

Original Research Article

Mechanism of cytotoxic effect of astaxanthin on HeLa cell line

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Abstract

Purpose: To investigate the roles that autophagy, oxidative DNA damage, and proapoptotic and anti-apoptotic proteins play in astaxanthin's cytotoxic action on HeLa cells.

Methods: HeLa cells were treated with varying concentrations of astaxanthin (2.5 - 40 µM). Untreated cells served as the negative control. Cell viability was assessed using XTT assay to determine the cytotoxic effects of the compound. The levels of B-cell lymphoma 2 (BCL-2), Bcl-2-associated X protein (Bax), caspase 3, poly (ADP-ribose) polymerase (PARP), and 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) were measured by ELISA using cell lysates collected after a 24-hour incubation with astaxanthin at IC50 concentration. Furthermore, immunofluorescence staining was performed to evaluate the expression of 8-oxo-dG and Microtubule-associated protein 1 light chain 3 beta (LC3β) in HeLa cells.

Results: Astaxanthin exhibited a concentration-dependent cytotoxic effect on HeLa cells, with an IC50 value of 18.47 µM after 24 hours. Treatment with IC50 dose significantly increased the expression of apoptotic proteins (cleaved caspase 3, Bax, cleaved PARP; $p < 0.001$) while leaving BCL-2 expression unchanged ($p > 0.05$). Furthermore, the use of this medicine resulted in an enhancement in the levels of 8-oxo-dG ($p < 0.001$), a well-recognized indicator that signifies oxidative harm to DNA. Additionally, there was an observed rise in LC3β levels ($p < 0.05$), an indicator closely linked to the process of autophagy.

Conclusion: Astaxanthin's cytotoxic activity against cervical cancer may be attributed to its capacity to induce both apoptotic and autophagic cell death pathways. This finding emphasizes the positive potential of astaxanthin as a cervical cancer therapy option.

Keywords: Anticancer, Astaxanthin, Apoptosis, Autophagy, Cervical cancer

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INTRODUCTION

Even with access to effective vaccines and screening, cervical cancer still represents a serious health issue on a global scale. Globally, more than half a million women receive a cervical cancer diagnosis every year, placing it as the fourth leading cancer affecting women.

Tragically, the mortality rate associated with this disease is substantial, affecting approximately half of those diagnosed. While early-stage cervical cancer can be successfully treated with surgery, chemoradiation, or a combination of therapies, advanced cervical cancer is frequently incurable and becomes relatively resistant to treatment upon recurrence. Despite cervical

cancer receiving less research funding compared to other women's cancers, significant advancements have emerged in recent years, leading to changes in clinical practice. Ongoing studies aim to enhance the care and outcomes for women affected by this disease. Chemotherapy is a commonly used approach in cancer treatment, employing drugs such as cisplatin, paclitaxel, and topotecan to destroy cancer cells [1].

Natural herbal medicines, known for their diverse therapeutic properties, have been acknowledged for their potential in fighting cancer, especially due to their anti-cancer properties. Numerous plant-derived bioactive compounds exhibit promising potential as therapeutic agents in cancer treatment. Their application could significantly enhance patient outcomes by extending survival rates, lessening harmful reactions, and boosting the general well-being of individuals undergoing cancer treatment [2]. The FDA has approved astaxanthin, a carotenoid pigment belonging to the xanthophyll group, as a coloring agent in feeds for fish and livestock. It is naturally abundant in algae and aquatic organisms, showcasing potent antioxidant properties. Prior studies support the therapeutic potential of astaxanthin across a wide range of diseases, offering significant advantages without any reported side effects or toxicity [3].

Astaxanthin is a reddish pigment from the carotenoid family, predominantly found in marine organisms such as salmon, krill, shrimp, and microalgae. It is recognized for its powerful properties that support overall health. Astaxanthin demonstrates multiple biological properties, such as promoting apoptosis, anticancer effects, antioxidant activity, and improvement of endothelial dysfunction. Nevertheless, its exact function in preventing, treating, and reversing malignant cells remains under study. Recent studies explored astaxanthin's potential role in skin cancer prevention. Notably, studies indicate that astaxanthin is capable of suppressing the transformation of JB6 P+ mouse epidermal cells. A five-day treatment with 50 μM astaxanthin significantly reduced cell viability, reaching up to 94 %. These findings are consistent with earlier work by Chen *et al*, which demonstrated a significant reduction in cell proliferation in two melanoma cell lines, A375 and A2058, upon exposure to astaxanthin. Specifically, Chen *et al* observed a 50 and 80 % reduction in living cell proportion in A375 and A2058 cells, respectively, when treated with 168 μM astaxanthin. The antiproliferative effects of astaxanthin have also been observed in various other cancer cell lines,

such as human hepatocarcinoma (CBRH-7919), rodent breast carcinoma cells (SHZ-88), and mouse Lewis's lung cancer cells. Notably, the human hepatocarcinoma cell line exhibited higher sensitivity to astaxanthin, with IC_{50} values around 39 μM . These results indicate that astaxanthin may possess therapeutic potential against cancer types, showcasing its ability to inhibit proliferation in different cell lines.

Although astaxanthin has an antiproliferative activity on HeLa cells [4], the mechanisms contributing to this effect have not been adequately studied. This study aimed to investigate the contribution of proapoptotic and anti-apoptotic proteins, oxidative DNA damage, and autophagy - a cellular degradation process - to the antiproliferative effect of astaxanthin in HeLa cells.

EXPERIMENTAL

Maintenance of cell cultures

The cervical carcinoma-derived HeLa cell line (CRM-CCL-2) was acquired from the American Type Culture Collection (ATCC). For cell culture purposes, HeLa cervical cancer cells were cultured using Dulbecco's modified Eagle's medium (DMEM) and sustained at a temperature of 37 °C. The cells were grown in a humidified environment supplemented with 5 % carbon dioxide (CO_2). The culture medium used in this study consisted of 1 % streptomycin/penicillin, 1 % L-glutamine, and 10 % fetal bovine serum (FBS) [5]. Astaxanthin was initially solubilized in dimethyl sulfoxide (DMSO) before being further diluted using the cell culture medium. To minimize potential DMSO-induced effects on cell viability and function, the final concentration of DMSO in the cell culture medium was strictly maintained below 0.1 %. All reagents and materials utilized in this study, except the cell line, were sourced from Sigma-Aldrich.

Assessment of cell viability

The XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay was performed to assess the effect of astaxanthin on HeLa cell viability. Prior to treatment, 1×10^4 cells per well were seeded and allowed to adhere overnight (24 hours). Subsequently, the HeLa cells were subjected to a range of astaxanthin concentrations (2.5, 5, 10, 20, and 40 μM). A control group consisting of untreated cells was included. At the end of the incubation period (24 h), a 50 μL XTT mixture was introduced to every well. This assay was

performed as described in the study by Dirik *et al* [6].

Enzyme-linked immunosorbent assay (ELISA)

To evaluate the levels of Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-oxo-dG in both HeLa cells with and without astaxanthin treatment, human ELISA kits specifically designed for these biomarkers were utilized. The ELISA kits were obtained from BT Lab Shanghai, China. To conduct the experiments, HeLa cells were placed into a 6-well plate and subjected to astaxanthin at a concentration of 18.47 μM , which corresponds to IC_{50} value, for a duration of 24 hours. Both exposed to astaxanthin and unexposed HeLa cells were gathered and mixed with phosphate buffer saline (PBS) after the treatment period. To induce cellular damage, the cells were subjected to repeated cycles of freezing and thawing. Quantification of marker levels in the cell lysates was performed using ELISA kits, adhering to the protocols supplied by the manufacturer. To determine the total protein quantities in the groups, the Bradford protein assay kit from Merck Millipore in Germany was utilized [7,8].

Immunofluorescence staining and imaging

Once prepared in suspension, cells were deposited onto a glass slide using a pipette. Subsequently, the transferred cells were allowed to dry for a period of 8 hours. Following the previous step, methanol fixation was performed at -20°C for 5 minutes. After the fixation step, the cells were rinsed using PBS (phosphate-buffered saline) to remove any remaining fixative. After washing, cells were permeabilized with 0.1 % Triton X-100 in PBS for 15 minutes at room temperature. Cells were then washed and incubated in a blocking solution (PBS with 2 % BSA (bovine serum albumin)) for 1 hour at room temperature. Following another wash, cells were incubated overnight at 4°C with primary antibodies: monoclonal anti-MAP LC3 β (Santa Cruz, sc-271625) and monoclonal anti-8-OHdG (Santa Cruz, sc-66036), both diluted 1/200 in antibody diluent. This assay was performed as described in the study by Ataseven *et al* [9]. During evaluation, the overall cell positivity in each field was assessed semi-quantitatively and classified as negative (0), mildly positive (1), moderately positive (2), or strongly positive (3).

Statistical analysis

Data are reported as mean $\pm$ standard error (SEM). The Statistical Package for the Social Sciences (Version 22) was used to conduct the

statistical analyses. One-way ANOVA with Tukey's post-hoc tests was used to analyze XTT assay data, while Student's t-tests were employed for ELISA and immunofluorescence staining results. Significance was defined as $p < 0.05$.

RESULTS

The antiproliferative activity of Astaxanthin in HeLa cells

HeLa cells were incubated with astaxanthin concentrations between 2.5 and 40 μM for 24 hours, after which cell viability was determined via XTT assay. No significant effect on cell viability was observed at concentrations of 2.5, 5, and 10 μM . However, a concentration-dependent anti-proliferative effect of astaxanthin was evident at 20 and 40 μM ($p < 0.001$; Figure 1). The IC_{50} value of astaxanthin, determined using GraphPad Prism, was 18.47 μM .

The effect of astaxanthin on apoptotic markers

ELISA was used to assess apoptosis-related protein levels in HeLa cells. This method was employed to analyze the expression levels of multiple proteins, including Bcl-2, cleaved caspase 3, Bax, and cleaved PARP. Treatment with astaxanthin at a dose of 18.47 μM for 24 hours resulted in a significant elevation in the levels of cleaved caspase 3, increasing from 524.76 ± 7.76 to 901.61 ± 42.54 pg/mg protein ($p < 0.001$; Figure 2).

Similarly, the levels of Bax showed a significant increase from 10.01 ± 1.02 to 19.27 ± 0.57 ng/mg protein ($p < 0.001$, Figure 2). Furthermore, the levels of cleaved PARP exhibited a significant rise from 505.67 ± 3.366 to 613.08 ± 5.109 pg/mg protein ($p < 0.001$, Figure 2) upon exposure to astaxanthin. In contrast, astaxanthin treatment did not lead to a meaningful difference in the BCL-2 level ($p > 0.05$; Figure 2).

Effect of astaxanthin on 8-oxo-dG level in HELA cells

To assess the potential DNA-damaging effects of astaxanthin, the expression of 8-oxo-dG in HeLa cells was measured using ELISA. Following exposure to astaxanthin at a dose of 18.47 μM for 24 hours, a substantial elevation in the quantity of 8-oxo-dG was observed, rising from 38.83 ± 4.25 to 73.01 ± 3.67 ng/mg protein ($p < 0.001$, Figure 3).

Effect of astaxanthin on 8-oxo-dG and LC3 β levels in HeLa cells

Six samples each from astaxanthin and control groups were stained. Astaxanthin and control groups consisted of a total of 6 stained samples. The analysis of the stained samples revealed significant differences in the levels of 8-oxo-dG and LC3 β between the two groups ($p < 0.05$), as depicted in Figure 4. The control group exhibited a mild expression of LC3 β and 8-OHdG, whereas astaxanthin group showed a severe expression of LC3 β and a moderate expression of 8-OHdG. The detected fluorescence positivity was located within the intracytoplasmic region.

DISCUSSION

Astaxanthin has garnered significant attention as a promising contender for cancer treatment in recent years. Researchers are actively confirming its anti-cancer properties, although they continue to explore and understand the exact mechanisms through which it exerts its effects. Employing in vitro models, this research explored the impact of astaxanthin on several cellular pathways. Specifically, its effects on cell growth, apoptosis, autophagy, and DNA damage were examined. HeLa cell line, which originated from cervical cancer cells, was utilized for this experiment. First, XTT studies were done to evaluate astaxanthin's concentration-dependent cytotoxicity on HeLa cells. The experimental data

revealed that astaxanthin markedly suppressed the proliferation of HeLa cells in a dose-dependent fashion. When HeLa cells were administered with astaxanthin for 24 hours, IC₅₀ value was found to be 18.47 μ M. Our results corroborate previous findings that astaxanthin possesses anti-proliferative activity against different types of non-small cell lung cancers. Likewise, Sowmya *et al*'s investigation explored the impact of astaxanthin on HeLa cells.

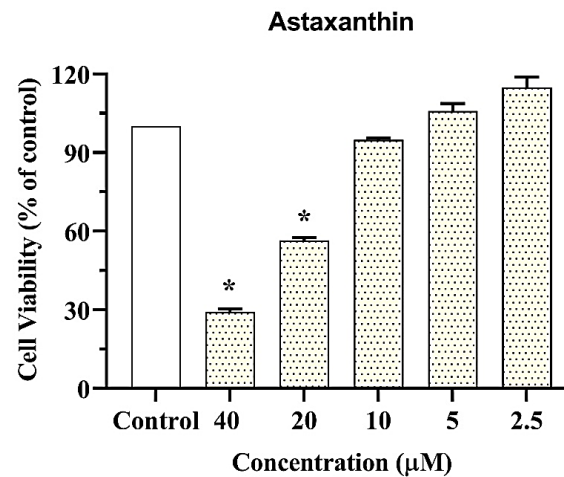


Figure 1: The effect of astaxanthin on HeLa cell proliferation. Cell viability was determined relative to a control group. Data is presented as the mean $\pm$ SEM from six independent experiments. * $P < 0.001$ vs. control group

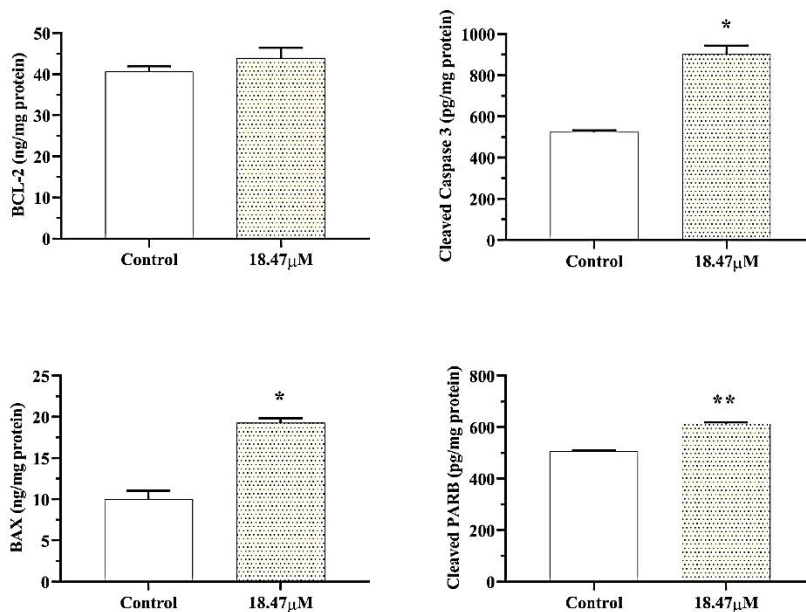


Figure 2: The effect of astaxanthin at a concentration of 18.47 μ M on the levels of Bcl-2, cleaved caspase 3, Bax, and cleaved PARP in HeLa cells. Treatment resulted in an increased apoptosis rate of HeLa cells. Data is presented as mean $\pm$ SEM from six independent experiments. * $P < 0.001$ vs. control group

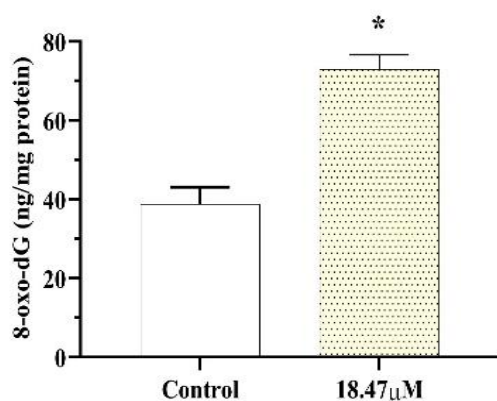


Figure 3: The effect of astaxanthin at a concentration of 18.47 μ M on the level of 8-oxo-dG in HeLa cells. The level of 8-oxo-dG was measured using an ELISA kit. Data is presented as mean $\pm$ SEM from six independent experiments. Treatment led to an augmentation of DNA damage. * $P < 0.001$ vs control group

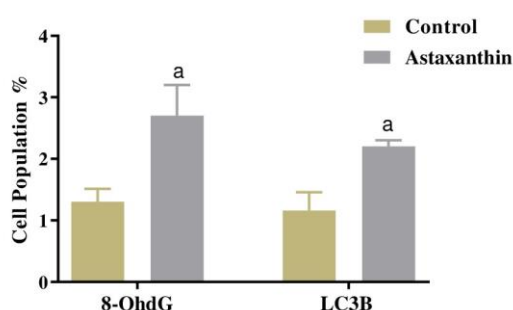
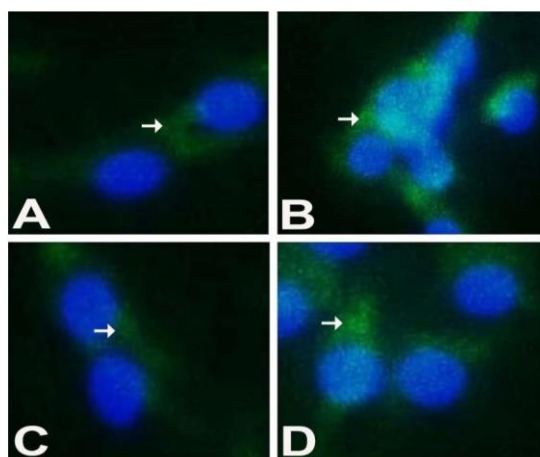


Figure 4: Immunofluorescence staining revealed distinct patterns of DNA damage and autophagy markers. (A) In control group, a mild level of 8-hydroxy-2'-deoxyguanosine (8-OHdG) positivity was observed (indicated by an arrow). (B) Conversely, astaxanthin-treated group exhibited a severe level of 8-OHdG positivity, suggesting increased oxidative damage. (C) Regarding autophagy, control group displayed mild LC3 β positivity, whereas (D) astaxanthin group showed moderate LC3 β positivity. $P < 0.05$ vs untreated cells

They found that astaxanthin significantly decreased cell viability dose-dependently after a 48-hour incubation period. Furthermore, administering astaxanthin resulted in a buildup of cells at the G2/M stage of the cell cycle. This finding suggests that astaxanthin may disrupt cell cycle checkpoints, potentially causing a halt in the cell cycle and thereby restricting cell multiplication [10].

Earlier findings have indicated that astaxanthin possesses antiproliferative properties against colon cancer cell lines HT-29 and HCT116, as well as the human hepatic cancer cell line HepG2. These effects were observed to vary depending on the duration of exposure and the concentration of astaxanthin [4]. Contrary to that, Wayakanon *et al* observed that astaxanthin did not demonstrate any adverse consequences on a non-malignant human oral keratinocytes (NOKs) cell line [11]. The significance of apoptosis within the intricate molecular landscape of cancer is well-established. Moreover, its regulatory influence profoundly affects the efficacy of therapeutic interventions like chemotherapy and radiotherapy. Comprehending the role of apoptosis in cancer pathogenesis is crucial for developing effective therapeutic strategies. By elucidating the intricate mechanisms governing apoptosis, researchers aim to identify novel targets for cancer therapy and develop interventions that trigger apoptosis specifically in cancer cells while sparing normal healthy cells [12].

An ELISA was carried out to assess the activity of astaxanthin on apoptosis in HeLa cells. These studies analyzed the levels of apoptotic and anti-apoptotic markers, including cleaved PARP, Bax, cleaved caspase 3, and BCL-2. By measuring the changes in these markers, researchers aimed to gain insights into the apoptotic pathways influenced by astaxanthin treatment in HeLa cells. ELISA assays provide quantitative measurements of these apoptotic markers, affording greater knowledge about the influence of astaxanthin on apoptosis at the molecular level. Bax, a pro-apoptotic protein, is a key mediator in the initiation of the disruption of the mitochondrial outer membrane. This crucial step causes cytochrome c to be released into the cytoplasm. Following its release, cytochrome c engages with Apaf-1 and caspase-9, assembling the apoptosome, a multiprotein complex. The apoptosome serves as a platform for the activation of effector caspases, thereby initiating the execution phase of apoptosis. In contrast, BCL-2, an anti-apoptotic protein, stabilizes the mitochondrial membrane, blocking cytochrome c escape and thus inhibiting apoptosis. Caspases,

a class of protease enzymes essential for apoptosis, exist initially as inactive precursors known as zymogens. These zymogens undergo a specific activation process during apoptosis. However, when apoptosis is triggered, these zymogens are cleaved, generating active caspases that facilitate the progression of apoptosis. Caspase 3, which holds a significant position among caspases, assumes a crucial role in the process of apoptosis. Upon activation, caspases trigger endonuclease CAD activation, leading to chromosomal DNA fragmentation and subsequent chromatin condensation. PARP, an essential enzyme in DNA repair mechanisms, plays a key role in fixing base excisions. Notably, targeting PARP through inhibition or cleavage exploits a critical vulnerability in DNA repair mechanisms, leading to selective cell death, particularly in cancer cells with defective DNA repair pathways [13].

Throughout the investigation, it was noted that the introduction of astaxanthin at a concentration of 18.47 μM exhibited a notable impact on enhancing the expression of Bax, as well as activating cleaved caspase 3 and cleaved PARP. This significant alteration in protein expression played a pivotal role in promoting cellular apoptosis. Our findings align with previous research demonstrating that astaxanthin possesses the capacity to enhance apoptosis across various cancer cell lines. These comprise colorectal, gastric, and breast cancer cells, as well as human hepatocellular carcinoma, lung cancer, melanoma, and ovarian carcinoma cells. In the context of colon carcinoma cells, astaxanthin has demonstrated its proapoptotic activity by modulating various key proteins and pathways. It downregulated the production of anti-apoptotic proteins, including Bcl-2, while upregulating pro-apoptotic proteins such as Bax, caspase-3, and PARP [14]. Exposure of prostate cancer cells to astaxanthin has been shown to increase caspase-3 levels and raise the population of apoptotic cells [15].

An experiment was conducted to evaluate DNA fragmentation in HeLa cells to examine a possible relationship between astaxanthin and its potential to induce cellular damage. These cells were treated with astaxanthin for 24 hours. To examine DNA fragmentation, we employed two widely used techniques: 8-oxo-dG ELISA and immunohistochemistry. These techniques are widely employed to quantify 8-oxo-dG, a proven biomarker that signifies oxidative DNA damage [16]. Our results demonstrated a substantial elevation in the levels of 8-oxo-dG in HeLa cells following treatment with astaxanthin. This finding further substantiates the apoptotic and cytotoxic

properties of astaxanthin. Elevated 8-oxo-dG levels indicate increased oxidative DNA damage, which may trigger DNA fragmentation and ultimately result in cellular demise. Previous preclinical studies have shown that metabolic stress in human tumors stimulates autophagy as a way for cells to meet the elevated energy needs during cancer development. If unfavorable metabolic conditions exceed a critical threshold necessary for cellular viability, autophagic cell death may ensue. This is a desirable outcome in the context of cancer treatment. Numerous anti-cancer therapies have been identified that trigger autophagy as a component of their mechanism of action [17]. Under normal cellular circumstances, the autophagy-related protein LC3 β displays a homogenous distribution throughout the cytoplasm, as revealed by immunofluorescence microscopy. However, upon initiation of autophagy, a distinct change in the appearance of LC3 β is observed. It adopts a granular or punctate pattern, indicative of its incorporation into the membrane of forming autophagosomes. This fusion process facilitates the degradation of the autophagosome contents, contributing to cellular recycling and maintenance. Examining LC3 β proteins under a microscope offers an effective way to evaluate autophagy stimulation [18].

This study observed a significant increase in LC3 β levels in HeLa cells following astaxanthin treatment. Elevated LC3 β levels are indicative of autophagy induction, indicating that the anti-proliferative impacts of astaxanthin may be partially carried out through the activation of autophagy. These results align with earlier studies showing that astaxanthin treatment increased the expression of autophagy-associated genes, such as LC3 and beclin-1, in the brain [19].

CONCLUSION

When HeLa cells are treated with astaxanthin, cell multiplication is inhibited in proportion to the concentration. This inhibition is concurrent with a significant upregulation of programmed cell death indicators such as Bax, cleaved caspase-3, and cleaved PARP. Furthermore, the administration of astaxanthin significantly increases the level of 8-oxo-dG, which acts as a dependable indicator of DNA oxidative damage. Moreover, treatment with astaxanthin elevates LC3 β level, which serves as a marker for autophagy. These findings indicate that astaxanthin has potential for use in managing cervical cancer. Nevertheless, additional confirmation through *in vivo* experiments and clinical trials is essential to verify these findings and comprehensively

evaluate the efficacy and safety of astaxanthin as a therapeutic option.

DECLARATIONS

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Ethical approval

None provided.

Use of Artificial intelligence/Large language models

We also declare that we did not use Generative artificial intelligence (AI) and AI-assisted technologies in writing the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors (Author's initials)

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Dilay Karademir conceived the original idea. Ayşegül Öztürk and Dilay Karademir supervised the project and experiment. Dilay Karademir wrote the manuscript with support from Ayşegül Öztürk, Bilal Şahin and Mustafa Özkaraca, who helped to supervise the project.

Availability of data and materials

The authors state that the datasets utilized and/or analyzed in this study can be obtained from the corresponding author upon request.

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