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Investigating The Effect of Astaxanthin on The Survival Rate of MCF-7 Breast Cancer Cells By MTT Method (Cytotoxicity Test)

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ABSTRACT

Cancer is known as a very dangerous and incurable disease, with symptoms such as pain and disability. In recent years, changes in the way people live have increased the prevalence of breast cancer in different societies. This terrible disease has increased research in this field. Astaxanthin is one of the most famous natural carotenoids, which, due to its strong antioxidant activity, can prevent cardiovascular diseases, strengthen the body's immune system, and prevent cell ageing. In this study, the effect of astaxanthin on MCF-7 cancer cells was investigated. After culturing the cells in 96-well plates, they were treated with different concentrations of astaxanthin and then evaluated using the MTT method. The results showed that astaxanthin can inhibit cell growth and induce apoptosis, and this effect is enhanced by increasing the concentration of astaxanthin, so that at a concentration of 100 μM, the rate of apoptosis increased to 35%. Therefore, it can be concluded that astaxanthin may be used as a control treatment for cancer.

1. Introduction

The word cancer is used for more than 200 different types of diseases in different parts of the body. What is common in all these diseases is a defect in the mechanisms regulating normal growth cell proliferation and death. Cancer cells can invade nearby tissues and eventually spread to other areas of the body. The fate of the cell at any time is precisely controlled by growth factors, environmental messages and some cellular proteins. Mutations that lead to the change of any of them can disrupt the order of proliferation and differentiation of cells and lead to the occurrence of cancer and the creation of tumour tissue. On the other hand, cancer cells are transferred to other parts of the body through blood and lymph, which is called metastatic tissue. The disease is mainly caused by the contact of people with the carcinogenic substance due to inhalation, eating and drinking and exposure in the workplace and environment. Personal habits such as smoking and diets play a greater role in the incidence of cancer than hereditary genetic factors (Qazvin University of Medical Sciences, 2014). Cancer can occur in various organs and cells, among which breast cancer is the most common cause of cancer death in less developed countries and ranks second after lung cancer in developed countries. Breast cancer is the most common malignancy in women and is one of the three most common cancers worldwide, along with lung and colon cancer (DeSantis, C.E, 2017). In South America, Africa and Asia, the incidence of breast cancer is increasing, probably due to lifestyle changes. For this reason, screening programs have been started (Torre LA, 2015).

During recent decades, changes in people's lifestyles have increased the incidence and prevalence of breast cancer and its risk factors worldwide. (Fredslund SO, 2012) Breast cancer is a multifactorial disease that has been proven to play a role in various risk factors. Based on the studies, these risk factors include family history, old age, menarche age under 12 years, menopause age after 54 years, age of first childbirth after 30 years, no history of childbirth, high density in mammography, high level of sex hormones, contact with ionizing radiation in childhood, race, economic status, body mass and lifestyle in terms of nutrition, physical activity, smoking and alcohol use (Karimi Z, 2012). It has also been pointed out in studies that the duration of breastfeeding has a positive relationship with the risk of breast cancer so the risk of breast cancer decreases with the increase in the duration of breastfeeding (Hernandez Aliva M, 2000).

After cardiovascular diseases, cancer is the second cause of death in human societies (DeSantis, C.E, 2017). Almost a quarter of all women's cancers are breast cancer. Breast cancer is the second leading cause of cancer death. Almost one out of every 8 women is diagnosed with breast cancer, which often leads to complete removal of breast tissue, chemotherapy, radiation therapy, and hormone therapy. In 2012, approximately 1.7 million patients were diagnosed worldwide and about half a million people died from this disease (Torre LA, 2015-Ferlay J).

The prevalence of breast cancer increases by approximately 2 per cent annually. So in the 70s, the probability of an American woman getting breast cancer was 1 in 13, in the 80s it was 1 in 11, in 2000 it reached 1 in 8, and in some studies, it reached 1 in 7 (Madigan, MP, 1995).). According to WHO criteria, the global prevalence of cancer is growing, fast and alarming (Elidrissi Errahhali, M, 2016). However the complete etiology of cancer is still unknown. In Iran, cancer is the third cause of death and one of the most common cancers among women (Masoompour SM, 2016). The prevalence of breast cancer among women in Iran has been reported as 97% and in America as 87.2% (Ataollahi MR, 2015). According to the report of the General Directorate of Disease Prevention and Control on the incidence of cancer in Iran in 2017, the most common cancer in Iranian women is breast cancer with a prevalence of 31%, and the highest incidence is in the fourth and fifth decades, which is 10 years less than the global

suffering. is that its rate has gradually decreased in age groups after 65 years (Masoompour SM, 2016).

Astaxanthin

Astaxanthin (3,31-dihydroxy, 1-carotene-4,41-dione) (ASX), a red carotenoid pigment was isolated from a crab by Kuhn and Sorensen (Kuhn, R, 1938). ASX is primarily biosynthesized by microalgae/phytoplankton and accumulates in zooplankton and crustaceans, and subsequently in fish, and is then added to higher levels in the food chain. ASX can be synthesized by plants, bacteria and microalgae. However, the chlorophyte algae Haematococcus pluvialis has the highest capacity for ASX accumulation (Boussiba, S, 2000). It should be noted that currently, 95% of ASX in the market is mass-produced using synthetic petrochemicals due to economic efficiency. There are safety concerns regarding synthetic ASX for human consumption, while ASX from H. pluvialis is the main source of several human applications including dietary supplements, cosmetics, and food (Y.F. Zheng, 2013- S. Zaripheh, 2002). Humans usually get astaxanthin from seafood such as salmon, trout, shrimp, crab, and fish roe, and it is used to add red colour to seafood and vegetable foods (Kuhn, R, 1938). AST has a molecular structure similar to beta-carotene and like other carotenoids (I. Higuera-Ciapara, L, 2006- M. Affandi, T, 2011). There are several types of ASX stereoisomers in nature, which differ in the configuration of the two hydroxyl groups on the molecule (I. Higuera-Ciapara, L, 2006). AST has ionic rings at both ends, giving it a good capacity to neutralize free radicals, and also has a non-polar region in the middle made up of a series of carbon-carbon double bonds called "conjugation". formed (Yuan, J.P, 2011 - Lorenz, R.T, 2000). In addition, ASX has several essential biological functions in marine animals, including pigmentation, protection against the effects of ultraviolet (UV) light, communication, immune response, reproductive capacity, stress tolerance, and protection against macromolecule oxidation (Lim, K.C, 2017). ASX is closely related to other carotenoids such as zeaxanthin, lutein and carotene. Therefore, it has many metabolic and physiological functions attributed to carotenoids. ASX has unique properties that support its potential use in promoting human health.

Unique properties of astaxanthin

ASX has received much attention due to its potential medicinal effects, including anticancer, antibiotic, anti-inflammatory, and antioxidant activities, as well as neuroprotective, cardiovascular, ophthalmic, and skin protective effects (Yuan, 2011). Extensive research over the past two decades has shown the mechanisms by which continued oxidative stress leads to chronic inflammation, which in turn increases chronic diseases, including neurological diseases, cancer, and skin damage. In a group of studies, it has been proven that the consumption of ASX reduces the production of nitrogen species caused by the reaction with ultraviolet rays, preventing the harmful effects of UV (Yoshihisa, 2014).

Laboratory studies on human lymphocytes have shown that astaxanthin consumption increases immunoglobulin in response to T-cell-dependent stimuli (Jyonouchi, 1995). ASX has increased NK activity, which acts as an immune surveillance system against tumours and virus-infected cells (Park, 2011, Chew, 2011).

ASX improves the DNA repair capacity of UV-exposed cells. In particular, ASX can minimize DNA damage and influence the kinetics of DNA repair (Santocono, 2006). For example, ASX inhibits UV-induced DNA damage and increases the expression of oxidative stress-reactive enzymes (Camera, 2009).

Over time, it became clear that cancer is a global problem and is not limited to industrialized countries. Although the death rate in industrialized countries is higher than in semi-industrialized countries. Cancer is a deadly disease associated with pain and disability in people's minds, therefore, the diagnosis of cancer is considered a serious crisis in a person's life and causes the balance of his life to be disturbed. The patient and his family react by remembering the word cancer, which negatively affects all life functions (Davinelli, 2018).
Breast cancer is the second leading cause of cancer death. Almost one out of every 8 women is diagnosed with breast cancer, which often leads to complete removal of breast tissue, chemotherapy, radiation therapy, and hormone therapy. (Mahreghi et al., 2017). Among the causes of death in breast cancer patients, the metastatic spread of cancer cells is the main known factor (Davinelli, 2018). However, there is still no cure for metastatic breast cancer (Olive Peart, 2017). Therefore, one of the important goals of cancer research is to identify and describe the mechanisms that cause metastasis, to facilitate the development of new therapeutic drugs to inhibit metastasis (Fassett, 2009, Davinelli, 2018). Breast cancer is one of the neoplasms that has been studied more than other types of cancer so due to its considerable prevalence, a factor or factors can be found to improve and fight it. In the present study, it has been tried to use the carotenoid substance astaxanthin to inhibit the growth of breast cancer in vitro. Many studies have been conducted on the unique properties of astaxanthin, but its effect on this particular cell line MCF-7 in breast cancer is a new work that has been addressed in this research.
Women, Khadijah Erani and Sanaz Ranapour researched the issue of breast cancer in women and the role of environmental factors in its development in 2010. In this research, a questionnaire including questions about the above factors was made available to 100 breast cancer patients and 100 healthy people. The results showed that pregnancy at an advanced age, abortion, use of birth control pills, and overweight increase the risk of cancer. Mrs Fatemeh Hosseini researched the anti-tumour and anti-inflammatory effects of the black seed plant on MCF-7 and CT26 tumour lines in 2016. In this study, tumour lines were treated with different concentrations of black seed extract. The results showed that black seed extract can not only reduce the growth of tumour cells but also significantly reduce cancer-related inflammation. Mrs Zahra Maqsoudi researched the effect of fruits and vegetables on the risk of breast cancer in 2017. By reviewing the keywords of vegetables and fruits and food groups and breast cancer on the PUBMED site, they found that the consumption of vegetables and fruits due to the presence of flavonoids, polyphenols, carotenoids, antioxidants and vitamins has a significant negative relationship with the risk of breast cancer. it shows. Mr Mohammad Reza Mati and his colleagues researched the issue of breast cancer risk factors in women of Golestan province in 2018. In this study, all breast cancer reports registered in the system of Golestan province in 2003-2005 were collected. The results showed that there is no significant relationship between the investigated variables and the chance of getting breast cancer, except for the age of menopause.

In 2017, Sayeh Bidaran et al studied the preventive and therapeutic effects of the carotenoid astaxanthin on multiple sclerosis in c57bl/6 mice. In this study, after the induction of EAE disease model in female mice, in the normal saline control group, the patient received astaxanthin prophylaxis for 21 days before disease induction, and the treatment group received astaxanthin at the onset of symptoms of multiple sclerosis. The results showed that the consumption of astaxanthin decreased the production of pro-inflammatory cytokines. In 2016, Fereshte Mirmohammed Rezaei et al. studied the protective effect of astaxanthin on urea and creatinine levels and kidney tissue changes following cadmium poisoning in male mice. In this

study, 42 male mice were divided into 7 groups receiving cadmium in different concentrations and at the end of the treatment, urea and creatinine levels were evaluated. Astaxanthin prevents kidney poisoning caused by cadmium by protecting kidney tissue and improving filtration. In 2016, Ms Azam Ghasemi studied the subject of evaluating the effects of astaxanthin on the viability and proliferation of stem cells derived from human adipose tissue. In this research, hADSCs were cultured for 72 hours in the presence of different concentrations of astaxanthin using MTT and trypan blue methods after isolating hADSCs and checking CD markers. The results showed that astaxanthin can increase the survival and proliferation of hADSC cells. This substance can be used in the treatment of MS. In 2017, Mrs Nafiseh Shokri and colleagues conducted research on the effect of astaxanthin supplementation on cognitive function and depression in patients with type 2 diabetes. This study was conducted on 44 patients with type 2 diabetes for 8 weeks, with daily consumption of 8 mg of astaxanthin. The results showed that the cognitive function score and the level of depression improved in the astaxanthin group.

Research Method

The effect of astaxanthin on cancer cells by MTT method

MTT assay, which stands for MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), is a colourimetric method based on the reduction and breaking of yellow tetrazolium crystals by succinate dehydrogenase enzyme and the formation of crystals. Purple colour is insoluble.

This study was conducted in a laboratory environment using an MCF-7 breast cancer cell line. MCF-7 cell line was obtained from the Pasteur Institute cell bank (Tehran, Iran). The cells of this cell line were cultured in 1640 RPMI culture medium containing 2 mM of L-glutamine, 10% FBS, 100 unit/mL penicillin and 100 µg/ml striatum at C37 temperature and 5% CO2 pressure. Astaxanthin extract was used to treat the cells. The stock solution of astaxanthin extract was prepared by dissolving this drug in 0.1% sterile dimethyl sulfoxide (DMSO).

We divided the stock solution into micro tubes and kept them at -20 degrees until use. To determine the optimal effects of the extract, two variables, dose and time, were considered in this research. Cancer cells were treated with concentrations of 50, 100 and 200 µM of astaxanthin extract and were studied after 12, 24 and 48 hours, respectively. Also, MCF-7 cells, which were not treated with the extract, were used as control group cells.

To perform the test, after passing twice, the cells were transferred to a 96-well plate (5000 cells per well). When the yellow MTT enters the living cells, it is regenerated by the dehydrogenase enzyme of these cells and creates a purple precipitate. In short, after the treatment of cells with glucose, 5ml/mg MTT solution in PBS was added to the wells of the 96-well plate and they were placed in an incubator at 37°C for 3 hours. After incubation, the plate containing the cells was centrifuged at 1000 g for 10 minutes. Then, the supernatant solution was emptied into the wells and 100 microliter DMSO (Dimethyl sulfoxide) solution was added to each and shaken for 10 minutes to dissolve the purple MTT deposits. Then, the optical absorption of the samples was measured at a wavelength of 570 nm by Elizaridan. To calculate the toxicity effect of astaxanthin extract on the survival of MCF-7 cells and to check the death rate of treated cells, the following formula was used:

$$\text{The rate of cell death (\%)} = \frac{\text{OD exp}}{\text{OD cont}} \times 100$$

In this formula, OD exp and OD cont represent the light absorption of treated cells and untreated cells (control), respectively.

findings

After performing the MTT test and reading the absorption value of each plate at a wavelength of 570 nm, the values were obtained in the spectrophotometer. The amount of absorption increases with the amount of colour produced, and on the other hand, the amount of colour produced has a direct relationship with the number of metabolically active cells (living cells). This experiment was repeated three times each time with two readings. The result of these readings included six times

Table 1: Mean and percentage of living cells treated with different concentrations of astaxanthin

	0	1/562	3/125	6/25	12/5	25	50	100
average	1/5	1/33	1/3	1/24	1/1	0/93	0/81	0/76
viability	100	88/64	84/65	81/87	74/41	71/36	64/21	57/49
stdev	0/059	0/021	0/038	0/099	0/033	0/055	0/058	0/037

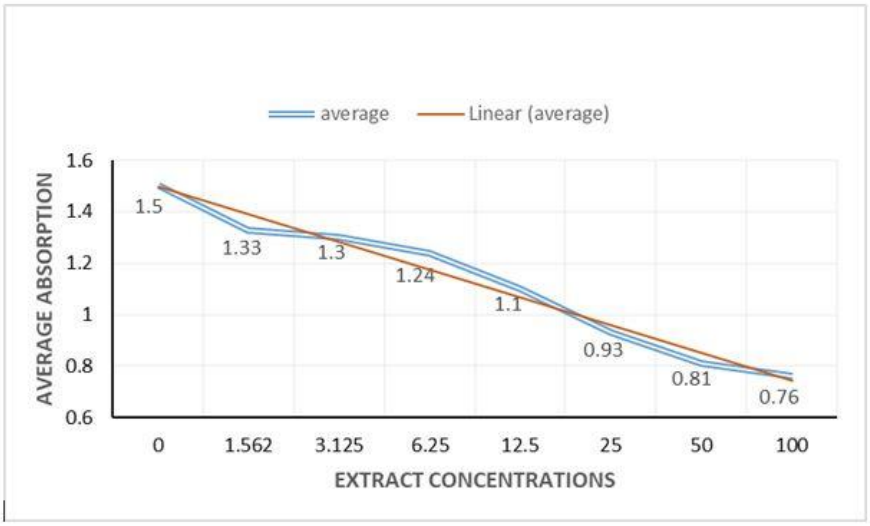

Chart 1: Percentage of living cells treated with different concentrations of astaxanthin

According to the table and graph, the survival rate of cells treated with 1.562 micromolar of astaxanthin decreased by 13%. By increasing the concentration of astaxanthin, this amount of death has decreased in MCF-7 cancer cells. For example, at a concentration of 50 mcmol, there is a 29% decrease, and at 100 mcmol of astaxanthin, only 65% of cell survival is observed, and 35% of cancer cells undergo apoptosis under the influence of astaxanthin.

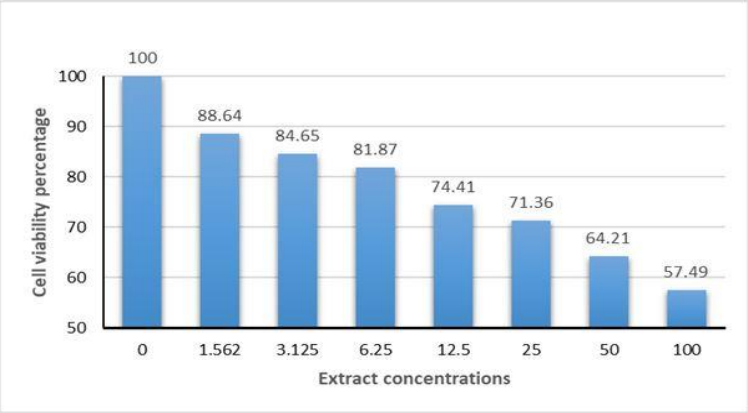

Chart 2: Percentage of living cells treated with different concentrations of astaxanthin in micromolar terms

Discussion

Breast cancer is known as the most common type of malignancy in women worldwide, and the prevalence of this disease is increasing in many countries, including Iran. Environmental factors such as air pollution, stress, lifestyle and diet are among the factors that are associated with the increase in cancer. Consumption of foods with antioxidant properties can play an important role in preventing and reducing cancer. Also, despite the use of different treatment methods such as surgery, chemotherapy and radiotherapy, the death rate in cancer patients is still high, which shows the ineffectiveness of these treatment methods. The harmful effects of chemotherapy and radiation therapy on healthy dividing cells are also another disadvantage of these treatment methods. For this reason, attention to the use of supplements and natural products with anti-cancer properties has increased in recent years. One of the nutritional supplements of interest in this field is astaxanthin extract, which recent studies have shown can enhance the growth of gastric cancer cells and induce apoptosis. Also, research has shown that astaxanthin has an anti-cancer effect against lung, bladder, prostate and colon cancer cells. In this study, it was observed that astaxanthin can inhibit the process of invasion and proliferation of cancer cells. In addition, astaxanthin can have anti-inflammatory and antioxidant effects that can be effective in preventing oxidative damage in healthy cells and also in reducing cancer-related inflammation. Therefore, the use of astaxanthin extract as a food supplement with anti-cancer properties can be used as a new method in the prevention and treatment of some types of cancer. But to confirm its positive effects on cancer cells and its side effects, we need to conduct more studies and clinical trials on cancer patients.

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