Antioxidant and Anti-skin cancer potential of a Ketocarotenoid pigment Astaxanthin isolated from a green microalga *Haematococcus pluvialis* Flotow

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Abstract: Universally, astaxanthin is known for its powerful antioxidant potential which occurs naturally in various marine and fresh water organisms. Haematococcus pluvialis, a unicellular green microalga is one of the efficient producers of astaxanthin but comparatively in high concentration. The skin cancer is a major crisis coupled with direct exposure of radiations from the sunlight due to ozone depletion. Especially, in Australia two/third of the people has been diagnosed skin cancer below the age of 70. In this present study, the isolated pigment astaxanthin has been appraised for the antioxidant and anti-skin cancer potential. About 90% of astaxanthin was extracted from the enriched biomass of green alga Haematococcus pluvialis. The IC50 value of astaxanthin towards antioxidant and anticancer activity was found to be 39.1 ± 1.14 µg/ml and 63 ± 0.22 µg/ml respectively. The gene expression fold of caspase 3 was enhanced further for different time periods of 0, 6 and 12 hours during the treatment of astaxanthin with the skin cancer cells. While investigating the gene expression of oncogene Bcl-2 and tumor suppressor gene p53, the IC50 concentration of astaxanthin was found to suppress and induce both the genes respectively. Hence, it has been proven that astaxanthin can prevent the proliferation of skin cancer cells.

Key Words: Haematococcus pluvialis; Astaxanthin; Skin Cancer; DPPH; MTT

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1. Introduction

The incidence and dispersion of melanoma and non-melanoma skin cancer (MSC and NMSC) is the major public health concern among people (Young *et al*., 2005). About 90% of the MSC and NMSC are caused by the carcinogenesis due to enormous UV exposure (Skin cancer foundation, 2008). The rate of NMSC was increased by 3-8% annually in Europe, US, Canada and Australia since 1960 (Diepgen and Mahler, 2002). Several epidemiological studies have linked the rising rates of NMSC with ozone depletion, increased exposure to UV light, changes

in clothing style, increased longevity, and increased outdoor activities (Diepgen and Mahler, 2002).

Astaxanthin is a symmetric ketocarotenoid, (3, 3'-dihydroxy-β, β'-carotene- 4,4'-dione) found predominantly in marine forms including microalgae, trout, salmon, shrimp, krill, crayfish and crustaceans (Breithaupt, 2007). Natural astaxanthin is usually found either conjugated to proteins or esterified with fatty acids. Since, the consumers of aquatic ecosystem are not capable of carotenoid *de novo* synthesis where the xanthophyll and other carotenoid pigment found in their bodies (e.g. canthaxanthin and lutein). Hence, microorganisms (producers) being the main source of carotenoid for them (Johnson and An, 1991). The carotenoid pigment isolated from the microalga *Haematococcus* sp. was named as "haematochrom" till 1944 and which was identified as astaxanthin a principle carotenoid pigment accumulated quantitatively high in the microalga *Haematococcus* sp. up to 4-5% cell dry weight (Tisher, 1944). In 1954, Droop described the conditions governing astaxanthin formation. He showed that the action of light and carbon dioxide were dependent on one another, but that of organic carbon (such as acetate) is independent of light.

Thus, astaxanthin formation could occur in the dark when energy is derived from organic carbon. Encystment and astaxanthin production can be induced by low nitrate or phosphate, high temperature or light, or the addition of sodium chloride in the culture medium (Boussiba and Vonshak, 1991; Kobayashi *et al.*, 1992; Fan *et al.*, 1994; Kakizono *et al.*, 1992). Studies have demonstrated that the significant role played by natural carotenoid in regulating immunity and disease etiology. Astaxanthin significantly reduces DNA damage, stimulates lymphocyte proliferation and increases natural killer cell cytotoxic activity produces increased number of T-cells and enhances the total number of antibody producing B-cells.

In addition, it also has pro-vitamin-A property more effective antioxidant than other carotenoids, decelerate age-related macular degeneration, immunomodulatory effects, etc. (Lorenz and Cysewsky, 2000; Dufosse *et al.*, 2005; Breithaupt, 2007). The salivary immunoglobulin A (sIgA) is an antibody plays a major role in the immunity; where the supplementation of astaxanthin to the soccer players have shown elevated levels of sIgA and hampers muscle damages by their anti-inflammatory potential (Baralic *et al.*, 2015).

Therefore, the astaxanthin is used in pharmaceutics, neutraceutics, cosmetics and food additives with a recent great success on the market (Dufosse *et al.*, 2005). During March 2010, researchers at Washington State University showed that astaxanthin inhibits cancer cell growth by decreasing free radical induced cellular damage, reducing inflammation and increasing immune response. Various drugs are being implied to treat skin cancer, but astaxanthin is a wonder drug due to its strong free radical quenching property. A431 skin cancer cell line is a model cell line (epidermoid carcinoma) used in biomedical research. They contain no functional p53, a potent tumor suppressor gene, and so are highly sensitive to mutagenic stimuli. A431 skin cancer cells were established from an epidermoid carcinoma in the skin/epidermis of an 85- year-old female patient ("A431 - Cytokines and Cells Online Pathfinder Encyclopedia"). In this present study, the isolated ketocarotenoid pigment astaxanthin from the green microalga *Haematococcus pluvialis* was evaluated for its antioxidant and anticancer activity against a skin cancer cell line A431.

2. Materials and Methods

2.1: DPPH free radical scavenging assay

The free radical scavenging potential of astaxanthin (test sample) was determined by the DPPH free radical scavenging assay (Blois, 1958). The DPPH free radical scavenging activity of the pigment astaxanthin was calculated in terms of percentage of inhibition of the free radicals by using the following formula.

$$\text{Percentage of inhibition (\%)} = \left(\frac{\text{Absorbance of Control - Absorbance of Sample}}{\text{Absorbance of Control}}\right) \times 100$$

2.2: Apoptotic cell-toxicity assay

The cell viability of the skin cancer cell line A431 was assessed against the test sample astaxanthin based on the MTT assay described by Mosmann in 1983. The absorbance of purple blue formazan dye was measured by Microplate reader at 570 nm (Biorad 680). The cytotoxicity was determined using Graph pad prism5 software. The 50% inhibitory concentration value (IC_{50}) of the test sample (astaxanthin) was evaluated and recorded.

2.3: DNA laddering assay

The A431 skin cancer cells were treated with different concentrations of astaxanthin (25 µl to 250 µl). Then the cells were nourished with DMEM medium and incubated for 24 hours. The genomic DNA was extracted and separated with 2 µl of DNA ladder electrophoretically using 1.2 % of agarose gel at 100 V. The separated DNA bands were observed at 312 nm in a UV illuminator.

2.4: Caspase activity assay

The activity of caspase-3 belongs to A431 skin cancer cells were determined chromogenically by cleavage of substrate: Ac-DEVD-7-amino-4-methylcoumarin pNA. (Short caspase specific peptides) according to described methods (Kohler *et al.,* 2002). Cleavage of the chromogenic peptide substrates was monitored by pNA release in a 405 nm scan II plate reader (Biorad, USA). Chromogenic units were converted to percentage by comparing the untreated control cells generated free pNA. Quantification of caspase activity was calculated as fold increase over control samples.

2.5: Gene expression assay

Total RNA was isolated using TRIZOL-(Sigma, India) according to the manufacturer's instructions. Frequently, the test sample (astaxanthin) in TRIZOL was repeatedly pipetted in and out to disrupt the A431 skin cancer cells. The test samples (astaxanthin) were incubated for 5 min. at room temperature to permit complete dissociation of nucleoprotein complexes. RT-PCR was performed in triplicate using SuperScriptTM two Step RT-PCR with platinum® Taq kit according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, U.S.A.).

For cDNA synthesis, Complementary DNA was synthesized from 1 µg total RNA from each sample in 20 µL of reaction buffer (contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM MgCl2) using SuperScript II reverse transcriptase enzyme (Genetech, RT-PCR mix-Germany) in a 20 µl volume reaction containing 10 mM dithiothreitol, 10 U RNase inhibitor (Promega, Madison, WI, USA), 1 mM dNTPs and 2.5 µM random hexamers. The cDNA (1 µl) was then amplified in 20 µl of reaction buffer for 35 cycles of denaturation (94°C for 30 s), annealing (50°C for 30 s), and extension (72°C for 30 s) using the following primers: Primers used were b-actin (FW 5' -ATGTTTGAGACCTTCAACAC- 3′ RW 5' -

CACGTCACACTTCATGATGG- 3') (expected product 489 bp). p53: FW, 5'-AGGGTTAGTTTACAATCAGC-3', RW, 5'-GGTAGGTGCAAATGCC-3'; bcl-2 FW, 5'-TCGATGTGATGCCTCTGCGAA GAAC-3'; RW, 5'- ATTGCACTGCCAAACGGAGCTG-3';

The cDNA amplified as described above were run on a 1.2% agarose/EtBr gel in 1X TAE (Tris-acetate-EDTA) buffer and then visualized under UV illuminator (Sambrook and Russel 2001). The density of each band on the agarose gel was measured using ImageJ pixel quantitation software. Background measurements were subtracted, and a relative number was assigned to each band intensity (Grifantini *et al.*, 2003).

3. Results

3.1. DPPH free radical scavenging activity

The 50 % inhibitory concentration (IC^{50}) of the astaxanthin required to hamper the generation of free radicals was found to be 39.1 ± 1.14 µg / ml (Fig. 1) when compared to a positive control (ascorbic acid). The free radical scavenging potential of the astaxanthin isolated from *H. pluvialis* was found auspicious to control the emerging free radicals.

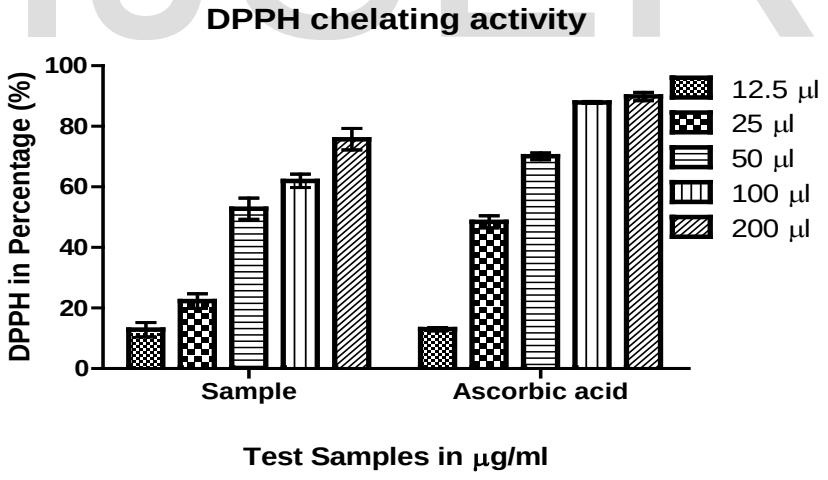

Fig. 1: DPPH free radical scavenging activity of astaxanthin in comparison with a positive control

3.2. Apoptotic cell-toxic activity

The apoptotic toxicity of the skin cancer cells remained significant with 22.080±4.760 % and 33.52 ± 2.32 % upon astaxanthin treatment with the concentration of 500 µg/ml and 100 µg/ml on A431 skin cancer cells respectively (Fig. 2). The inhibitory concentration IC^{50} and IC^{30} values to prevent the proliferation of skin cancer cells were found to be 63 µg/ml and 37.8 µg/ml respectively.

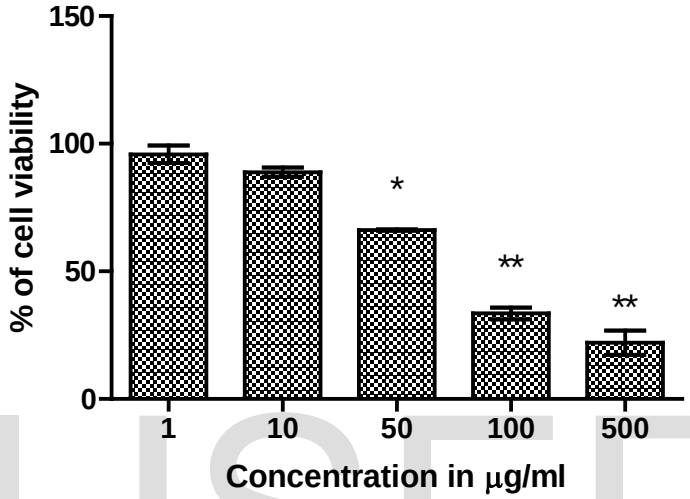

Fig.2: Apoptotic cell-toxicity assay of astaxanthin on A431 skin cancer cell lines

3.3. DNA Laddering Assay

The experiment was characterized by the activation of endogenous endonucleases with subsequent cleavage of the chromatin DNA into inter-nucleosomal fragments of roughly 50 base pairs (bp) and multiples thereof (100, 150 etc.). This method of DNA laddering assay can be used to detect the apoptosis of cancer cells due to the treatment of the test compound. The gel image (Fig. 3) particularly lane 3 and 4 shows the fragmented and drifted DNA due to the apoptotic activity of cancer cells by the treatment of the astaxanthin extracted from the green microalga *Haematococcus pluvialis.*

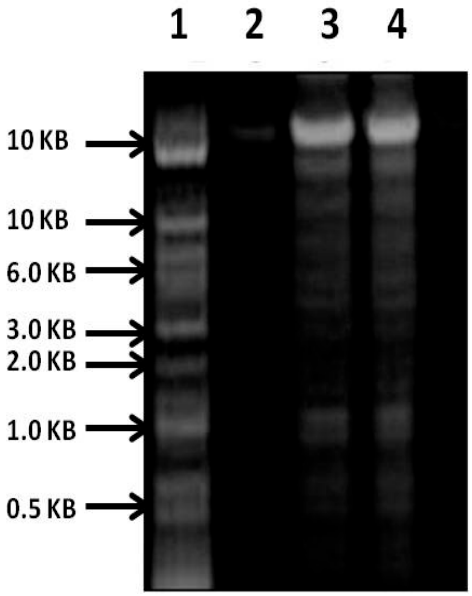

Fig. 3: The agarose gel image showing bands of DNA, lane 1: DNA ladder; lane 2: normal A431 skin cancer cell's DNA; lane 3 and 4: A431 skin cancer cell's DNA treated with the test compound astaxanthin

3.4. Caspase activity assay

The mRNA levels of caspase 3 were evaluated for better understanding the action of astaxanthin over apoptosis of skin cancer cells. The mRNA expression fold of caspase 3 was compared with the control cells (untreated cells) and analyzed for 0, 6 and 12 hours and the values were 1.05±0.05, 1.37±0.03 and 1.795±0.075 respectively (Fig. 4). Hence, the result confirmed that the caspase 3 expression was elevated while increasing the time of incubation with the test compound astaxanthin.

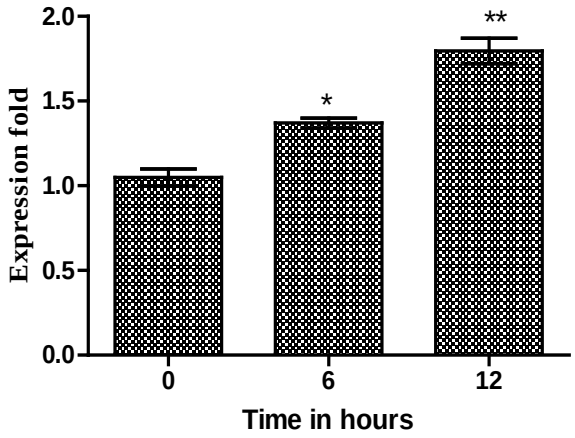

Fig. 4: Caspase activity assay showing the expression fold of caspase 3 gene

3.5. Gene expression assay

A group of genes P-53, Bcl-2, NFkB and Beta actin were chosen for the gene expression studies. The gene expression was compared with the house keeping gene beta actin as an internal control (Fig. 5). The gene expression of p53, Bcl-2 and NFkB were compared between the astaxanthin treated and the untreated cells of A431 skin cancer cells. The activity of the tumor suppressor gene p53 was increased to two folds while Bcl-2 is an oncogene whose activity is reduced and NFkB is a gene which is responsible for cell survival and there is no such considerable change in the astaxanthin treated A431 skin cancer cells (Fig. 6). Therefore, the test compound astaxanthin is determined to be effective against the skin cancer cell line A431.

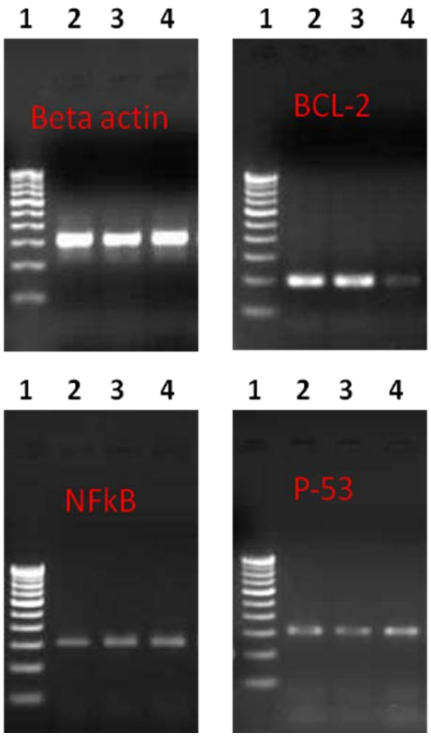

Fig. 5: The agarose gel image with bands showing the expression fold of different genes, lane 1: DNA ladder; lane 2: Control (normal cDNA genes) lane 3: bands from A431 skin cancer cells and lane 4: bands belongs to A431 skin cancer cells treated with astaxanthin

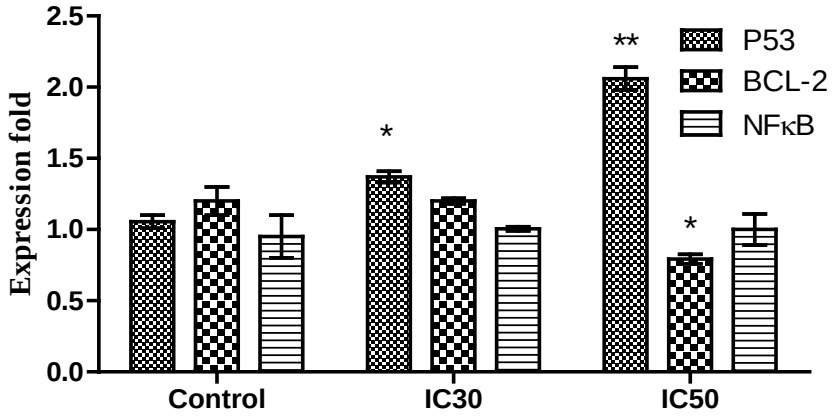

Fig. 6: The IC^{30} and IC^{50} values of astaxanthin treated A431 skin cancer cells showing expression fold of three genes p53, Bcl-2 and NFkB, where the suppression of Bcl-2 and over-expression of p53 was obtained at IC^{50}

4. Discussion

Due to strong free radical scavenging property of astaxanthin, it has been proved as a neutraceutical and pharmaceutical agent against free radical supported diseases including cardiovascular diseases, neuro-degenerative diseases, oral, colon and liver cancers (Lorenz and Cysewski, 2000; Guerin *et al*., 2003). During such high oxidative stress conditions, the astaxanthin and its esters has proven to be a strong free radical scavengers which can restore the inactivated antioxidant enzymes. The ROS quenching enzymes such as superoxide dismutase, catalase, peroxidase and thiobarbituric acid reactive substances (TBARS) were obtained in high concentration in the blood plasma of rat while feeding with biomass of *H. pluvialis* as a source of astaxanthin (Ranga Rao, 2010). The astaxanthin proved against the hydroperoxides and superoxide free radicals and which is even high in diabetic patients (Hashimoto *et al.*, 2013). The age related macular degeneration was treated with astaxanthin and resulted that the cells were well protected against the oxidative stresses (Li *et al*., 2014).

The oral supplementation of astaxanthin protects the retinal photoreceptors of eyes when exposed to UV (Tso and Lam, 1996); prevents UV-induced photooxidation and protect skin and eggs of salmon fish (Connor and Brien, 1998). In this present study, the DPPH free radical scavenging assay has significantly proven the antioxidant potential of the test compound astaxanthin isolated from green microalga *Haematococcus pluvialis*. The antioxidant results from the present investigation paved the way to analyze the cell viability of the skin cancer cells using the MTT assay using a skin cancer cell line A431. Hence, the cell viability of the skin cancer cells was found to be decreased by increasing the concentration of the astaxanthin which significantly reveals the enhancement of apoptosis of skin cancer cells.

The Caspase-3 is commonly activated by numerous death signals and cleaves a variety of important cellular proteins among the caspases identified. Failure of caspase mediated apoptosis is one of the main contributions to tumor development and autoimmune diseases (Kumar, 2007 and Wang *et al.*, 2005). The agarose gel image from this present study has shown the fragmentation of DNA by DNA Laddering assay which indicates the activity of the caspase activated DNase (CAD) during astaxanthin treatment with the skin cancer cells. Therefore, the mRNA expression fold of caspase 3 was found to be enhanced by the treatment of astaxanthin and further increased by increasing the duration of the treatment. As a result from this present study, the apoptotic cells may form a ladder of DNA fragments during electrophoretic

separation. Thus, the astaxanthin has proven to be a potent antitumor drug to cause DNA damage to the cancer cells (Jamieson and Lippard, 1999).

The different types of carotenoid pigments have already been reported to hamper the propagation of human breast cancer MCF-7 cell line in vitro (Li *et al*., 2002) especially, astaxanthin was reported to minimize the occurrence of pre-neoplastic lesions and neoplasm in mice with bladder cancer. The benign prostate cancer was caused due to the over expression of 5-α-reductase enzyme which was suppressed by the supplementation of astaxanthin (Anderson *et al*., 2001). Bcl-2 is unique among proto-oncogenes, being localized to mitochondria and interfering with programmed cell death independent of promoting cell division (Hockenbery *et al.*, 1990). Bcl-2 inhibits most types of apoptotic cell death, implying a common mechanism of lethality. Bcl-2 is localized to intracellular sites of oxygen free radical generation including mitochondria, endoplasmic reticulum and nuclear membranes (Hockenbery *et al.*, 1993). Whereas, NFkB (nuclear factor kappa beta) is a transcription factor that plays important roles in the immune system (Bonizzi and Karin, 2004) and a ubiquitous transcription factor involved in proliferative signaling and tumor promotion and is activated by oxidants and other stimuli known to generate ROS (Dhar *et al.*, 2002) Moreover, pathological dysregulation of NFkB is linked to inflammatory and autoimmune diseases as well as cancer.

This present investigation upon the evaluation of astaxanthin towards the apoptosis of skin cancer cells was focused on the expression of such genes including p53, Bcl-2 and NFkB. The astaxanthin upon the treatment of skin cancer cells has enhanced the expression of the p53 gene responsible for the suppression of tumor induction. At the same time the expression of an oncogene Bcl-2 was hampered and there is no significant modulation in the expression of gene NFkB responsible for autoimmune system. Furthermore, animal model studies are much needed to evaluate the potential activity of astaxanthin on skin cancer treatment.

5. Conclusion

Therefore, the overall investigation infers that the ketocarotenoid compound astaxanthin isolated from a green microalga *Haematococcus pluvialis* is very potent to suppress the proliferation of the skin cancer cells and thereby to enhance the apoptosis of skin cancer cells.

6. Conflict of interest

According to the authors there is no conflict of interest.

7. References

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