Protective effect of *Spirulina platensis* on cyclophosphamide-induced toxicity in experimental mice

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*Spirulina platensis* (SP) is a blue-green alga used as a dietary supplement as it possesses antioxidant, anti-inflammatory and hepatoprotective effects with clinical importance in several disorders. The aim of this study was to evaluate the ameliorating effects of SP extract on toxicity induced mice by treatment with the anticancer drug cyclophosphamide (CTX). To this end, SP was cultured under optimal conditions of growth at pH 9 for 9 days using Zarrouk’s medium. The active ingredients including pigments, phycobiliproteins and total soluble proteins were estimated in SP suspensions which were then dried to a powder form. To assess anti-toxicity, adult male albino mice were treated with interperitoneal (i.p.) injection of PBS, single dose of CTX (4mg/mouse), CTX (i.p.) followed by subcutaneous (s.c.) injection of G-CSF (5μg/mouse) daily for 5 consecutive days or CTX (i.p.) followed by oral administration of 0.5 gm/mouse SP extract for 7 consecutive days. After 7 days of initial treatment with CTX, mice were sacrificed; blood, spleen, bone marrow and liver were harvested to assess CBC, total count for spleen and bone marrow and liver for biochemical analysis. The results showed that SP showed the highest yield of dry weight and pigment content C-phycocyanin (CPC), allophycocyanin (APC), phycoerthrin (PE) and total phycobiliproteins (phycobilins) at the 9th day of growth at pH 9 in culture. Oral administration of SP induced amelioration of CTX induced leucopenia in blood, bone marrow and spleen comparable to those of G-CSF. It also ameliorated the dysfunction in the liver enzymes ALT and AST. Interestingly, however, SP showed higher antioxidant effects than those of G-CSF as reflected by the higher activities of the anti-oxidant MDA and GSH. **Conclusion:** SP showed potent antitoxic effect through antioxidant activity and thus could be a useful co-adjuvant agent against chemotherapeutic drugs toxicity including cyclophosphamide.

**Keywords:** Anti-oxidant, CTX, G-CSF, Leukocyte, Liver, *Spirulina platensis*, Toxicity

**INTRODUCTION**

Recently, much attention has been focused on the microalgae as sources of novel, biologically active compounds such as phycobiline, phenols, terpenoids, steroids and polysaccharide (Li et al., 2007; Abd El Baky et al., 2008). *Spirulina platensis* (SP), which is a blue green alga, contains significant amounts of vitamin B12, pro-vitamin A and vitamin E as well as mineral content, including iron, calcium, magnesium, manganese, potassium, and zinc and few amounts of glycolipids and sulfolipids (Bensehaila et al., 2015). The body surface of SP is smooth and without covering so it easily digestible in the body by simple enzymatic systems. Studies conducted on SP suggest that...
it is safe in healthy subjects. Several preclinical studies have proved the importance of SP to be commercialized for therapeutic purposes (Palaniswamy et al., 2018). Many experimental studies have attributed the beneficial effects of SP with its antioxidant capacity (Chu et al., 2010; Kim et al., 2010; Ponce-Canchihuaman et al., 2010). Consequently, it has been considered as a functional food, because of its ability to provide medical or health benefits, including the prevention and/or treatment of diseases.

Recent studies have shown the importance of SP to protect against toxicities experimental animals including nephrotoxicity induced by the antibiotic gentamicine (Karadeniz et al., 2008), toxicity induced by lead acetate (Eltantway, 2016) and urotoxicity induced by CTX (Eltantawy et al., 2018). These protective effects of the SP extract have been suggested to be attributed to the anti-oxidant effects of its active constituents. This may explain the in vitro anticancer effect that has been suggested to be mediated by modulating the activity of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-2 (IL-2) (Choi et al., 2013).

Cyclophosphamide (CTX), a DNA-alkylating agent is widely utilized to treat cancer and some types of autoimmune diseases. It is also considered an immunosuppressant since it causes blocking the replication of DNA in cells and consequently prevents cells from dividing, leading to cell death (Berd and Mastrangelo, 1988). Despite its therapeutic benefits, CTX has been found to have several side effects as particular systemic leucopenia. Detailed analysis of this leucopenia in mice showed that CTX decreased the absolute number of leukocytes in the peripheral blood (PBL) at days 3-15, and in the spleen at days 3-6 and came back to normal range at the recovery phase (day 9) (Salem et al., 2009; Salem et al., 2010a and b; Salem et al., 2016). These leucopenia effects of CTX make it a reliable model to study the ameliorating effects of certain compound on it. Granulocyte colony-stimulating factor (G-CSF), a hematopoietic growth factor is considered as an adjuvant therapy during anti-cancer to correct leukopenia. It stimulates the immune response through activating the release of neutrophils from bone marrow to blood (Salem et al., 2016). G-CSF regulates granulocyte production and is produced by a diversity of cells in response to inflammation and infection (Gross-weenge et al., 1997, Rubinstein et al., 2013).

This study was designed to compare the ameliorating effect of SP against leucopenia and toxicity induced by CTX to those of a G-CSF as a reference drug.

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**MATERIALS AND METHODS**

**Reagents and antibodies:**

CTX was purchased from Sigma (Sigma Aldrich, USA) and reconstituted in phosphate buffered saline (PBS) in a stock solution and kept at -80° C until use. G-CSF (Neupogen) was purchased from a local pharmacy. G-CSF was diluted in PBS to the required concentration before injection. Anti-CD11b, Allophycocyanine (APC) was purchased from BD Pharmingen (San Diego, CA).

**Spirulina platensis (SP) cultivation**

SP strain was obtained from National Research Center (NRC) and cultured in the lab using Zarrouk medium under optimum conditions of growth (Zarrouk, 1966). Erlenmeyer flasks (250 ml) contained 150 ml of Zarrouk medium were sterilized in an autoclave at 1.5 atm for 20 min. After cooling the flasks were inoculated with 15 ml of pre-culture organisms and incubated under continuous fluorescent light of 2500 lux. The cultures flasks were aerated with sterile air mixed with 3% CO2 to accelerate cyanobacterial growth. The rates of gas were regulated by a mean of a plastic valve (Zarrouk, 1966).

**Determination of growth parameters:**

**Optical density**

The growth of SP was determined by measuring the optical density of the algal suspension at 560 nm as recommended by (Fatma et al., 1994)
Dry weight
A definite volume of SP suspension (200ml) was centrifuged at 6000 rpm for 10 min. The precipitated cells were washed twice with distilled water to eliminate salts, dried overnight in an oven at 65 °C till constant weight and kept in desiccators for 20 min for cooling and weighed. The data were given as g/100ml.

Extraction of pigment
Chlorophyll a and carotenoids were determined according to the spectrophotometric method recommended by (Mackinney 1941). Phycobiliproteins contents were determined according to the method described by (Bennett and Bogorad 1973).

Estimation of total soluble proteins
After pigment extraction, algal cells were extracted with 1N NaOH in a boiling water bath for 2 hrs as described by Payne and Stewart. Total soluble proteins were measured determined using the method described by Bradford.

Animals
Adult male Swiss albino mice weighting 22 ± 2 g were purchased from National Cancer Institute (Cairo, Egypt). Animals were housed (n=8) at the animal house at Zoology Department, Faculty of Science (Tanta University, Egypt) in clean and dry plastic cages, the mice were maintained in a control environment under standard condition of temperature and humidity with an altering light and dark cycle throughout the experimental period; animals were fed with a balanced commercial diet and water and tap water ad libitum. This study was performed in accordance to the guidelines for the use of experimental animals in research at Zoology Department, Faculty of Science, Tanta University, Egypt.

Treatment protocol
Animals were divided into four equal groups (n=10) control group in which mice were received intraperitoneal (i.p.) injection of PBS (Phosphate buffer saline) as control, once i.p. injection of CTX (4 mg/mouse) for induction of leucopenia, subcutaneous (s.c.) injection two days after CTX injection (the time point where leucopenia that occurred (Salem et al., 2016) of 5 µg/mouse G-CSF (Neupogen, CA, USA) daily for 5 consecutive days. To test the impact of SP on leucopenia, mice were treated for 7 consecutive days orally using stomach gavage of 500 mg/mouse starting on the day of CTX injection.

Preparation and counting of peripheral blood mononuclear cells
At the end of the experiment, mice were anesthetized by inhalation of isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethylether; Hospira, Inc. Lake Forest, IL, USA). Fasting blood samples were withdrawn from the retro-orbital vein of each animal using a glass capillary tube after fasting period of 12 h. Blood was analyzed for the total number of leukocytes, using an automated instrument for complete blood counts (Vet-Scan HM2TM Hematology System, Abaxis, Union City, CA) to determine white blood cells (WBCs), platelets, relative and absolute number of neutrophils and lymphocytes.

Serum collection and tissue preparation
After blood collection, mice were sacrificed by decapitation. Liver was rapidly excised from each animal; homogenized and prepared for estimation of biochemical parameters using heparinized micro hematocrit tubes into 1.5 mL Eppendorf® tubes. Spleen and bone marrow were trimmed of the surrounding tissues and single cell suspensions were prepared and counted using a hemocytometer with trypan blue dye.

Preparation of tissue homogenate
The frozen tissues were cut into small pieces and homogenized in 5 ml cold buffer (0.5 g of Na2HPO4 and 0.7 g of NaH2PO4 per 500 ml deionized water (pH 7.4) per gram tissue, then centrifuged at 4000 rpm for 15 minutes at 4°C. The supernatant was removed and used in estimation of biochemical parameters (Manna et al., 2005).

Estimation of serum liver functions
The sera were used for estimation of serum liver according to manufacturer protocol. Alanine amino- transferase (ALT) and aspartate amino-
transferase (AST) activities were determined colorimetrically according to (Reitman and Frankel, 1957).

**Estimation of liver oxidant and antioxidant**
Liver malondialdehyde (MDA) CH2(CHO)2 was determined according to Uchiyama and Mihara (1978), liver reduced glutathione (GSH) was determined according to the method described by (Beautler et al., 1963).

**Flow cytometry**
Fresh single-cell suspensions of leukocytes from blood were prepared. Briefly, peripheral blood samples were collected by bleeding (about 5 drops) each mouse from retro-orbital plexus ant RBCs were lysed with ammonium chloride-potassium chloride (ACK; Invitrogen, Carlsbad, CA) buffer. About 1x106 cells were stained with anti-flurochrome-conjugated mAbs against CD11b and Ly6G for 5 min on ice. Cells were then incubated for 30 min on ice. The cells were washed twice and re-suspended in 0.3 ml 0.5% BSA, 0.02% sodium azide solution. Cells were analyzed by flow cytometry using the Cell Quest software package.

**Statistical analysis:**
Results in this study were represented in the form of mean ± standard deviation; the current data were analyzed using one-way ANOVA, version 16 of SPSS. When p value is < 0.05, it was considered a significant difference.

**RESULTS**

**Optimal growth conditions for Spirulina platensis**
To obtain the maximum growth of SP cultivated in Zarrouk's medium was obtained at optimal of light, pH and incubation period as shown in Table 1. The different growth parameters measured as dry weight, total soluble protein and pigment content as shown in Table (1). The 9th day of incubation of SP at pH 9 showed the maximum pigment content that are considered to be the most bioactive ingredient of SP.

**Effect of SP on CTX-induced leukopenia in blood, spleen and bone marrow**
Toxicity induced by CTX treatment decreased the absolute number of leukocytes in the peripheral blood (PBL) and in the spleen and bone marrow caused significant leucopenia as demonstrated by decreases in the total numbers of leucocytes bone marrow and spleen as compared to their corresponding control animals. Administration of 500mg/kg/day SP or 5ug/mouse G-CSF into CTX-treated mice resulted in significant recovery from the induced leukopenia with comparable effects compared to CTX- treated mice Figure 1 & Figure 2.

As regard to the immune cell subset, CTX caused significant decreases in the relative and absolute numbers of neutrophils accompanied with decreases in the number of lymphocytes when compared to naive mice. Co-treatment of CTX-treated mice with SP ameliorated the relative numbers of neutrophils and leucocytes to values close to their control values (Table 2).
Figure (2): Total count of leucocytes of spleen in different studied groups. a, significant differences at P < 0.05 as compared to the control group. b Significant difference at P < 0.05 compared to the CTX group.

Data also showed increasing the mobilization of CD11b\(^+\) cells by G-CSF and slight ameliorating noticed in SP compared to CTX treated mice Figure (3)

**Effect of SP treatment on ALT and AST activities**

CTX toxicity caused significant increases in the liver enzymes ALT and AST compared to their corresponding controls, P < 0.05. Administration of SP supplement as well as G-CSF to CTX-intoxicated animals revealed a significant decrease in ALT and AST activities compared to CTX treated mice, P< 0.05, Table (3).

The effect of SP supplementation on the liver oxidant-antioxidant capacities were shown in Table (4). CTX increased the hepatic oxidative stress marker MDA and significantly decreased GSH when compared to control group levels. Administration of SP or G-CSF into CTX-treated mice significantly (P<0.05) induced elevation of the antioxidant activity of GSH close enough to the normal capacity and significantly reduced the MDA levels. Interestingly, however, the resultant effects of SP were higher than those of G-CSF.

Figure (3): Representative histogram shows expression of CD11b\(^+\) in Peripheral blood in different studied groups.
Table (1): Effect of optimum culture conditions on SP contents

| Optimal conditions | Dry weight (g/100 ml) | % of soluble protein | Pigment concentration |
|--------------------|-----------------------|----------------------|----------------------|
|                    |                       |                      | Chlorophyll A | Carotenoids | C-phycocyanin | Alliphycocyanin | Phycoerythrin |
| Days (9) Mean ±SD  | 0.28 ±0.09            | 61.3%                | 6.7 ±0.76      | 2.35±0.01  | 45.5±0.3     | 35.5±0.33     | 10.3±0.22    |
| pH (9) Mean ±SD    | 0.25±0.02             | 59.5%                |                      |            |             |              |              |

Significant difference at P < 0.05

Table (2): Cell counts in peripheral blood in different studied groups

| Parameters | Lymphocyte (X10^3) | Total number of leucocyte (X10^9) | Platelets thousands / cmm | Absolute numbers of neutrophils (X10^3/ μl) | Relative numbers of neutrophils % |
|------------|--------------------|----------------------------------|---------------------------|--------------------------------------------|----------------------------------|
| Control    | 7.4 ±0.29          | 7.9 ± 0.47                       | 519 ±1.4                  | 5 ±0.15                                    | 37.2 ±0.59                       |
| CTX        | 1.2±0.19           | 1.6±0.13                        | 483±1.2                   | 0.63±0.14                                  | 5.3±0.29                         |
| CTX +G-CSF | 7.9±0.37           | 7.5±0.37                        | 1062±1.9                  | 4.9±0.29                                   | 28.3±0.41                        |
| CTX +SP    | 2.4±0.33           | 5.8±0.22                        | 536±1.3                   | 3.1±0.18                                   | 25.2±0.32                        |

* Significant difference at p < 0.05 compared to the control group, † Significant difference at p < 0.05 compared to the CTX group, ‡ Significant difference at P < 0.05 compared to the SP group.

Table (3): Serum functions ALT and AST levels in different studied groups.

| Parameters | Serum ALT (I/U) | Serum AST (I/U) |
|------------|-----------------|-----------------|
| Control    | 26 ±0.67        | 32 ±0.36        |
| CTX        | 56±0.70         | 81±0.77         |
| CTX+G-CSF  | 34±3.1          | 40±0.50         |
| CTX+SP     | 42±0.54         | 48±0.28         |

* Significant difference at p < 0.05 compared to the control group. † Significant difference at p < 0.05 compared to the CTX group.

Table (4): Liver oxidant and antioxidant levels in different studied groups.

| Parameters | Liver MDA (nmol/g tissue) | Liver GSH (mg/g tissue) |
|------------|----------------------------|-------------------------|
| Control    | 10±0.09                    | 18±0.22                 |
| CTX        | 45±0.35                    | 4.9±0.12                |
| CTX+G-CSF  | 24±0.38                    | 11.8±0.20               |
| CTX+SP     | 29±0.20                    | 14±0.33                 |

* Significant difference at p < 0.05 compared to the control group. † Significant difference at p < 0.05 compared to the CTX group.
DISCUSSION

Given that SP is an excellent source of potent antioxidant and anti-inflammatory constituents such as phycocyanobilin which is a strong antioxidant, gamma-linolenic acid, alpha-lipoic acid, ergothioneine, carotene, zeaxanthin, vitamin E, vitamin C, selenium, and zinc (Bhat and Madyastha, 2001), it has demonstrated antioxidant (Finamore et al., 2017), anti-inflammatory (Wu et al., 2016), hypolipemic and antihypertensive (Carrizzo et al., 2019), antidiabetic (Szulinska et al., 2017), anticancer (Czerwonka et al., 2018), immunestimulant, antimicrobial (Marangoni et al., 2017), hepatoprotective (Khafaga et al., 2018), neuroprotection (Pabon et al., 2012), and antileucopenic effects (Patel et al., 2013). As such, it has shown several beneficial therapeutic implications in due to these properties.

SP cultivated in Zarrouk’s medium was obtained at optimal of light, pH and incubation period as shown in Table 1. The most bioactive ingredients of SP is pigment content (chlorophyll a, C-phycocyanin, allophycocyanin and phycoerytherin) have been obtained at the 9th day of incubation at pH 9. These conditions also showed the maximum dry weight and total soluble protein. These results are consistent with those of El-Sheekh et al., (2014) who recorded specific growth rate, biomass and protein contents of SP.

In the present study, the effects of supplementation with SP were evaluated to test its anti-toxic leucopenic, antioxidant and hepatoprotective effect. *Spirulina platensis* effect on hematological indices has shown significant ameliorating effect on leucopenia induced by CTX evidencing by enhancing of total leucocytes count in bone marrow and spleen. Moreover, increasing the relative, absolute numbers of neutrophils, number of leucocytes of bone marrow and spleen compared to CTX group. G-CSF has been regarded as immune stimulators due to their role in granulocyte and myeloid hematopoietic and immune function (Martins et al., 2010).

Recently, SP known to have anti-cancer, antiviral properties and immunomodulatory functions through facilitating the releasing of cytokines, antibodies, activating macrophages and enhancing splenocytes (Hirahashi et al., 2002). CTX is cytotoxic and lymphoablative, in this study it was observed that compare to control group, CTX treated mice showed a significant decrease in the total number of leucocytes in both of bone marrow and spleen. Treatment with G-CSF after CTX administration ameliorate the side effect of CTX by increase the level of the total leucocytes up to 40*10^6 per mouse. Treatment with SP after CTX administration ameliorated these leucopenic effects; however, the SP effect was less than the effect of treatment with G-CSF as shown in Figure 1 and Figure 2. These results were in concomitant with (Proietti et al., 2012) and (El-Naggar et al., 2018) who suggested that CTX has severe immune suppression effect.

CTX is often administered together clinically with other chemotherapeutic agents to enhance its anticancer effects or with growth factors such as G-CSF to enhance HSC mobilization and correction of leucopenia. Our data revealed that mice treated with G-CSF showed significant increase in total numbers of leucocytes, relative and absolute numbers of neutrophils, number of leucocytes of bone marrow and spleen compared to CTX group. G-CSF has been regarded as immune stimulators due to their role in granulocyte and myeloid hematopoietic and immune function (Martins et al., 2010).

G-CSF stimulate the immune stimulatory effects on neutrophils has direct and indirect immunomodulatory effects on other immune cells. *In vivo* administration of G-CSF in both animal models and humans has concordantly shown immunomodulatory effects, suppressing the production of pro-inflammatory cytokines in peripheral blood mononuclear cells, inducing tolerant DCs, and enhancing IL-4 but reducing interferon γ production in lymphocytes (Mannon et al., 2009).

*In vitro*, G-CSF inhibits Toll-like receptors (TLR) that motivate the release of inflammatory cytokine like tumor necrosis factor (TNF),
interleukin-12 (IL-12) and interleukin-8 (IL-8) in monocytes macrophages and neutrophils (Fukuzono et al., 2010). Treatment with G-CSF cause induction of type 2 dendritic cells (DCs) that stimulate T cells to express IL-4 and IL-10. On the other hand, G-CSF found to block the production of IL-12 from bone-marrow derived DCs cultured in vitro. Therefore, G-CSF has immunomodulatory effects on cells of the monocyte lineage by both systemically mobilizing DCs and locally by prohibiting inflammatory cytokine production (Rutella et al., 2004).

Also G-CSF administration postchemotherapy (CTX) could alter mobilizing effects on leukocytes. The highest effect appeared when treatments started two days after CTX, but remained so even if therapy began five days post-CTX. However, again, effects of the G-CSF were short-lived. In this study G-CSF showed immune stimulating effect resulted in ameliorating effect on leucocytes count in bone marrow and spleen, also increasing the lymphocytes count, relative and absolute numbers of neutrophils. These results were consistent with those from (Salem et al., 2016), (Demetri and Griffin 1991) who noted G-CSF administration induced increases in WBC counts by acting as a potent hematopoietic growth factor.

CTX treatment could induce an state of immune-depression by direct cytotoxic effects that severely reduce the number of T lymphocytes or through of a heterogeneous population of immature myeloid cells able to suppress immune cells proliferation upon activation by those immature CD11b’Ly6G’CD31` cells (Angulo et al., 2000). This hypothesis is in close agreement with our data that indicate that lymphopenia induced by CTX lead to decrease in myeloid cells that express CD11b’ significantly enhanced immunoregulatory effects. Such results have already been reported in agreement with (Neyrinck et al., 2017). Altogether, those data indicate an improvement of the immune function of mice after oral intake of SP. Furthermore, other studies also showed anti-cancer effects of the bioactive compound phycocyanin from spirulina on differentiation and morphological and cytochemical changes of human myeloid leukemia cell lines, U937 and HL-60 cells as well as the enhanced the expression of monocytic surface antigens CD11b and CD14 on human premonocytic U937 cells (Hayashi 2012), which are generally used for the studies of cell differentiation (Hayashi 2012).

The activities of ALT and AST are indicators of hepatotoxicity (D’Souza Sunil et al., 2009). In the present study, there was a significant increase of both of ALT and AST levels in CTX treated group as compared to control group. These results were in contaminant with (Grigorian and O’Brien 2014). CTX is a chemotherapeutic drug that produce hepatic injury in the liver, CTX toxicity leads to free radical damage by two pathways: (1) the generation of reactive oxygen species (ROS) evaluated by MDA levels as a final product of lipid peroxidation and (2) the depletion of antioxidant reserves.

Our results indicated that CTX resulted in lipid peroxidation in the liver associated with reduction in the antioxidant status, resulting in highly significant increase of MDA levels and significant decrease of GSH. SP with CTX also co-treatment led to decreasing the oxidative stress (MDA) and ameliorating the antioxidant activity (GSH) as compared to G-CSF group. The protective effect of SP may be due to the radical scavenging activity of its components.

Treatment of CTX-intoxicated animals with Spirulina platensis supplement showed anti-inflammatory and antioxidant effect, while the antioxidant effect was more pronounced in the group of SP treated group more than G-CSF treated group.

Administration of SP attenuates serum liver functions, hepatic oxidant/antioxidant levels. Concomitant with our results (Mendiola et al., 2010) found that whole supplement of SP results in significant decrease in oxidation products such as malondialdehyde (MDA) and increase in levels antioxidants by maintaining the efficiency of the cellular antioxidant enzymes such as glutathione peroxidase (GPx) and glutathione reductase (GR) and by increasing reduced glutathione in cells versus...
oxidative stress induced by iron. SP is able to eliminate the free radical-mediated cell damage by discarding superoxide anion, and 2,2′-azobis-(2-aminodipropanone) dihydrochloride free radicals and protect effects on erythrocytes against H2O2-that support DNA damage caused by oxidation. In addition to reducing the MDA level, serum liver enzymes activity and increase in liver GSH level. Restoration of activities of antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione-S-transferase to near normal level in mercuric chloride intoxicated mice (Bedirli et al., 2009). Increased activities of these enzymes indicated changes in the membrane functions and permeability leading to a destruction of hepatocytes and cellular leakage as a result of the powerful effect of CTX in inducing hepatocellular toxicity. The mechanism by which CTX or its main metabolite acrolin affect liver tissue found to be by overproduction of reactive oxygen species (ROS) (Jeelani et al., 2017) causing sever hepatotoxicity.

In conclusion, the results of this study demonstrate that *Spirulina platensis* supplement exhibits marked and multiple effects as antioxidant, antitoxic and immune-boosting effect, suggesting that spirulina could be used as adjuvant therapy with CTX. Further studies are necessary to determine the optimum dose and timing to get the maximum beneficial action of *Spirulina platensis*.

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