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Original Article

Evaluation of C-phycocyanin Effects with Drug Purity on the Immune System through Its Effect on Interferon-gamma (IFN-γ)

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Abstract

Introduction: Spirulina (Arthrospira) exerts a wide spectrum of pharmacological activities that are largely attributed to its phycobiliprotein content, mainly to C-phycocyanin. C-Phycocyanin is a natural blue pigment with many commercial applications in foods, cosmetics, and medicines.

Methods: In this study, the stimulatory effect of C-phycocyanin on the immune system was investigated using blood tissue and Peripheral Blood Mononuclear Cells (PBMC) and the measurement of inflammatory cytokine, interferon-gamma, which has important effects on dedicated immune responses. For this purpose, extraction of PBMC blood cells from blood tissue The range of purified C-phycocyanin extract concentrations with drug puritywas: 1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml. Then, the response of PBMC cells to C-phycocyanin at the protein level was investigated. Finally, interferon-gamma was measured using the culture supernatant and ELISA sandwich method.

Results: Descriptive analysis of the read concentrations results by ELISA technique showed that C-phycocyanin is dose-dependent and the results of the effect of C-phycocyanin on PBMC cells in blood tissue showed the strengthening of the immune system by increasing the amount of inflammatory cytokines. According to the results of the analysis of variance, it is observed that the p-value is less than 0.05. This means there is a significant difference between the mean read concentration of ELISA in different concentrations of cytokines.

Conclusion: The results of the experiments demonstrate that C-phycocyanin activates PBMC cells in a manner that is consistent with the recruitment of diverse populations of leukocytes in response to inflammatory and infectious signals.

Keywords: C-phycocyanin, Peripheral Blood Mononuclear Cells, IFN Gamma, Phycobiliprotein, Inflammatory Cytokine, Cell Culture

Introduction

Spirulina (Spirulina platensis) is a member of the blue-green alga family and it is inhabited by carbonate-rich lakes that are attracting considerable interest as a dietary supplement.[1,2] It contains large amounts of protein (70 % dry-weight), carotenoid (4000 mg/kg), omega-3 and omega-6 polyunsaturated fatty acids, Gamma Linoleic Acid (GLA), sulfolipids, glycolipids, polysaccharides, provitamins; vitamin A, vitamin E, vitamins B, mineral such as magnesium, iron, calcium, manganese, potassium, selenium and zinc.[3] The results showed the highest content of total protein in Spirulina sp.1 (46.08 ppm) and total carbohydrates in Chlorella sp. (48.01 ppm) The basics.[4] Since the early 1970s, spirulina has been used as a feed additive in aquaculture and poultry nutrition and has been found to have a positive effect on the health status of farm animals. It has been utilized as a source of protein and vitamin supplements and has been sold as a health drink or pills in tablet form for more than 10 years without any undesirable effect on humans.[5] Spirulina is a potent mixture of antioxidants and most of Spirulina's health benefits are associated with its antioxidant pigments. These are carotenoids (mixture of carotenes and xanthophylls), chlorophyll, and the unique blue pigment phycocyanin.[6] These observations led to numerous in vitro and in vivo studies in attempts to characterize the biological effects of spirulina and its pharmacological mechanism

of action.[7] Hayashi et al.[8] were among the first to report that mice on a spirulina diet showed an enhanced primary immune response to sheep red blood cells. These investigators also noted that a water extract of the blue-green alga increased the proliferation of spleen cells in culture and enhanced interleukin-1 (IL-1) production by peritoneal macrophages. Leukocyte activating effects of spirulina were subsequently confirmed by other laboratories. Dietary spirulina was found to enhance humoral and cell-mediated immune functions in chickens as reflected in increased phagocytic activity of macrophages and higher antibody titers.[9,10] Its safety for human consumption has also been established through numerous toxicological studies. Recently, Spirulina has been speculated to be associated with modulation of the host immune system.[11] The identity of the constituents accounting for the immunostimulatory effects has not yet been established. There has been an extensive amount of research on the Arthrospira Platensis species, more commonly known as "Spirulina". Spirulina exerts a wide spectrum of pharmacological activities that are largely attributed to its phycobiliprotein content, mainly to C-phycocyanin. C-phycocyanin is the major phycobiliprotein in spirulina. This Cyanobacterium contains mainly two phycobiliproteins, namely, C-phycocyanin (C-PC) and allophycocyanin (APC) approximately at a ratio of 10:1.[12] C-PC and APC are water-soluble, brightly colored, and highly fluorescent proteins. They are mainly used as fluorescent markers in biomedical research,[13] nutrient ingredients, and natural dye for food and cosmetics,[14] and also as a potential therapeutic agent in oxidative stress-induced diseases.[15] Bot et al. reported that phycocyanin is a potent peroxyl radical scavenger with an IC (50) of 5 micro mols that the covalently linked chromophore, phycocyanobilin, is involved in the antioxidant and radical scavenging activity of phycocyanin.[16] Further, the studies suggest that the covalently-linked tetrapyrrole chromophore phycocyanobilin is involved in the radical scavenging activity of C-PC. The Electron Spin Resonance (ESR) spectra of C-PC indicate the presence of free radical active sites, which may play an important role in its radical scavenging property. Thus, the electron spin resonance activities of C-phycocyanin without disturbance can cause the formation and arrangement of radicals and thus help to inhibit free radicals.[17] Also, the anti-inflammatory activity of phycocyanin has been demonstrated in part by inhibiting the formation of pro-inflammatory cytokinin, inhibiting the induction of nitric oxide synthase, and reducing the detection and expression of cyclooxygenase-2 in many in vitro and in vivo studies.[18] Remirez et al. stated that the inhibitory effects of phycocyanin were dose-dependent. Taken together, our results suggest that inhibition of allergic inflammatory response by phycocyanin is mediated, at least in part, by inhibition of histamine release from mast cells.[19] In other studies, to study the immunom-odulation caused by selenium-enriched phycocyanin (Se-PC) from spirulina, microalgae have been investigated. Se-PC dosage made up to 450 mcg per rat daily that corresponded to 5 mcg of selenium. Rats receiving Se-PC demonstrated significantly increased specific IgG response.[20] Finally, spirulina increases the activation of cellular antioxidant enzymes, inhibits lipid peroxidation and DNA damage, sweeps free radicals, and increases the activity of superoxide dismutase and catalase.[21] Furthermore, Spirulina improves oxidative stress markers and NK activity in healthy subjects and CD_4^+ count in HIV^+ patients.[22] Here we show that the protective effect of C-phycocyanin on the immune system by Select the most effective immune-related tissue, namely blood tissue, by extracting blood PBMC cells and performing the cell Culture technique, in a dose-dependent manner, to inducing inflammatory cytokine INF-γ in treated PBMC cells. These findings support the proposition that the immunostimulatory effects of C-phycocyanin can be attributed to the ability of its high molecular weight protein fraction to stimulate the production of cytokines involved in immune and inflammatory processes. Most of them are currently secreted by active macrophages or dendritic cells, which are considered as an indicator of NK cell activity. Therefore, interferons can play an effective role in the antiviral activity of NK cells. The relationship between NK cells and interferon cells is a complementary and reinforcing relationship that reinforces each other's actions and effects and increases the strength of the immune system.

Materials and Methods

Blood Samples

Peripheral blood (10 ml) was collected into sterile tubes containing EDTA or heparin from healthy

adults. Volunteers gave informed written consent, and the studies were approved by the local ethics committee. All experiments were performed in duplicate.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Five ml of peripheral blood was slowly added by the sampler through the wall on the ficoll (2.5 ml) and centrifuged at 650 rcf for 15 minutes at 6 °C. Then, the ring of mononuclear cells was isolated slowly. Bafficoat was drawn to the volume of 10ml with PBS and centrifuged with a balanced wash unit of 450-500 g for 6 minutes at 4 °C. After washing twice, the PBMC were increased to 1ml with DMEM, and cell count was performed with a Neubauer chamber.

Culturing Peripheral Blood Single Cell

At this stage, six houses were used so that in each well 3 ml of culture medium (FBS 20% and DMEM) and 4×10^5 cells were used. Then, five wells per plate, one without treatment as control, and the rest of the houses were treated with the concentration range of 1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml of purified C-phycocyanin extract, respectively. The samples were then placed in a CO_2 incubator for 24 hours. After incubation, the contents of each well were exposed to a centrifuge of 6000 rpm for 10 minutes at 4 °C. Its supernatant was transferred to -20 °C until the ELISA test.

Assay for IFN-γ-inducing Activity

To measure the level of IFN-γ, Human IFN-γ Standard Kit ABTS ELISA Development Kit (Cat No: 900-K27) was used. This method is based on the standard sandwich ELISA, in which rabbit monoclonal antibodies. Dilute capture antibody with PBS to a concentration of 1 µg/ml. Immediately, add 100 µl to each ELISA plate well. Seal the plate and incubate overnight at room temperature. Then wash and add 300 µl block buffer (1% BSA in PBS) to each well. Incubate for at least 1 hour at room temperature. Then wash the plate with wash buffer (0.05% Tween-20 in PBS). Immediately add 100 µl of standard or sample to each well. Incubate at room temperature for at least 2 hours. Dilute detection antibody in the diluent to a concentration of 1 µg/ml. Add 100 µl per well. Incubate at room temperature for at least 2 hours. Avidin-HRP Conjugate: Dilute 5.5 µl of Avidin-HRP conjugate 1:2000 in diluent for a total volume of 11 ml. Add 100 µl per well. Incubate 30 minutes at room temperature. Add 100 µl of substrate solution to each well. Incubate at room temperature for color development. 10 minutes after adding the ABTS solution, the absorbance was read with an ELISA reader at 405 nm. According to the concentration of standards and their optical absorption values and by using the Expert Curve software, the standard curve was drawn and the concentration of each sample was calculated using the read optical absorption.

Statistical Analysis

To assess the effects of C-phycocyanin and the analysis of dose-response and time course studies, one-way analysis of variance (ANOVA) and Bonferroni post-test were used, respectively.

Results

The descriptive results of ELISA test analysis are presented in Table 1. The mean ± SD concentrations, by testing different concentrations of cytokines are provided. According to the results of the analysis of variance, the *P*-value is less than 0.05. Therefore, there is a significant difference between the mean read concentrations of ELISA at different concentrations of cytokines. Bonferroni post-test was used to determine which concentrations differed significantly. The results are reported with letters in Figure 1 and Table 1. The groups that show the same letters are not statistically different in the reading concentration of the ELISA.

Table1. Statistical Analysis of the Results of the ELISA Test

			Descriptive Results		
Concentrations	**Control**	**1**	**10**	**100**	**250**
Mean ± sd	69.62±12.98[a]	98.02 ± 22.07[ab]	114.84 ± 29.11[ab]	147.47 ± 22.31[b]	251.78 ± 41.82[c]
			Analysis of Variance Results		
	Sum of Squares	**df**	**Mean Square**	**F**	***P*-value**
Between group	99164.048	4	24791.012	33.056	<0.001
Within-group	14999.407	20	749.970		
Total	114163.455	24			

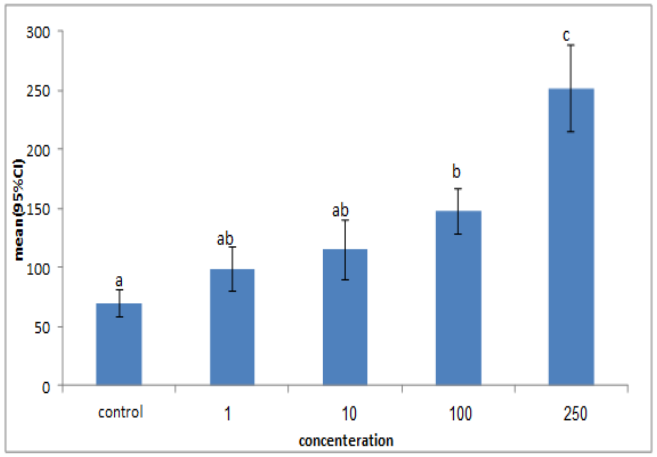

Figure 1. Mean of different concentrations of reading cytokine in treatment with different concentrations of C-phycocyanin.

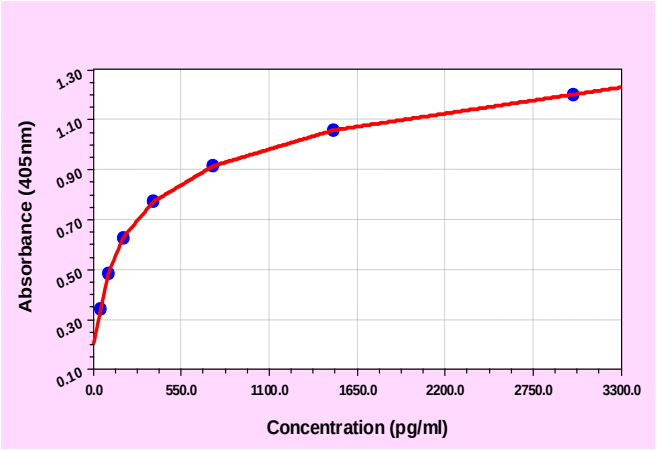

Figure 2. Standard curve INF-γ. The standard curve is obtained when either O.D. reading does not exceed 0.2 units for the zero standard concentration, or 1.2 units for the highest standard concentration.

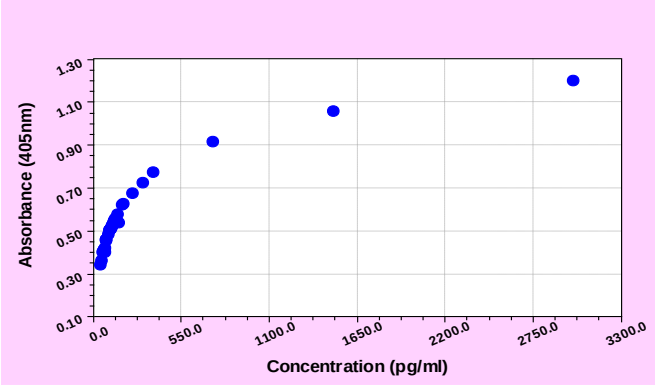

Figure 3. Evaluation of the effect of c-phycocyanin on IFN-γ protein levels in PBMC cells measured by the ELISA method.

The groups that show the same letters are not statistically different in the reading concentration of the ELISA. Therefore, the control group and concentrations of 1 and 10 are not significantly different. There is no significant difference between the concentration of groups 1 and concentrations of 10 and 100. Concentrations of 100 and 250 were also significantly different and concentration of 250 with other levels of concentration, a significant difference.

Figure1 shows the mean of different concentrations read by ELISA reader in treatment with different concentrations of C-phycocyanin.

According to the concentration of standards and their optical absorption values, the standard curve was drawn using the Expert Curve software (Figure 2). Thus, the concentration of each sample was calculated using the read light absorption.

In addition to the order of concentration of each sample and using the read light absorption, the effect of C-phycocyanin on the level of interferon-gamma protein in the treated PBMC was measured and its diagram was drawn according to Figure 3. Therefore, the density of points in the curvature of the sigmoid curve shows its effect on the immune system.

Discussion

In recent years, Spirulina has gained more and more attention from medical scientists as a nutraceutical and a source of potential pharmaceuticals, although its other potential health benefits have attracted much attention. Oxidative stress and dysfunctional immunity cause many diseases in humans, including atherosclerosis, cardiac hypertrophy, heart failure, and hypertension. Thus, the antioxidant, immunomodulatory, and anti-inflammatory activities of these microalgae may play an important role in human health. Clinical trials show that Spirulina prevents skeletal muscle damage under conditions of exercise-induced oxidative stress and can stimulate the production of antibodies and up or down regulate the expression of cytokine encoding genes to induce immunomodulatory and anti-inflammatory responses. Therefore, in this project, first, the protective effect of C- phycocyanin on the immune system by selecting the most effective immune-related tissue, namely blood tissue, and secondly by extracting blood PBMC cells and performing Cell Culture technique, the effect of C-phycocyanin on the level of interferon-gamma protein in treated PBMC cells was measured and its effects on the immune system were studied.

Cytokines are polypeptides that are produced in response to microbes and other antigens that direct and

regulate immune or inflammatory reactions. Some of these cytokines are inflammatory cytokines. The cytokines in this group work either by stimulating innate defense factors or specific defense factors, or both.[23] Interferon-gamma is an inflammatory cytokine that exerts its effect on the immune system, mostly through enhancing growth, regulation, and delayed-type hypersensitivity. TCD_4^+ lymphocytes not only affect the function of other cells by secreting lymphokines and specific mediators, but also enhance their activity. For example, IL-2 stimulates the growth of other T cells and B lymphocytes, or in IL-4, which has opposite effects to IL-2, it causes T lymphocytes to play a regulatory role in the immune system. While TCD_4^- TCD_8^- cells are usually γ type, they produce IL-2, IL-4, and IFN-γ when activated.[24] The results of our study showed that the treatment of mononuclear cells with C-phycocyanin increases IFN-γ levels. Increased levels of IFN-γ may indicate an increase in T lymphocyte activity. In addition to enhancing the growth and regulatory role of T lymphocytes in the immune system, this can lead to the production of other cytokines such as IL-2 and IL-4. T helper cells (Th) are also divided into two groups, Th1 and Th2. Th1 cells produce the cytokines IL-4, IFN-γ, and the lymphotoxin TNF-β (tumor necrosis factor), while Th2 cells produce IL-4, IL-5, IL-6, and IL-10. Th1 cells seem to play a more important role in the fight against intracellular pathogens and Th2 cells in the fight against extracellular pathogens.[25] Therefore, based on the fact that Th1 cells are produced by IFN-γ, and on the other hand, Th1 cells play a more important role in the face of intracellular pathogens, reviewing the results and graphs obtained from the effect of different concentrations of our purified extract on IFN-γ and its increasing trend, it may be concluded that purified C-phycocyanin extract with the mentioned drug purity, in the treatment of diseases caused by cell growth abnormalities, such as tumors and cancers, which are considered as intracellular pathogens, play an effective role by activating the immune system.

The study of the practical properties of lymphocytes has led to the identification of a new class of these cells called inherently lethal lymphocytes (natural killer cells). These cells unlike T and B lymphocytes, are not antigen-specific and after initial contact with the tumor or virus-infected cells, no memory is created. Therefore, since these cells do not require prior contact with antigen for a response, they seem to play an important role in defense against viral infections in the early stages before the production of antibodies and the emergence of specific cells. NK cells in addition to their innate lethal activity, exhibit antibody-dependent cellular cytotoxic activity, which by this mechanism, cytotoxic cells can kill targets of antibody-covered. NK cells in addition to cytotoxic activity, exhibit other immunological activities. For example, these cells can produce IFN-γ, TNF, and serine esterases. Interferon cells also increase the cytotoxic activity of NK cells. Therefore, interferons can play an effective role in the antiviral activity of NK cells.

Thus, the relationship between NK cells and interferon cells is complementary and reinforcing so that each other's actions and effects are enhanced and increase the strength of the immune system.[26] In this regard, it has been shown that lymphocytes strengthen and differentiate monocytes by secreting lymphokines such as IL-2, IL-3, IL-4, IFN-γ, and CSFs.[23] Therefore, increasing the level of interferon-gamma leads to the strengthening and differentiation of monocytes, which is consistent with the results of this study.

Monocytes also boost the immune system in several ways. Firstly, adult monocytes have large amounts of monocyte-specific antigens (CD14), but they lose these antigens when the monocytes become macrophages. The most important property of monocyte-macrophage is the ability to swallow particles smaller than 0.2 microns by pinocytosis and swallow particles larger than 0.2 microns by phagocytosis.[24]

Monocytes-macrophages in humans, contain antigens of MHCII, interferon-gamma receptor. Besides, human monocytes also contain MHCI antigens. Therefore, these cells can deliver antigens to both TCD_4^+ and TCD_8^+ lymphocytes.[23]

Conclusion

By increasing the amount of interferon-gamma, which leads to the strengthening and differentiation of monocytes and since the monocytes strengthen the immune system in two ways: unsafe(non-specific) and safe or specific as mentioned in the discussion section, the results of this study are in line with increasing and strengthening the immune system. On the other hand, monocytes-macrophages act as a deputy cell or Antigen-Presenting Cell (APC) by delivering antigen to T lymphocytes, and on the other hand, lymphocytes

also affect the activity and differentiation of monocytes and macrophages by factors secreted. Noting that the increase in gamma interferon levels has strengthened and differentiated monocytes, and subsequent, delivery done of antigen to T lymphocytes by monocytes-macrophages, and in contrast, the intensifying effect of lymphocytes on the activity and differentiation of monocytes and macrophages occurs. Therefore, by studying the results of the effect of this drug extract on PBMC cells, and considering the increase in the effect of this protein on interferon-gamma levels and the interaction of cytokines on each other, it seems that this drug has an important role in increasing the immune system in humans. The results of this study could be useful in opening a new perspective on C-phycocyanin as a new treatment strategy for diseases of the immune system.

Conflict of Interest

The authors declare that they have no conflicts interest.

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