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Immunomodulating properties of astaxanthin extracted from shell discards of Arabian red shrimp *Aristeus alcocki* (Ramadan, 1938)

S. Sindhu* and P. M. Sherief[1]
Government Vocational Higher Secondary School, Kadamakudy, Pizhala, Kochi 682 027, Kerala, India.
[1]Director (Retd), School of Aquatic Food Products Technology, Kerala University of Fisheries and Ocean Studies, Panangad, Kochi 682 506, Kerala, India.

*Correspondence e-mail: sindhu.prasadnair@gmail.com

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Original Article

Abstract

Industrial shrimp processing discards are a potent source of natural carotenoids. This study was aimed at investigating the immunomodulating properties of astaxanthin, the major carotenoid extracted from shell discards of Arabian red shrimp Aristeus alcocki. The immunostimulatory effect of astaxanthin was determined by assaying the Splenic T-lymphocyte mitogen response, bone marrow cell proliferation assay, plaque formation cell assay, circulating antibody titre, and alpha-naphthyl acetate esterase activity in bone marrow cells at the end of 45 days feeding of the control mice with normal diet and the test animals with normal diet containing astaxanthin @ 10 mg/kg feed. In the astaxanthin treated animals, the spleen cell proliferation was found to be stimulated in the presence of mitogen. Enhanced proliferation of bone marrow cells was also observed in treated animals compared to control animals. These findings indicate induction of proliferation of bone marrow stem cells either directly or indirectly, stimulating the release of factors that are involved in the regulation of hemopoesis. The treated animals also showed an increase in number of plaque forming cells in the spleen, and antibody titer in the circulation which are the functions of B-cells. Bone marrow cells positive for non specific esterases were found to increase after astaxanthin treatment which indicates increased maturation of cells of lymphoid linkage. The present study thus reveals that oral administration of astaxanthin from A. alcocki shell discards stimulates immune function in mice through enhanced proliferation and function of immunocompetant cells.

Keywords: *Arsteus alcocki, astaxanthin, immunomodulating property*

Introduction

Industrial shrimp waste is one of the cheapest sources of natural carotenoid (Shahidi *et al.*, 1998 and Venugopal, 2008). This waste finds very little practical application at present and is categorized as a major environmental contaminant. The major component of carotenoids of shrimp and crab shell backs are mono and diesters of astaxanthin (Shahidi *et al.*, 1992), a very potent antioxidant with some unique properties suitable for use as a drug or food supplement in the treatment of cardiovascular, immune, neurodegenerative diseases and cancer. Astaxanthin is reported to show a number of immunomodulatory effects. Positive effects of astaxanthin on immune response mechanisms in aquatic invertebrates have been demonstrated by Kawakami *et al.* (1998). Astaxanthin is considered as a vital vitamin that is essential for the proper development and survival of juveniles of salmon (Christiansen, 1995). In the case of mammals also astaxanthin has been shown to significantly influence the immune function in a number of *in vitro* and *in vivo* assays using animal models. Studies on the immunological significance of the astaxanthin and effects on the antitumour effector activity of natural killer cells suggested that astaxanthin improves antitumour immune responses by inhibiting lipid peroxidation (Kurihara, 2002).

Astaxanthin enhances *in vitro* antibody production by mouse

spleen cells stimulated with sheep red blood cells done (SRBC) by use of mitogen responses to spleen cells, thymocyte proliferation, interleukin 2 production and antibody production in response to sheep red blood corpuscles (SRBC) (Jyonouchi, 1991) partly by exerting actions on T-cells especially T-helper cells (Jyonouchi *et al*., 1993). Astaxanthin can also partially restore decreased humoral immune responses in old mice Jyonouchi (1994). Studies on human blood cells *in vitro* demonstrated that astaxanthin can enhance immunoglobulin production in reponse to T-dependent stimuli (Jyonouchi *et al*., 1995). These immuno modulating properties are not related to pro vitamin A activity, because astaxanthin, unlike β-carotene does not have such activity in mammals (Jyonouchi, 1991). Astaxanthin has been found effective in preventing development of symptoms in autoimmune-prone mice (Tomita, 1993). Astaxanthin was found to suppress interferon gamma (IFN-gamma) production by cloned murine Th1 cells and increased the number of antibody secreting cells in primed and unprimed spleen cells. In the culture of Th2 clone astaxanthin enhanced the number of antibody secreting cells (Jyonouchi, 1996). In a study with *in vivo* modulatory activity of beta-carotene, canthaxanthin and astaxanthin, mice fed with astaxanthin and beta-carotene had enhanced phytohemagglutin induced lymphoblastogenesis compared to unfed ones. Astaxanthin also enhanced lymphocyte cytotoxic activity (Chew *et al*., 1999). It has also been suggested that astaxanthin's immunomodulating functions may be related to its antitumour activity (Jyonouchi *et al*., 2000). The immununomodulating functions capacity of astaxanthin was superior to that of β-carotene and canthaxanthin (Jyonouchi *et al*., 1995; Jyonouchi *et al*., 1996). In an *in vitro* cell culture experiment with different carotenoids, astaxanthin caused significant stimulatory effect on cell proliferative response of spleen cells and thymocyte in Balb/c mice. Cytokine inducing activity was highest for astaxanthin (Okai and Okai, 1996). The present study reports the immunostimulating action of astaxanthin. Astaxanthin treatment stimulated proliferation of spleen cells in the presence of mitogen, proliferation of bone marrow cells, increase in number of plaque forming cells in the spleen, and antibody titer in the circulation which are the functions of B-cells.

Material and methods

Chemicals

Acetone, petroleum ether (B.P.40-60oC), DMSO, ethylene glycol monoethyl ether, were purchased from Merck, India Ltd, Mumbai. SDS, Dimethyl formamide were purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai. Alpha naphthyl acetate, alloxan, Pararosaniline were purchased from Loba Chemie Pvt. Ltd., Mumbai. Tissue culture media RPMI-1640, Foetal Bovine serum (FBS) and agarose were purchased from Himedia Laboratories, Mumbai. Phytohaemagglutinin (PHA) and PBS were obtained from Genei, Bangalore. MTT was purchased from Sigma chemicals, USA. All other reagents were of Analytical Reagent quality.

Extraction of astaxanthin

Shell discards from the deep sea shrimp *Aristeus alcocki* was collected from the processing plants RF Exports Pvt. Ltd, Chandiroor and Caps Seafoods Pvt. Ltd, of Vypeen, Kerala, India. The waste was transported to the laboratory in an insulated box in iced condition. They were packed in polyethylene bags and stored at -20ºC until used. The wet waste was homogenized in a laboratory mixer (Crompton Greaves, India) and used for the extraction of astaxanthin according to the method of Barbosa *et al.* (1999). A known weight of homogenized wet sample was extracted with acetone. The carotenoid extract was filtered using Whatman No.42 filter paper. The recovered shrimp discards were repeatedly extracted with fresh solvent until the filtrate was colourless to a maximum of three times. The pooled extract was collected for the quantification of astaxanthin. In the case of acetone extraction the pooled extract was collected in a separating funnel, 12.5 ml of petroleum ether (BP 40-60ºC) and 9.4 ml of 0.73 % (w/v) NaCl solution were added. After thorough mixing, the epiphase was collected. To the lower phase an equal volume of water was added, mixed and the epiphase was collected, the pooled epiphase was evaporated to dryness under a stream of nitrogen. A known volume of the extract was evaporated to dryness and the residue was dissolved in hexane and astaxanthin was quantified by measuring absorbance at 470 nm using the equation of Kelley and Harmon (1972). Analysis of different components in the shrimp shell extract was done using thin layer chromatography (TLC) based on the method of Kobayashi and Sakamoto (1999); Sindhu and Sherif (2011).

Animals

Male Balb/c mice 4-5 weeks old weighing 20-25 g purchased from Small Animal Breeding Centre, Kerala Agricultural University, Mannuthy, Thrissur, Kerala, India were used for the study. The animals were housed under hygienic conditions in polypropylene cages under 12 hour light and dark cycle. All procedures involving animal care and experiments were in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and with the approval of Institutional Animal Ethics Committee (IAEC).

Experimental design

The animals (male Balb/c mice) were housed in groups of two in polypropylene cages with a 12:12 light/dark cycle. Sufficient number of control groups and test groups were maintained so that at least 6 animals were available for each assay. The animals in the control group (Group I) were fed

on normal diet purchased from College of Veterinary and Animal Sciences, KAU, Thrissur, Kerala. The animals of the test group (Group II) were fed on normal diet containing astaxanthin @ 10 mg/kg feed. They were provided with food and water *ad libitum*. The experimental duration was 45 days. At the end of experimental period animals were sacrificed and spleen, bone marrow cells and blood were collected for the following assays.

Splenic t-lympocyte mitogen response

At the end of the feeding study, the animals were sacrificed; spleen removed asceptically and made into single cell suspension by passing through a wire mesh. The cells from both the control and test animals were cultured (10^6 cells/ml) in the presence and absence of mitogen phytaheamagglutinin PHA (5μg/ml) in RPMI-1640 medium containing 10% FBS (final volume 1 ml) and antibiotics in a CO_2 incubator at 95 % RH and 5 % CO_2 at 37ºC. After 48 h, 50μl MTT dye [3-(4, 5-Dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] was added and further incubated for 4h. MTT solution was dissolved in RPMI-1640 medium at 4 mg/ml. It was passed through 0.45μ filter, to remove any amount of insoluble residue. After 4 h the liquid contents of the well were discarded and added 100 μl MTT lysis buffer (2g SDS dissolved in 5ml dimethyl formamide and 5 ml distilled water) to each well to solubilize the formazan crystals to produce a solution suitable for measurement of absorbance. After adding MTT lysis buffer the plates were incubated further for 2h in dark. The results were reported as absorbance measured at wavelength 570 nm (Singh *et al.*, 2007).

Bone marrow cell proliferation assay

Bone marrow cell proliferation assay was carried out by following the method of Kumar *et al.* (1999). Total bone marrow cells were collected from control and test animals and made into single cell suspension in RPMI-1640 as described in section 2.5. The cells (106 cells/ml) were cultured in the presence and absence of mitogen PHA (5μg/ml) in RPMI-1640 medium containing 10% FBS (final volume 1 ml) and antibiotics in CO_2 incubator at 95 % RH and 5 % CO_2 at 37ºC in a humidified atmosphere of 5 % CO_2 at 37ºC. After 48 h, 50μl MTT dye [3-(4, 5-Dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] was added and further incubated for 4h. MTT solution was dissolved in RPMI-1640 medium at 4 mg/ml. It was passed through 0.45μ filter, to remove any amount of insoluble residue. After 4 h the liquid contents of the well were discarded and added 100 μl MTT lysis buffer (2g SDS dissolved in 5 ml dimethyl formamide and 5 ml distilled water) to each well to solubilize the formazan crystals to produce a solution suitable for measurement of absorbance. After adding MTT lysis buffer the plates were incubated further for 2h in dark. The results were reported as absorbance measured at wavelength 570 nm.

Plaque formation cell assay

Preparation of SRBC suspension: Collected 10 ml of sheep blood to 10 ml of Alsever's solution. Mixed gently and kept at 4ºC till used. Removed the supernatant and washed with physiological saline. Resuspended in saline and centrifuged at 700g for 5 min. Noted the volume of packed cells and made in to desired suspension in normal saline.

Plaque assay: Modified slide technique of Jern's Plaque assay was adopted for plaque formation cell assay (Mehrotra, 1992). At the end of experimental period the control and test animals were immunized with 1 ml of 5 % SRBC intraperitoneally. The spleen was collected from the sacrificed animals on the 5th and 10th day following immunization. A single cell suspension of the spleen cells was prepared in PBS (8x106 cells/ml). To 0.5 ml of 0.5% agarose prepared in PBS, 50 μl of 10% SRBC and 50 μl of spleen cell suspension were added, mixed well and poured over a glass slide. The slides were allowed to solidify and then incubated with fresh human serum as a source of complement for 1 h at 37ºC. The plaques formed were counted using a colony counter and represented as plaque forming cells (PFCs /million spleen cells).

Circulating antibody titre

Blood was collected from the immunized animals on the 3rd and 5th day following immunization. The blood was allowed to clot and serum was separated by centrifugation. Two-fold serial dilution of the sera samples were made in physiological saline and mixed (1:1) with 1% SRBC in physiological saline. Agglutination was noted after incubation at room temperature for 3 h (Nelson and Davey, 1992).

Alpha-naphthyl acetate esterase activity in bone marrow cells

Naphthyl acetate esterase activity in the bone marrow is an indicator of maturation of stem cells to monocytes-macrophages. Total bone marrow cells from control and test animals were made in to a single cell suspension. A smear was prepared, dried and stained according to the method of Nelson and Davey (1992). The slides were incubated in a reaction mixture containing 44.5 ml phosphate buffer (pH 7.6), hexazotized pararosaniline (1.2 ml 5 % pararosaniline and 1.2 ml 4% NaNO2) alpha-naphthyl acetate (50 mg/2.5 ml ethylene glycol monoethyl ether) for 45 min. It was washed and counter stained with haematoxylin. A total of 4000 cells were counted in triplicates and number of cells positive for esterase was counted.

Statistical analysis

The statistical analyses of all the experiments were done using the statistical package SPSS. Student's t test were carried out for statistical analyses. The observations are expressed as mean $\pm$ SD. The level of significance was selected at $P \leq 0.05$.

Results

Determination of different components in astaxanthin extract by thin layer chromatography

Thin layer chromatographic separation of carotenoid extracts from *A. alcocki* yielded three distinct bands (Fig. 1). The Rf values for the three bands were respectively 0.33, 0.60, 0.78 which corresponded to astaxanthin, astaxanthin monoester and astaxanthin diester. Spectrophotometric quantification of three bands showed that the extract contained astaxanthin astaxanthin monoester and astaxanthin diester in the ratio 1:1:2 showing the predominance of astaxanthin diester (Sindhu and Sherif, 2011).

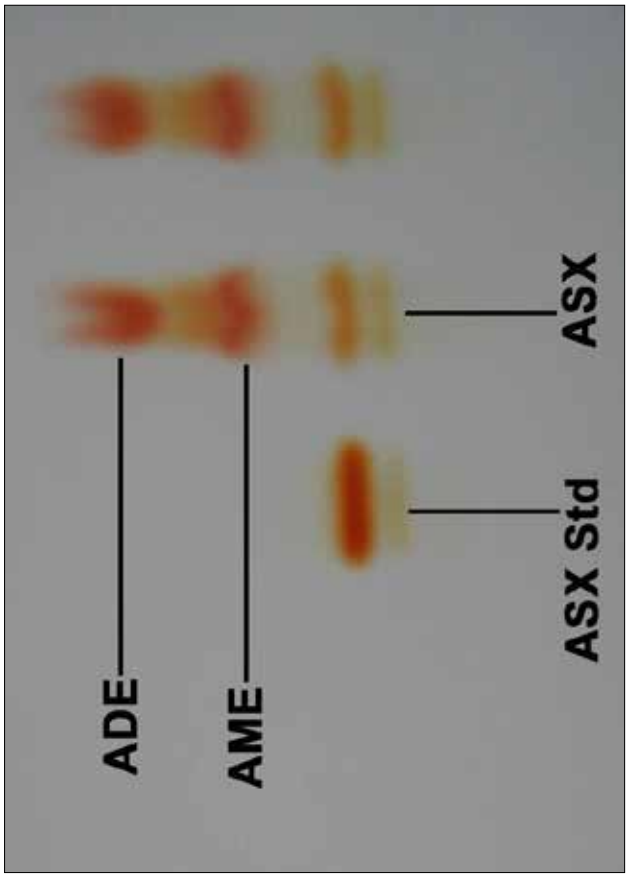

Fig. 1. Thin layer chromatography plate showing astaxanthin, astaxanthin monoester and astaxanthin diester in the carotenoid extract from *Aristeus alcocki* shell waste.

Splenic T-lymphocyte mitogen response

The effect of feeding astaxanthin on the proliferation of spleen cells is shown in the Table 1. Student's t test showed that in the absence of mitogen, there was no significant difference in the OD between the control and the test group (Table 1). Feeding astaxanthin @ 10mg/kg feed for a period of 45 days showed a two fold increase in the OD when the spleen cells of mice are stimulated with PHA, compared to the control group receiving no astaxanthin (Table 2).

Bone marrow cell proliferation assay

The results of student's t test showed that there was a significant increase in the proliferation of bone-marrow cells of rats fed on astaxanthin @ 10mg/kg feed compared to the control (Table 3). Bone marrow cells of astaxanthin fed group showed a four times increase in proliferation as indicated by the results (Table 4). Rate of proliferation is not affected by mitogen treatment as the bone marrow cells did not have any mature T-cells.

Plaque formation cell assay

Student's t test for the number of plaque forming cells showed that there was a significant increase in the number of plaque forming cells in mice fed on astaxanthin @ 10mg/kg feed (Table 5). The plaque forming cells were maximum on the 5th day and it was more than double that of the control (Table 6).

Circulating antibody titre

Feeding astaxanthin enhanced the production of antibody in normal mice. There was a 16 times increase in the circulating antibody titre in the serum of test animals on 5th day following immunization (Table 7).

Table 1. t test for the proliferation of spleen cells in the absence and presence of mitogen

Source of variation	Degrees of freedom	t-critical	t-value
Spleen cells without mitogen	18	2.10	0.72
Spleen cells PHA (5μg/ml)	18	2.10	8.18*

Table 2. Effect of feeding astaxanthin extracted from shrimp shell waste of *Aristeus alcocki* on splenic T-lymphocyte mitogen response

Treatment	Spleen cells (Mean OD$\pm$SD)	
	No mitogen	PHA (5μg/ml)
Control	0.131 $\pm$ 0.04	0.23 $\pm$ 0.11
Treatment	0.141 $\pm$ 0.02	0.56 $\pm$ 0.08*

* $P<0.05$, n =10

Table 3. t test for the proliferation of bone marrow cells in the absence and presence of mitogen

Source of variation	Degrees of freedom	t-critical	t-value
Bone marrow cells without mitogen	18	2.10	5.84*
Bone marrow cells PHA (5μg/ml)	18	2.10	11.69*

Table 4. Effect of feeding astaxanthin extracted from shrimp shell waste of *Aristeus alcocki* on bone marrow cell mitogen response

Treatment	Bone marrow cells (MeanOD$\pm$SD)	
	No mitogen	PHA (5μg/ml)
Control	0.14 $\pm$ 0.01	0.15 $\pm$ 0.01
Treatment	0.58 $\pm$0.25*	0.63 $\pm$ 0.12*

* $P<0.05$, n =10

Table 5. t test for the number of plaque forming cells on 5th and 10th day

Source of variation	Degrees of freedom	t-critical	t-value
5th day	10	2.22	39.95*
10th day	10	2.22	26.22*

Table 6. Effect of feeding astaxanthin extracted from shrimp shell waste of *Aristeus alcocki* on plaque forming cells

Treatment	No of PFCs / million spleen cells on (Mean ± SD)	
	5th day	10th day
Control	177 ± 9	157 ± 5
Treatment	440 ± 138*	358 ± 18*

* $P<0.05$, n =10

Table 7. Effect of feeding astaxanthin extracted from shrimp shell waste of *Aristeus alcocki* on circulating antibody titre

Treatment	Circulating antibody titre (Mean ± SD)	
	3rd day	5th day
Control	16 ± 1	16 ± 2
Treatment	64 ± 2	256* ± 6

n=6

Table 8. Effect of feeding astaxanthin extracted from shrimp shell waste of *Aristeus alcocki* on esterase activity in bone marrow cells

No of cells Treatment	Cells with positive staining in total of 4000 cells (Mean ± SD)	% increase
Control	616 ± 18	-
Test	788 ± 23	28

n=3

Alpha-naphthyl acetate esterase activity in bone marrow cells

The effect of feeding astaxanthin to mice on esterase activity is presented in Table 5. The group fed on astaxanthin showed an increase in the number of cells with esterase activity. The percentage increase in the cells positive to esterase was 28 % compared to control (Table 8).

Discussion

Sahidi *et al.* (1992) and Sachindra *et al.* (2005) have reported that astaxanthin and its esters are the major carotenoids in the marine crustaceans. Breithaupt (2004) observed that homogenous diester astaxanthin was the predominant compound, followed by mixed diester astaxanthin in the carotenoid extract from *Pandalus borealis*. Sachindra *et al.* (2006) reported that astaxanthin and its mono and diesters (63.5-92.2%) were the major carotenoids in *A. alcocki* and *Solonocera indica*, two important deep sea species from Indian waters. A quantitative study of carotenoid distribution in those species have revealed a higher proportion of esterified astaxanthin than the free form. *A. alcocki* had a higher proportion of astaxanthin esters (61.7-70.8%) compared to *S. indica* (43.8-58.4%).

Immunomodulating effects of astaxanthin in the present study were assessed based on the evaluation of immune system using different assays. Immune response cells are more sensitive to oxidation and antioxidants like astaxanthin can act as a positive effector in protecting those cells from free radical attack. Due to the various antioxidant capabilities of astaxanthin it can be beneficial in many pathological conditions by its immunopotentiating action. In the study by Kurashige *et al.* (1990) and Miki (1991) astaxanthin supplementation at 1 mg per 100mg feed was found to be effective in providing promising results. The present study was also assessed by evaluating the immune system in mice fed with astaxanthin @ 10 mg/kg feed. Calculated amount of astaxanthin was thoroughly mixed directly with the feed.

Feeding astaxanthin caused a significant increase in the proliferation of spleen cells. Lymphocyte proliferation assay using MTT is a rapid colorimetric assay and has a number of advantages over the conventional assays. The test is based on the capacity of mitochondrial enzymes succinate dehydrogenase to transform the tetrazolium salt of MTT in to a blue color product formosan that can be quantified spectrophotometrically (Bancroft and Cook, 1994; Mossmann, 1983). T-cells contribute the major effector mechanisms in cell mediated immunity (Bounous *et al.*, 1992). Astaxanthin was found to stimulate the spleen cell proliferation in presence of mitogens. Immune cells respond to mitogens such as PHA by rapid blastogenesis (Kumar *et al.*, 1999). Immunomodulating properties of astaxanthin observed in present study are in agreement with previous studies done in mice. Jyonouchi *et al.* (1991) studied the immunomodulating effects of carotenoids on mouse lymphocytes *in vitro* by assay of mitogen responses of spleen cells, thymocyte proliferation, interleukin 2 production and antibody production *in vitro* in response to sheep red blood cells. Proliferation rate of splenocytes were increased in presence of astaxanthin. Chew *et al.* (1999) reported that enhanced phytohemagglutin induced lymphoblastogenesis was noted in mice fed with astaxanthin.

Administration of astaxanthin to mice in the present study also enhanced the number of plaque forming cells in the spleen and antibody titre in the circulation which are functions of B-cells. Jyonouchi *et al.* (1991) has also reported that spleen cells produced significantly more antibody forming cells (plaque forming cells, immunoglobulins M and G) in presence of astaxanthin. A significant increase in the production of antibodies *in vitro* in response to SRBC was also observed in presence of astaxanthin (Jyonouchi *et al.*, 1991). Astaxanthin produces a significant effect on the antibody production even at lower concentrations compared to other carotenoids like βcarotene (Okai and Okai, 1996).

Bone marrow cells from astaxanthin fed mice also showed an enhanced proliferation rate in vitro. This indicates that astaxanthin administration stimulates the formation of blood forming cells. Bone marrow cells positive for non specific esterases were found to increase after astaxanthin treatment which indicates increased maturation of cells of lymphoid linkage (Nelson and Davey, 1992).

Astaxanthin was reported to show more significant effects in the bioassays compared to other carotenoids like β-carotene. Findings of Okai and Okai (1996) proved that astaxanthin shows considerable immunomodulating activities in *in vitro* cell culture experiments. Carotenoids like beta-carotene, canthaxanthin and astaxanthin caused significant stimulatory effects on cell proliferative response of spleen cells and thymocytes from Balb/c mice. Astaxanthin exhibited highest activity on the polyclonal antibody production of murine spleen cells. The different mechanisms responsible for the immunomodulating properties can be explained based on the earlier studies. Astaxanthin enhanced the *in vitro* antibody production to T-cell dependent antigen and maximum enhancing action was exerted when it was present at the initial period of antigen priming. The direct interactions between T-cells and B-cells are required for the activation of antibody production by astaxanthin. Carotenoids like astaxanthin without pro vitamin A activity augment *in vitro* specific antibody production to T-cell dependent antigen, partly through affecting the initial stage of antigen presentation without facilitating polyclonal B-cell activation or autoantibody production (Jyonouchi *et al*., 1993). Jyonouchi *et al.* (1995) found that astaxanthin has a significant effect on human immunoglobulin production in response to T-dependent stimuli. Jyonouchi *et al.* (1996) also reported that astaxanthin enhances murine T-helper (Th) cell clone mediated antibody production. It also suppresses interferon-gamma (IFN-gamma) production by Th cells and increased the number of antibody secreting cells.

The present study thus reveals that oral feeding of astaxanthin from *A. alcocki* shell discards stimulates immune function in mice through enhanced proliferation and function of immunocompetant cells. Immunomodulating effects of astaxnthin evidenced in the present study is described based on the unique structural feature of astaxanthin with repeated isoprenoid units and their antioxidant properties. Natural astaxanthin from algal sources have long been found to be a principal ingredient in many of the functional foods and nutraceuticals. The antioxidant property of astaxanthin should be further investigated to study its physiological role as an immunostimulant from a pharmaceutical point of view.

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