International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2023.114.121

Research Article
Investigation of Antitumor Activity of Phycocyanin Obtained from *Spirulina* (*Arthrospira*) *platensis* in Mice

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Abstract

Background and Objective: *Spirulina* contains two types of biliproteins, i.e., C-phycocyanin and allophycocyanin. In this current *in vivo* study, phycocyanin's antitumor impact was examined following its administration by gavage feeding to BALB/c mice with Ehrlich ascites tumor (EAT). **Materials and Methods:** Phycocyanin was administered to three different experimental groups, treatment, control and prophylaxis in different concentrations and the antitumor activity in each group was detected in terms of a dosage effect. Statistical analyses were performed using SPSS 21 software, with the results expressed either as standard deviation or percentage. **Results:** Total antioxidant level (TAL) of the prophylaxis group was found to be statistically significant compared with the control group. When the prophylaxis groups were statistically compared with each other, a significant increase was observed in the values as the dose increased, while a statistically significant difference was found in terms of antioxidant activity between the prophylaxis group 1, which was administered the lowest phycocyanin dose and the prophylaxis group 4, which was administered the highest phycocyanin dose. When the TAL, total oxidant level (TOL), Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) values of the treatment groups were statistically compared with the control group 2, it was detected that treatment group 4 had the highest TAL value, with a statistical significance. **Conclusion:** In the pathological evaluations, the highest dose of phycocyanin was found to be effective against EAT in both treatment and prophylaxis groups.

Key words: Phycocyanin, Ehrlich ascites tumour, phytotherapy, antitumor

Citation: Oğuzkan, S.B., İ. Güler, B.K. Güroy, K. Bakır and M. Özaslan, 2023. Investigation of antitumor activity of phycocyanin obtained from *Spirulina* (*Arthrospira*) *platensis* in mice . Int. J. Pharmacol., 19: 114-121.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Microalgae are among the important producers of many novel compounds with biological activity that can be used as a useful therapeutic agent or an active ingredient. Although microalgae were used in aquaculture in previous years, they are currently preferred in studies due to their ability to contain various high-value molecules such as proteins, chlorophylls, carotenoids and lipids[1,2]. Microalgae such as *Spirulina* contain many bioactive components such as proteins, carbohydrates, gamma linolenic acid, fatty acids, carotenoids, vitamins B1 and B2. *Spirulina* contains two types of biliproteins, namely C-phycocyanin and allophycocyanin, with antioxidant properties. Approximately 20% of this microalgae's protein fraction consists of phycocyanin, a blue pigment that dissolves in water[3,4]. Phycocyanin is a water-soluble, blue-colored, photosynthetic pigment with high antioxidant and strong fluorescent properties[4]. *Spirulina* is a ubiquitous phycobiliprotein variety with a high economic value. Phycocyanobilin chromophore, which is accepted as the prosthetic group of phycocyanin and which is responsible for the characteristic blue color of the protein, binds to the cysteine amino acid from different regions causing the formation of a phycocyanin structure[5]. Phycocyanin has gained recognition as a natural pigment in the food, pharmaceutical and cosmetic industries to replace the synthetic pigments suspected to be carcinogenic[6]. It has been reported by many researchers that purified phycocyanin obtained from cyanobacteria with different nutraceutical and pharmaceutical uses has antioxidant and radical scavenging activity and therefore, that the purified C-PC has a potential for nutraceutical and pharmaceutical use[7,8]. It was determined that phycocyanobilin, which constitute a major part of phycocyanin, are responsible for the destruction of hydroxyl radicals[9]. The antioxidant potential of C-PC (C-Phycocyanin) isolated from *Spirulina* was investigated and it was found to be capable of removing peroxy and hydroxy radicals[10]. The anti-inflammatory, antioxidant and hepatoprotective effects of *Spirulina* phycocyanin have been shown in many studies[11,12]. The structure of the phycocyanin varies depending on the microorganism to which it is attached, environmental conditions and the amount of heat and light used in production. Therefore, in this current study, phycocyanin with an amount >4 obtained from *Spirulina platensis* produced in Turkey was used. In this study, the EAT tumor model was used to evaluate the antitumor activity of phycocyanin. Ehrlich ascites tumor is a transplantable tumor model and it is specific to mice[13,14], studying EAT facilitates tumor evaluation and monitoring. In addition, different experimental models have been made regarding the anticancer activities of phycocyanin produced at different locations and under different production conditions.

Antitumor activity of phycocyanin was performed by solid EAT tumor modeling in previous studies while its activity against EAT tumors in liquid acid form was first demonstrated in this study.

MATERIALS AND METHODS

This study was approved by the local ethics committee of animal experiments with protocol number 62. Gaziantep University BAP- 2018. SHMYO was financially supported by the project with code number 18.01.

Experimental animals: In this study, Ehrlich acid tumor (EAT) was obtained from the Istanbul University Experimental Animal Center. The EAT fluid collected from the mouse was injected into the peritoneum of 8-10 weeks old BALB/c mice weighing between 25-30 g which were grown at the Gaziantep University Experimental Animals Center and the peritoneal fluid obtained was used in this study. The 0.2 mL of EAT was administered intraperitoneally to the animals in the experimental group to induce tumor formation.

Experimental groups and phycocyanin application: All animals were divided into three groups (treatment, prophylaxis and control) and each group was divided into four sub-groups in order to determine the impact concentration level of phycocyanin. There were 6 animals in each group. Then, all animals were weighed to determine the baseline body weights. Phycocyanin (50, 100, 150 and 200 mg kg^{-1}) to be administered was given by gavage in buffer solution (acetate buffer (50 mM sodium chloride+0.002 M sodium azide)). Purified water and buffer solution administered to the control groups were given by gavage. In the prophylaxis groups, animals were given phycocyanin for 5 days and EAT was administered to the animals once on day 6 of the study and the study was terminated after 15 days. In the treatment groups, animals were administered EAT once on the first day of the study and starting from the sixth day of the study, phycocyanin was given daily for 10 days and the study was completed within a period of 15 days. The administrations in the control groups continued for 15 days.

Experimental design: On the following day (day 1), EAT bearing mice were randomly divided into three groups (n = 6 each) as follows:

Group 1: Prophylaxis groups
- **1:** 50 mg kg^{-1} phycocyanin (in 0.3 mL buffer) (for 5 days)+0.2 mL EAT (day 6)
- **2:** 100 mg kg^{-1} phycocyanin (in 0.3 mL buffer) (for 5 days)+0.2 mL EAT (day 6)
- **3:** 150 mg kg^{-1} phycocyanin (in 0.3 mL buffer) (for 5 days)+0.2 mL EAT (day 6)
- **4:** 200 mg kg^{-1} phycocyanin (in 0.3 mL buffer) (for 5 days)+0.2 mL EAT (day 6)

Group 2: Treatment groups
- **1:** 0.2 mL EAT+50 mg kg^{-1} phycocyanin (in 0.3 mL buffer) (from day 6, for 10 days)
- **2:** 0.2 mL EAT+100 mg kg^{-1} phycocyanin (in 0.3 mL buffer) (from day 6, for 10 days)
- **3:** 0.2 mL EAT+150 mg kg^{-1} phycocyanin (in 0.3 mL buffer) (from day 6, for 10 days)
- **4:** 0.2 mL EAT+200 mg kg^{-1} phycocyanin (in 0.3 mL buffer) (from day 6, for 10 days)

Group 3: Control groups
- **1:** 0.3 mL of purified water (for 5 days)+0.2 mL of EAT (day 6)
- **2:** 0.2 mL of EAT+0.3 mL of purified water (from day 6, for 10 days)
- **3:** 0.3 mL of purified water (for 15 days)
- **4:** 0.3 mL of buffer solution (acetate buffer (50 mM sodium chloride+0.002 M sodium azide)) (for 15 days)

Assessment of blood samples: At the end of the treatment, cardiac blood was collected from every animal using a heparinized syringe to determine the oxidants and antioxidants. Serum was obtained from blood samples collected and the total oxidant status (TOL) and total antioxidant status (TAL) were studied for determination of the oxidant level in the serum. The TAL and TOL levels were studied using the TAL-TOL Assay Kit of Rel Assay Diagnostics[15,16]. The ALT and AST parameters were evaluated on the serum obtained, using the kit, in order to determine the blood values indicating liver damage.

Histopathological analysis: Subjects were sacrificed under ether anesthesia and kidney, stomach, small intestine and large intestine tissues were removed and fixated in 10% formaldehyde. After tissue monitoring, incisions were made and stained using the "Hematoxylin-Eosin" method and were examined under light microscopy to determine any tumor growth.

Evaluation of data: Statistical analyses were performed using SPSS 21 software, with the results expressed either as standard deviation or percentage. Paired t-test was used to determine the differences between prophylaxis, treatment and control groups. The Friedman test, a non-parametric test, was used to reveal the differences between the groups. The p-values in all of the statistical tests were two-tailed p-values and $p<0.05$ was accepted as statistically significant.

RESULTS

In the present study, which was designed to include three groups, i.e., prophylaxis, treatment and control groups, each group was divided into four subgroups. In the subgroups, phycocyanin, the effect of which was intended to observe, was given to each group in escalating doses between 50 and 200 mg and the dosage difference was evaluated.

When the results from each group were compared with those of control group 1, it was detected that the TAL results of prophylaxis 1 and prophylaxis 4 groups were statistically significant compared to the control group 1 ($p<0.05$). There were no differences between TOL and AST and ALT values ($p>0.05$) as shown in Table 1.

When the prophylaxis groups were statistically compared with each other, a significant increase was observed in the values as the dose increased, while a statistically significant difference was found in terms of antioxidant activity between the prophylaxis group 1, which was administered the lowest phycocyanin dose (50 mg) and the prophylaxis group 4, which was administered the highest phycocyanin dose. It was detected that the amount of phycocyanin, which was determined to be the highest dose, statistically increased the antioxidant activity compared to the lowest dose group.

To determine whether it has therapeutic properties against the antitumor activity, phycocyanin was given for 15 days to mice, in which the tumor was induced by EAT injection for 5 days, increasing the doses (50, 100, 150 and 200 mg) from day 6. The control group to be compared with the treatment group was also injected with EAT cells for 5 days and the experiment was completed by administering distilled water for 15 days. The mean values of TAL, TOL, ALT and AST of the treatment groups were provided in Table 2.

When the standard mean values of the groups were compared statistically with those of control group 2 by paired t-test, TAL value was found to be the highest value in treatment group 4 (200 mg) and it was found to be significant ($p<0.05$).

Table 1: Results of TAL, TOL, ALT and AST values of control group 1 and prophylaxis groups

Parameter	Prophylaxis group 1 (50 mg)	Prophylaxis group 2 (100 mg)	Prophylaxis group 3 (150 mg)	Prophylaxis group 4 (200 mg)	Control group 1
TAL	1.589	1.858	2.115	3.222	2.020
TOL	0.236	0.278	0.345	0.265	0.333
ALT	33.3	33.0	32.5	31.66	30.6
AST	29.6	32.5	29.16	31.6	30.16

Table 2: Results of TAL, TOL, ALT and AST values of control group 2 and treatment groups

Parameter	Treatment group 1 (50 mg)	Treatment group 2 (100 mg)	Treatment group 3 (150 mg)	Treatment group 4 (200 mg)	Control group 2
TAL	1.626	1.712	1.755	1.977	1.606
TOL	0.516	0.640	0.470	0.684	0.662
ALT	49.1	49.5	42.8	30.8	47.5
AST	27.5	28.3	31.6	30.0	32.5

Table 3: TAL, TOL, ALT and AST values of the control group 3 and prophylaxis groups

Parameter	Prophylaxis group 1 (50 mg)	Prophylaxis group 2 (100 mg)	Prophylaxis group 3 (150 mg)	Prophylaxis group 4 (200 mg)	Control group 3
TAL	1.589	1.858	2.155	3.222	2.211
TOL	0.236	0.278	0.345	0.265	0.486
ALT	33.3	33.0	32.5	31.6	28.3
AST	29.1	32.5	29.16	31.6	30.8

Table 4: TAL, TOL, ALT and AST values of the control group 3 and treatment groups

Parameter	Treatment group 1 (50 mg)	Treatment group 2 (100 mg)	Treatment group 3 (150 mg)	Treatment group 4 (200 mg)	Control group 3
TAL	1.626	1.712	1.755	1.977	2.211
TOL	0.516	0.640	0.470	0.684	0.486
ALT	49.1	49.5	42.8	30.8	28.3
AST	27.5	28.3	31.6	30.0	30.8

Table 5: TAL, TOL, ALT and AST values of control group 4 and control group 3

Parameter	Control group 4	Control group 3
TAL	1.716	2.211
TOL	0.413	0.486
ALT	25.8	28.3
AST	32.5	30.8

Likewise, there was a statistically significant difference between treatment group 4 and control group 2 in terms of the ALT values and the same results were also found to be similar during comparison by dosage difference.

The experimental group named control group 3 was given purified water for 15 days and was not exposed to any tumoral agent. The values compared between this group and 4 different prophylaxis groups were shown in Table 3.

As a result of the statistical evaluation, a significant difference was found in terms of the antioxidant capacity of prophylaxis 1, 3 and 4 groups and the control 3 group ($p<0.05$). The same significance was found in the TOL values between the prophylaxis 4 group and the control 3 group. There was no statistical difference between the ALT and AST parameters.

In the comparison of biochemical parameters between control group 3 and the treatment group, there was a difference in terms of antioxidant parameters between all treatment groups and control group 3. Significance was also found between treatment groups 1, 2 and 3 and control group 3. There was no statistically significant difference between TOL and AST values ($p>0.05$) as shown in Table 4.

During the administration of the active ingredient phycocyanin by gavage method, since the gavage was performed by dilution in the buffer solution used during the extraction of phycocyanin, the control group 4 formed to evaluate the effect of the buffer was given acetate buffer (50 mM sodium chloride+0.002 M sodium azide) for 15 days.

In order to evaluate the effect of the buffer, a comparison was made with control group 3, which was given water for 15 days. While the TAL values were detected to be lower in control group 4 given the buffer solution in comparison to control group 3, no statistical difference was found between TOL, ALT and AST parameters ($p>0.05$) as shown in Table 5.

Histopathological examination of EAT: After all, groups were sacrificed under ether anesthesia, the stomach, kidney, colon and small intestine tissues of the mice were removed as sampled. All samples were incubated for 24 hrs in 10% formaldehyde, then passed through alcohols and xylols of different concentrations and assayed within 24 hrs. Samples stained with hematoxylin were evaluated under a Nikon light microscope after being rendered transparent in xylol.

Preparations prepared from tissues were histopathologically examined. These examinations revealed that the tumor invaded the kidney and stomach tissues. Tumor presence was determined by scoring. Tumor invasion (+) was detected in kidney and stomach tissues when tissue samples from the prophylaxis groups and control group 1 were evaluated as seen in Fig. 1a-b.

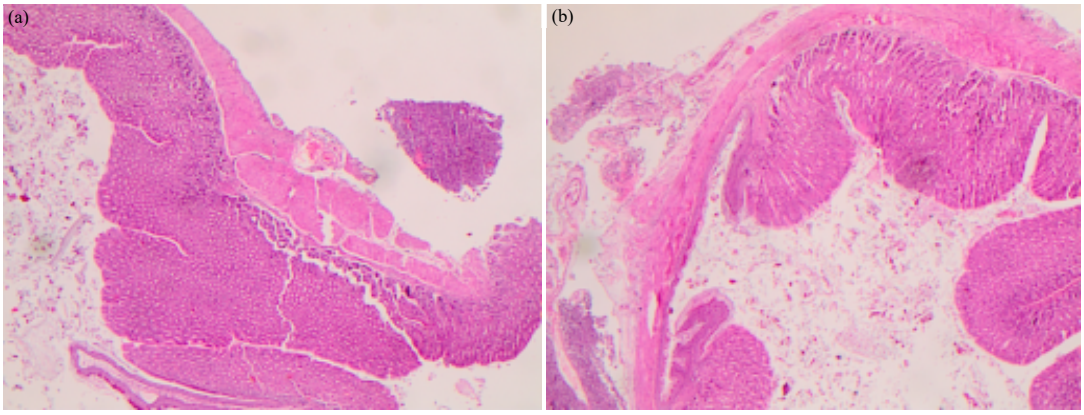

Fig. 1(a-b): Stomach tissue of (a) Control group 2 and (b) Prophylaxis group

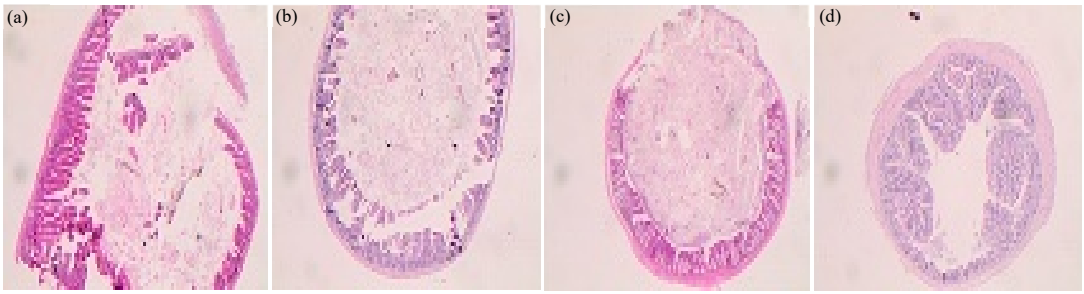

Fig. 2(a-d): (a) Prophylaxis group small intestine, (b) Treatment group small intestine, (c) Prophylaxis group large intestine and (d) Treatment group large intestine

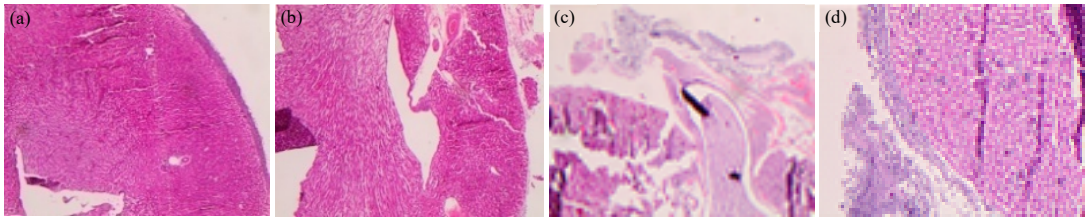

Fig. 3(a-d): Tumor formation in renal tissues of treatment groups by dosage difference (a) 50 mg, (b) 100 mg, (c) 150 mg and (d) 200 mg

Pathological examination of the fibrotic tissue showed the response characteristics of the tumor and no tissue was found to be positive. Formation of new vessels, i.e., neovascularization, after tumor formation was not detected in the prophylaxis or treatment groups or the control groups were given tumors.

Lymphocytes demonstrated the environmental response to tumor formation have been imaged in the small intestine and large intestine tissues of some of the prophylaxis and treatment groups as shown in Fig. 2a-d.

Intra tumoral and lymphatic infiltration was also evaluated. Because the first reactions of the organism to the emerging neoplastic formation are observed in these regions in many cancer types. Intratumoral lymphocyte involvement was also observed in the gastric tissue of the prophylaxis groups.

Intra tumoral lymphocyte tissue involvement of the gastric tissue of both groups was also detected. It was seen in the kidney tissues in Fig. 3 that phycocyanin applied in increasing doses of 50-200 mg does not have a regressive effect on treatment or tumor. When the histopathological samples were assessed on the basis of dosage difference, the amounts of phycocyanin administered at increasing concentrations were found to have no impact on tumor inhibition or treatment as shown in Fig. 3a-d.

DISCUSSION

Spirulina has been used as a food supplement for many years as plankton, algae or cyanobacteria[17]. *Spirulina* is widely studied in the field of pharmacology due to its anti-inflammatory, antioxidant and anti-cancer effects[18,19]. In this study, both treatment and protective effects of phycocyanin obtained from *Spirulina* (*arthrospira*) *platensis* were evaluated together with the control group according to the dose difference. It is quite significant that it has a good protective effect at high doses and a decrease in oxidant values it. In terms of its anti-tumor effect, phycocyanin inhibits cell viability in tumor cells and has little effect on normal cell lines. It has been determined in this study that phycocyanin has a high antioxidant effect, especially at maximum doses and causes a decrease in oxidant parameters at the same rate. In this study, both therapeutic and prophylactic groups were evaluated together in order to show that phycocyanin has high antioxidant activity both as a treatment and as a preventative and to show its usability not only in the diagnosis of disease but also in the prevention of diseases. Likewise, antioxidant activity was found to be high in the protection groups depending on the dosage increase. The antitumor activity of marine algae was first investigated by a researcher in aqueous extracts[20]. The polysaccharide content of aqueous extracts was reported to be associated with antitumor activity[20]. Noda *et al.*[21] examined the antitumor activity of polysaccharide and lipid fractions of 24 species of red, green and brown algae in terms of *Ehrlich carcinoma* and argued that *Scytosiphon lomentaria*, *Lessonia nigrescens* and *Laminaria japonica* demonstrated a considerable impact. Specific compounds with cytotoxic and antitumor effects obtained from algae are studied and reported[22,23]. Therefore, the antitumor activity of phycocyanin, the active ingredient of *Spirulina*, in mice were studied in this project.

In a study of animal experiment models, Yogianti *et al.*[24] have found that the antitumor activity of *Spirulina* is effective in UV-B induced skin cancers. In another report, it has been found to be chemo preventive against 7,12-dimethylbenzene-induced breast cancer[25]. The antitumor potential of phycocyanin isolated from *Spirulina platensis* is well-established. *In vivo* administration of phycocyanin purified from *Spirulina* (5.0 mg kg^{-1} b.wt.) has been found to suppress tumor transparency and increase the average survival time of tumor-bearing hamsters[26]. Phycocyanin, a natural product purified from *Spirulina*, has been found to effectively inhibit *in vitro* pancreatic cancer cell proliferation and xenograft tumor growth *in vivo*[27]. According to data from the literature, phycocyanin has a strong anti-cancer effect in various cancer cell types, both *in vitro* and *in vivo*, including lung cancer[28], colon cancer, breast cancer[29] and bone marrow cancer[30]. In addition, high doses of phycocyanin up to 0.25-5.0 g kg^{-1} b.wt. (w/w) do not produce remarkable symptoms of toxicity and mortality in animals[31].

These studies suggested that phycocyanin has therapeutic potential in the treatment of cancer. It has been reported that phycocyanin shows antitumor activity by various mechanisms in different tumor cell lines and that DNA synthesis in tumor cells causes interference in the recommended intervals. Mechanically, phycocyanin exerts an anti-cancer effect by modulating apoptosis and cell proliferation[32]. Phycocyanin induces apoptosis in tumor cells through ROS production and down-regulates the expression of Bcl-2, a well-known anti-apoptotic molecule and induces PARP cleavage by induction from cytochrome c from mitochondria to cytosol[32,33]. Phycocyanin can induce apoptotic cell death by upregulation of caspase 3 and caspase 8 activities. The anti-cell proliferative effects of phycocyanin are mediated by the inactivation of BCR-ABL signaling and the PI3K/Akt pathway[34,35].

CONCLUSION

The dose-dependent data obtained in this study which examined the antioxidant and hepatoprotective effects of phycocyanin, a natural product purified from *Spirulina*, to determine its antitumoral activity *in vivo* were quite significant. In terms of inhibition of tumor formation, phycocyanin inhibits cell viability in tumor cells while it has little effect on normal cell lines. It has been established in this study that phycocyanin shows high antioxidant properties, particularly at maximum doses, while also causing a proportional decrease in the oxidant parameters. The high antioxidant activity of phycocyanin, both therapeutic and prophylactic, has shown its benefit not only in the diagnosis but also in the prevention of diseases. Detection of its significant effect on liver enzyme parameters in the tumor-induced treatment group in this study has demonstrated that it also has a hepatoprotective impact.

SIGNIFICANCE STATEMENT

In this study, the anti-tumoral activity of phycocyanin was studied *in vivo* in animal experiments by forming an EAT tumor. Both serum parameters and pathological evaluation were performed and antioxidant activity and hepatotoxic effect were evaluated together. The results are very important in terms of comprehensively showing the *in vivo* effect of phycocyanin obtained from *Spirulina* (*Arthrospira*) *platensis*.

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