.88±0.06
methotrexate50 mg/kg	3.34±0.43	2.85±0.04	400.32±6.43	4.95±0.87
Methotrexate50 mg/kg+*Spirulina* 250 mg/kg	4.90±0.95	4.03±1.05	353.76±5.90	3.96±0.94
LSD	1.02**	1.53**	14.76**	1.90^{NS}

Values are presented as mean ± SD. **Significant difference between groups at P < 0.05. ^{NS} not significant

As shown in Table 5, immunological biomarkers revealed that MTX caused a significant decline in IL-1 levels (3.34 ± 0.43 pg/mL) compared to the control group (5.75 ± 1.09 pg/mL), whereas *Spirulina* supplementation enhanced IL-1 production to 4.90 ± 0.95 pg/mL. Similarly, IL-6 levels were reduced by MTX to 2.85 ± 0.04 pg/mL compared to control (4.96 ± 0.78 pg/mL), and treatment with *Spirulina* restored IL-6 toward normal levels (4.03 ± 1.05 pg/mL). In contrast, TNF-α levels were significantly elevated following MTX administration (400.32 ± 6.43 pg/mL), and *Spirulina* mitigated this effect, reducing TNF-α to 353.76 ± 5.90 pg/mL. C-reactive protein (CRP) levels showed a non-significant change among groups, which aligns with the review by Shahraki *et al*. (2025), suggesting no linear correlation between *Spirulina* treatment and CRP levels, possibly due to anti-inflammatory compounds such as omega-3 fatty acids and polyphenols.

The immunomodulatory effects of *Spirulina* observed in this study are consistent with its reported bioactive properties. Cytokines such as IL-1, IL-6, and TNF-α are essential in immune regulation, and their reduction following MTX treatment is likely due to methotrexate's folate antagonism, which suppresses the production of inflammatory mediators (Youssef *et al*., 2023). The increase in TNF-α after MTX may also be explained by MTX-induced injury to the small intestine, activating the NF-κB signaling pathway and enhancing TNF-α expression (Wang *et al*., 2024). Administration of *Spirulina* restored cytokine levels, likely through its bioactive components, including β-carotene, vitamins, and selenium, which possess immune-stimulatory and protective properties. Additionally, the lipopolysaccharide fraction of *Spirulina* may activate NF-κB via TLR4 signaling, further enhancing immune responses (Khafaga and El-Sayed, 2018). Similar results were reported by Salahuddin *et al*. (2025) and Wu *et al*. (2020), who observed restoration of IL-6 and TNF-α levels and enhancement of both innate and adaptive immunity following *Spirulina* administration.

The effects of *Spirulina* on inflammatory cytokines are multifaceted and context-dependent. Some studies suggest that it may stimulate pro-inflammatory cytokine production, whereas others highlight its anti-inflammatory properties, particularly via inhibition of NF-κB. Variations in study design, dosage, and experimental models likely contribute to these differences. Further research is needed to fully elucidate how *Spirulina* modulates inflammatory cytokines, especially under immunosuppressed conditions. Investigating its effects in cancer models or tumor cell cultures may provide additional insight into its role in enhancing immune responses against malignancies.

CONCLUSIONS

From the present study, it can be concluded that *Spirulina* exerts multiple beneficial effects on overall health. The data demonstrated that *Spirulina* is a potent antioxidant due to its rich phytochemical composition, which helps regulate various physiological processes, including liver function by normalizing AST, ALT, and ALP enzyme levels. In terms of immunomodulatory activity, *Spirulina* exhibited promising anti-inflammatory effects, mitigating the adverse effects of methotrexate (MTX), a widely used anticancer drug known to impair oxidative balance. Therefore, co-administration of *Spirulina* with MTX could be a potential strategy to reduce drug-induced oxidative stress and support immune function during tumor treatment. Additionally, incorporating this alga into the diet may enhance overall health by reducing free radical-induced cellular damage associated with environmental and physiological stress.

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The authors have no acknowledgment to declare.

NOVELTY STATEMENT

The study proved that Aqueous Extract of Spirulina platensis has high therapeutic and preventative efficacy against the pathogens caused by Methotrexate in rats.

AUTHOR'S CONTRIBUTION

The authors contributed to the study as follows: Study conception and design were carried out by SMMA-C and AIS; data collection was performed by SMMA-C; analysis and interpretation of results were conducted by RZF, NRJ, and SSR; and draft manuscript preparation was undertaken by RZF. All authors reviewed the results and approved the final version of the manuscript.

Ethics statement

This study required ethical approval, which was obtained from the Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq (Ref. No. E.B.18; Date: 02/01/2024).

Generative AI and AI-assisted technology statement

The authors declare that no genrative AI was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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