Astaxanthin induces apoptosis and increases activity of antioxidant enzymes in LS-180 cells

Maryam Hormozia\textsuperscript{a,⁎}, Shadi Ghoreishi\textsuperscript{b} and Parasto Baharvand\textsuperscript{c}

\textsuperscript{a}Department of Biochemistry, Lorestan University of Medical Science, Khorramabad, Iran; \textsuperscript{b}Student Research Committee, Lorestan University of Medical Sciences, Khorramabad, Iran; \textsuperscript{c}Department of Community Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran

ABSTRACT
Astaxanthin, a Xanthophyll carotenoid, has strong antioxidant properties. Some studies have shown the effectiveness of this compound on the prevention and treatment of cancer. Therefore, the aim of this study was to evaluate the effects of astaxanthin on induction of apoptosis and antioxidant activity in the LS-180 cell line. In this experimental study, after the treatment of LS-180 50, 100 and 150 \textmu m of Astaxanthin for 24 h, the expression levels of Bax, Bcl2 and Caspase3 genes were investigated by Real-time PCR. Also, the level of malondialdehyde, as an indicator of oxidative stress and activity of anti-superoxide dismutase enzymes, catalase and glutathione peroxidase was investigated by colorimetric methods. The results showed that astaxanthin increases the expression of Bax and Caspase3 genes and decreases that of Bcl2, thereby, inducing apoptosis and inhibiting growth and proliferation of the cells. Additionally, reduction in the levels of malondialdehyde was evident with a significant elevation in antioxidant activity mediated by the action of superoxide dismutase, catalase and glutathione peroxidase. These results suggest that astaxanthin has the potency to induce apoptosis in LS-180 cells by increasing the expression of apoptotic genes and activity of antioxidant enzymes. Thus, astaxanthin has potential in the prevention and treatment of cancer.

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Introduction
Various studies have shown that the use of exogenous antioxidants can be effective in the prevention and treatment of various diseases such as diabetes, hypertension, atherosclerosis, cardiovascular disease, Alzheimer’s, Parkinson’s disease and cancer. Astaxanthin, belonging to the family of carotenoids, has a very strong antioxidant activity, often called the “king of carotenoids”, due to its significant impact on human health, by strengthening the immune system, preventing and treating various types of cancers and ageing process and including the prevention of cardiovascular disease \cite{1–3}. \textit{Hematococcus pluvialis} (\textit{H. pluvialis}) is a microalga known to be the main source of natural astaxanthin. Other natural sources of astaxanthin include; Zantophyllumiss dendorhousis yeast, Pfaffia rhodozyma, and from marine organisms such as Ghezel Alla, Crab and Shrimp \cite{4,5}. Astaxanthin does not elicit side-effects even at higher concentrations, which adds to its significance \cite{6,7}. Researches have failed to report any adverse effects of astaxanthin consumption in humans or animals \cite{5,8,9}. Apoptosis or cell death is a mechanism for the removal of unwanted or damaged cells. Disturbance in this process eliminates the balance between cell proliferation and cell death and plays a critical role in the development of cancer. Therefore, the induction of apoptosis in pre-cancerous and cancerous cells is one of the most important goals in the prevention and treatment of cancer \cite{10}. Henceforth, the purpose of this study is to evaluate the effect of astaxanthin on induction of apoptosis, expression of the respective genes in this process, and the activity of antioxidant enzymes in the LS-180 cells of human colorectal cancer.

Materials and methods

Cell culture

Human LS-180 colorectal cancer cell line was purchased from the Pasteur Institute and cultured in a DMEM containing 1% antibiotic (penicillin and streptomycin) and 10% FBS in an incubator containing 5% CO$_2$ at 37°C. When the cells reached their logarithmic phase, they were treated with the drug.

MTT assay

MTT technique was used to determine the time and dose of the drug on colorectal cancer cells. This method is a mitochondrial metabolic competitive test and is based on the fragmentation of tetrazolium salt by mitochondrial Succinate dehydrogenase and reduction of yellow tetrazolium salt by mitochondrial enzymes of cancer cells to form purple formazan by mitochondrial succinyl dehydrogenase and release formazan in the medium.
dehydrogenase enzyme in living cells. Herein, we added 100 μl culture medium (containing 10^4 cells) per well in a 96-well plate. After 24-h incubation of the cells with different concentrations of astaxanthin and determining cell proliferation after 24, 48 and 72 h, based on the protocol of the MTT assay (Jena Bioscience), Cell survival was calculated according to the following formula:

\[
\text{Cell survival rate} = \left( \frac{\text{optical absorption test}}{\text{optical absorption control}} \right) \times 100 \quad (1)
\]

**Astaxanthin treatment**

Depending on the results of MTT, the LS-180 cells were treated with 50, 100 and 150 μm astaxanthin for 24 h.

**RNA extraction and cDNA synthesis**

RNA extraction was performed using the Jena Bioscience kit based on the relevant protocol (Jena Bioscience, Germany) and the concentration of RNA extracted was determined, followed by cDNA synthesis using a microgram of extracted RNA.

**Evaluation of gene expression using Real-Time PCR**

Analysis of gene expression was performed using the Jena Bioscience kit based on the relevant protocol according to the following program (Jena Bioscience). The first stage characterized by DNA denaturation and activation of the polymerase enzyme at 95°C for 2 min proceeded by proliferation reaction which was conducted in 40 cycles at 95°C for 15 s and 60°C for 40 s. The primers sequences are mentioned in Table 1.

**Measuring the activity of antioxidant enzymes**

After treatment of the cells with different concentrations of astaxanthin and washing with PBS, 800 μl of lysis buffer was added, and cells were mixed several times. In the next step, using an ultrasonic device, the solutions were sonicated 2 to 3 times, each time for 30 s. Finally, centrifugation was performed at 20°C for 10 min. The supernatant was stored at −80°C until the activity of the enzymes was measured.

**Total protein measurement**

The Bradford method was used to measure the total protein content in cell lysate samples and BSA was used to plot the standard curve after serial dilutions.

**Measuring malondialdehyde**

To measure malondialdehyde, spectrophotometric measurements were conducted based on the reaction of a thiobarbituric acid substrate in (nmol/mg) protein [11].

### Table 1.

| Gene name | Sequences          | Product size |
|-----------|--------------------|--------------|
| BCL2-F    | TGGCCCTTGGATGACTGA | 134 bp       |
| BCL2-R    | CAGAGACAGCCAGGAATCA| 195 bp       |
| Bax-F     | TGCCAGCTGGATGACTGA | 134 bp       |
| Bax-R     | TGGCCAGCTGGATGACTGA| 195 bp       |
| Cas3-F    | TACCTGTGGCTGTGTACG | 134 bp       |
| Cas3-R    | TCAGTGTTCCATGTGACCT | 167 bp      |
| B2M-F     | ACTGAAATCCACCCCACTGA| 134 bp       |
| B2M-R     | AAGCAAGGCAAGGCAATTGGA| 167 bp      |

**Results**

According to the results of MTT assay, 50, 100 and 150 μm of astaxanthin were used for 24 h to treat LS-180 cells (Figure 1).
Gene expression results

Effect of astaxanthin on caspase 3 gene expression
Expression of caspase 3 at the concentration of 100 μM AST was significantly elevated as compared to control cells \((P < .01)\) as those treated with 50 μM AST \((P < .01)\) (Figure 2).

The effect of astaxanthin on the expression of bax gene
The results of the study on LS-180 cells at concentrations of 100 and 150 μM showed an increase in the expression of Bax gene expression in the treated groups in variance with the control one, nonetheless, this increase was not statistically significant \((P > .05)\) (Figure 3).

The effect of astaxanthin on the expression of bcl-2 gene
Bcl-2 gene expression was significantly suppressed in the treatment at various concentrations \((P < .01)\) (Figure 4).

Effect of astaxanthin on malondialdehyde and antioxidant enzymes activity
The results showed that treatment of the cells at all concentrations of astaxanthin reduced the level of malondialdehyde in the LS-180 cell line \((p < .05)\). Among different concentrations of the drugs, significant difference was found between the activity of 50 and 150 μM in the reduction of malondialdehyde levels. Similarly, astaxanthin significantly increases the activity of superoxide dismutase in contrast to the control group \((p \text{ values} < .05)\). This activity was statistically different between the lowest and highest concentration of the drug used in this study. 150 μM AST was more effective to elevate superoxide dismutase activity. (Table 1)

The average catalase activity in all treatments was significantly increased compared to the control group \((P < .05)\). Furthermore, the increase in mean catalase at 150 μM of astaxanthin was significantly greatest as compared to other concentrations. (Table 1). Similarly, increased in glutathione peroxidase activity was statistically evident at all the concentrations \((P < .05)\) as compared to control and significant difference was also noted when compared at different concentration value (Table 2).

Discussion
Colorectal cancer can be progressive and fatal, however, substantial advancements have been made to prevent and treat it. Studies have shown effects of astaxanthin to eradicate colorectal cancer [15]. Here we present its effects on cell cycle leading to cell death and instigation of anti-oxidation activity.

Various studies indicate the antitumor effects of *H. pluvialis* extract, which is rich in astaxanthin, being a potent growth inhibitor under laboratory conditions [16]. The results of the present study show anti-proliferative effects of astaxanthin seen by the increase in the expression of *caspase 3*, *Bax*, antioxidant activity conversely, decreasing the expression of *bcl2*, and the level of malondialdehyde in the LS-180 cell line [17,18]. A study reported that daily administration of astaxanthin could be effective in preventing colon cancer [19]. Studies have shown that astaxanthin reduces the proliferation of cancer cells by halting the progression of the cell cycle at the G0/G1 phase, inhibiting the expression of cyclin D1, which in turn increases p53, p21WAF-1/CIP1 and p27 simultaneously, thus controlling the progression of the cell cycle [20]. Studies have shown that cyclin D1 is an oncogene and its expression has been observed in several cancer cell
The results of the Jingjing Li study in 2015 on the human hepatoma cell lines LM3 and SMMC-7721 showed that astaxanthin, through NF-κB p65 and Wnt/β-catenin pathways causes apoptosis of tumor cells and inhibits cell proliferation [29]. This result suggests that astaxanthin inhibits PI3K, Akt phosphorylation, ERK, aiding binding of Wnt protein with its receptor, and thus reducing phosphorylation of IKKα/β ( Ser176/180) and GSK-3β (Ser9). Low concentrations of β-catenin and p-NFκB reduce Bcl-2 transcription and alter Bax/Bcl-2 ratios. As a result, caspase associated with mitochondrial apoptosis is activated for cell death and ultimately inhibits cancer cell growth. Oxidative stress and the formation of reactive oxygen species (ROS), or disturbance in the production and function of antioxidants chiefly contribute to the development of cancer. ROS, as a secondary messenger, takes part in activating and maintaining some signaling pathways [30]. Endogenous and exogenous antioxidants like astaxanthin appear to function in a similar manner. The results of Li Zhang and Handong Wang’s study in 2015 showed that astaxanthin reduces intracellular production of O2 by activating anti-oxidant SOD and CAT enzymes in U937 cells. Wu et al. showed that astaxanthin, by improving the activity of GPx and SOD, increases GSH content and reduces MDA levels, hence, can reduce the ageing of the brain in rats. Hashimoto, Choi, et al. also obtained that astaxanthin, is a potent antioxidant, due to its ability to increase the activity of superoxide dismutase and other antioxidant enzymes. Increasing the activity of antioxidant enzymes in astaxanthin-treated cells may have altered the antioxidant defense system, thereby inducing anti-proliferative effects and inducing apoptosis in LS-180 cells of colorectal cancer.

Conclusion

This study provides molecular evidence in regard to apoptotic and antioxidant potencies of astaxanthin. Results indicate that astaxanthin can inhibit proliferation and induce apoptosis in LS-180 cell lines by promoting the activity of antioxidant enzymes, reducing the production of malondialdehyde, and increasing the expression of genes that are effective in apoptosis. However, studies with further details, including interrelated molecular pathways can provide greater pieces of evidence.

Disclosure statement

No potential conflict of interest was reported by the authors.

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