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Effects of Astaxanthin-Containing Microalgae Extract on Viability and Proliferation of HEK293T Cells

Abstract : Microalgae have become a major area of research in the fields of biotechnology and health sciences in recent years due to the bioactive compounds they contain.Among these microalgae, Haematococcus pluvialis is significant microorganisms for its astaxanthin content, which has a notably high antioxidant capacity.The aim of this study was to investigate, in vitro, the effects of an algal extract derived from Haematococcus pluvialis on cell viability and proliferation in HEK293T cells, a human embryonic kidney cell line.

In the experiments, HEK293T cells were cultured under appropriate conditions and seeded into 6-well culture plates. The algal extract was prepared in DMSO and applied to the cells at various doses ranging from 3–30 µg/mL. Only DMSO was used in the control groups. Cell viability was assessed 24 and 48 hours after application using the Trypan blue exclusion method. The obtained data were validated through technical and biological replicates. The results demonstrated that the algal extract reduced cell viability and proliferation in HEK293T cells in a dose- and time-dependent manner. At low doses, cell viability remained close to that of the control groups; however, a significant decrease in cell count was observed at high doses, particularly in measurements taken at 48 hours. In the control groups, however, cell proliferation was found to continue normally. These findings suggest that the Haematococcus pluvialis extract may have inhibitory effects on cell proliferation. This study is expected to contribute to the understanding of the cellular effects of microalgae-derived natural compounds and to provide a basis for future mechanistic studies.

Keywords: Haematococcus pluvialis, microalgae, anticancer, astaxanthin.

Purpose

The aim of this project is to evaluate the effects of an algal extract derived from Haematococcus pluvialis on cell viability and proliferation in a human embryonic kidney cell line under in vitro conditions, in a dose- and time-dependent manner. Although microalgae are known to be rich in bioactive compounds, the cellular effects of these compounds on human cells have not been sufficiently elucidated, particularly through experimental studies at the high school level. Although the biochemical composition of microalgae has been examined in detail in the current literature, it is observed that data based on direct cell viability analyses using cell lines are limited. This study aims to address this shortcoming by quantitatively determining the effect of algal extract applied at different concentrations on cell viability. In this context, the effect of algal extract applied to HEK293T cells on cell proliferation, and how this effect changes in a dose- and time-dependent manner, has been experimentally determined. Comparative analyses with control groups were conducted to assess whether the observed effect was solvent-induced. The R&D content of the project includes an experimental approach based on cell culture, dosing, time-dependent analysis, and viability measurements. It is intended that the findings obtained will contribute to the understanding of the cellular effects of natural compounds derived from microalgae and provide preliminary data for future mechanism-based studies.

Introduction

In recent years, microalgae have been attracting increasing attention not only because of their high nutritional content but also because they support a healthy diet. The first efforts to utilize microalgae industrially began in the 1950s when Burlew proposed algae as an alternative protein source for plants and animals. Later, algal cultivation began to be actively utilized in animal feed and aquaculture. Additionally, microalgae have become an important raw material source in the pharmaceutical and cosmetic industries due to the bioactive compounds they contain. Furthermore, microalgae possess undeniable potential for the production of bioplastics and biofuels. Microalgae are suitable for industrial production due to their rapid biomass growth, ability to convert CO_2 through photosynthesis, and lack of need for specific environmental conditions. Their natural habitats are very extensive; microalgae can be found in many different ecosystems such as oceans, rivers, and lakes. For example, Haematococcus pluvialis is a resilient microalgal species capable of growing even under harsh environmental conditions, such as the Arctic waters of the White Sea. Among microalgal species, Haematococcus pluvialis is one of the most important for natural astaxanthin production, and the number of scientific publications on this species and astaxanthin has been increasing in recent years.

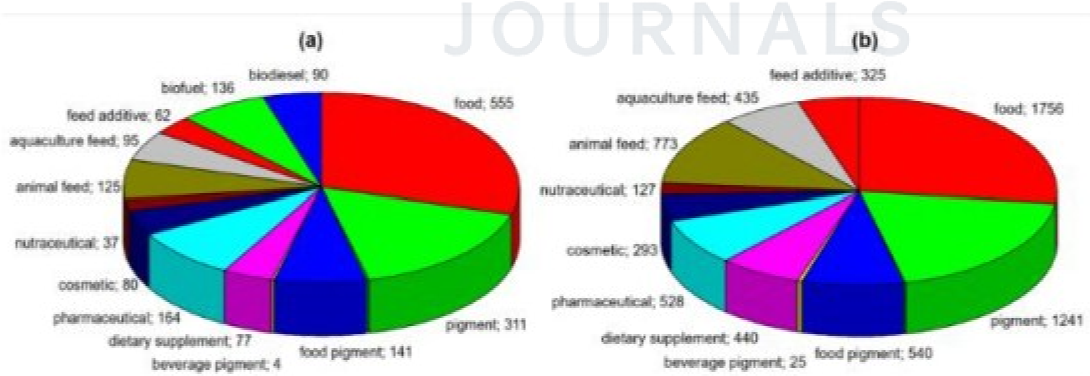

Figure 1. Potential applications of (a) *Haematococcus pluvialis* and (b) astaxanthin derived from this microalga (according to Web of Science, accessed August 3, 2020).

A significant portion of the applications of both microalgae and astaxanthin is related to human nutrition and health. In this context, microalgae are extensively used as natural pigments in foods, dietary supplements, and beverages, as well as in the pharmaceutical and nutritional supplement sectors. Research has focused on the potential applications of astaxanthin in human and animal nutrition and treatment, highlighting its antioxidant, anti-inflammatory, anti-diabetic, and anti-cancer properties.

Cell Morphology of Haematococcus pluvialis

Haematococcus pluvialis is a green, unicellular microalga that inhabits freshwater and belongs to the class Chlorophyceae and the order Volvocales. Its cell structure resembles that of other Volvocales single-celled green algae species and is characterized by four basic cell types throughout its life cycle: macrozooid (zoospore), microzooid, palmella, and hematocyst (aplanospore) (Hazen, 1899; Elliot, 1934; Shah et al., 2016). Among these morphological forms, the macro- and microzooids and the palmella stage are defined as the "green vegetative phase," while the hematocyst stage is defined as the "red cyst phase" (Shah et al., 2016). The cell morphology of Haematococcus pluvialis is highly sensitive to environmental conditions and exhibits a flexible life cycle, transforming from thin-walled, green, flagellated vegetative cells to thick-walled, red, astaxanthin-rich cyst cells. This morphological flexibility is not only a key adaptation enabling the species to survive under adverse environmental conditions but also holds significant biotechnological importance in terms of natural astaxanthin production (Shah et al., 2016; Wayama et al., 2013).Recent studies have shown that the biochemical components found in microalgae are rich in antioxidant and anti-inflammatory properties. Polysaccharides such as starch, cellulose, and β-1,3-glucan; lipids such as hydrocarbons and polyunsaturated fatty acids; bioactive compounds with antibacterial, antifungal, and antiviral effects; as well as enzymes, amino acids, polypeptides, and pigments such as carotenoids and chlorophylls; find extensive applications in the food, pharmaceutical, cosmetic, chemical, and agricultural sectors (Borowitzka, 2013; Oslan et al., 2021; Plaza et al., 2009).

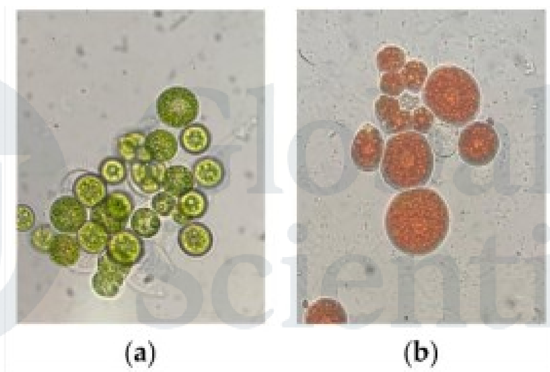

Figure 2. Cell morphology of *Haematococcus pluvialis* in (a) the green phase and (b) the red phase

Macrozooids are motile cells measuring 8–20 µm in size and possessing two flagella. Adverse environmental conditions cause the cells to lose their flagella, first transforming into the palmella form and subsequently into the thick-walled aplanospore form. Aplanospores are more resistant to environmental stress, and astaxanthin accumulation occurs most extensively during this stage. For this reason, the effects of factors such as nutrient limitation and light intensity on cell morphology and astaxanthin production have been examined in numerous studies. Optimizing culture conditions contributes to the higher-yield production of desired metabolites.

Culture of Haematococcus pluvialis

The cultivation of Haematococcus pluvialis occurs in two stages. The first stage is the green phase, during which cell division leads to an increase in biomass. Cell proliferation occurs when appropriate temperature, pH, and light conditions are provided. The second stage is the red phase, which emerges under stress conditions, and during this phase, the cells begin to accumulate carotenoids, primarily astaxanthin, in high concentrations. Different growth methods (photoautotrophic, heterotrophic, mixotrophic) can be used under culture conditions. Other factors affecting yield include medium composition and light-dark cycles. In industrial production, bioreactors or open-pond systems are used, and cost, water availability, and contamination risk play a major role in the selection of the production method.

The production of natural astaxanthin is limited worldwide, and a large portion of the astaxanthin available on the market is obtained through chemical synthesis. This situation is attributed to the high production costs of natural astaxanthin and the complexity of the production processes. Consequently, numerous studies are being conducted to reduce production costs and optimize culture conditions (Borowitzka, 2013; Shah et al., 2016). However, there is a risk of heavy metal accumulation in microalgal cultures grown in wastewater, which necessitates additional purification steps before the products can be used for food or feed purposes. The need for purification is considered a significant factor that further increases production costs (Markou & Georgakakis, 2011; Wang et al., 2018).

Biochemical Composition of Haematococcus pluvialis

Biochemical components in Haematococcus pluvialis exhibit significant changes depending on the cell's life stage. While cells in the green phase contain high levels of protein, in the red phase—which forms under stress—protein levels gradually decrease, while carbohydrate content increases rapidly, particularly in the cyst form. This represents an energy storage adaptation mechanism that enables the cell to survive prolonged exposure to stress. Lipid accumulation also increases under the influence of stress factors such as nitrogen limitation, high light intensity, and temperature. Some studies show that lipid accumulation increases more than twofold under stress conditions compared to the control group. (Damiani et al. 2010) For this reason, Haematococcus pluvialis is considered an important biomass source for both oil production and astaxanthin. In terms of carotenoids, lutein and β-carotene are prominent in the green phase. In the red phase, astaxanthin constitutes the majority of total carotenoids. Since astaxanthin accumulates only in the red phase, the culture process must be managed correctly. Overall, optimizing culture conditions in terms of protein, lipid, and carotenoid content is of great importance for both biofuel production and the production of animal feed, food supplements, and astaxanthin.

Fatty Acids	Kim Kim *et al.*, 2015 [19]	Lorenz, 1999 [39]	Scodelaro Bilbao *et al.*, 2016 [40]	Lei Lei *et al.*, 2012 [41]
C12:0 lauric	None	0.1	None	0.28
C14:0 myristic	0.1	0.5	1.99	0.65
C15:0 pentadecanoic acid	0.1	None	None	0.25
C16:0 palmitic	13.7	29	22.9	12.7
C16:1 palmitoleic	0.5	0.6	0.35	0.7
C16:2	0.4	None	None	None
C16:3	3.5	None	None	None
C16:4	3.3	None	None	None
C17:0 margaric	None	0.2	None	0.23
C17:1 margaroleic	None	1.3	None	0.0
C18:0 stearic	0.7	2.1	1.15	4.79
C18:1 oleic	4.9	25.9	16.3	11.2
C18:2 linoleic	24.9	20.8	23.9	13.0
C18:3 linolenic	39.7	12.8	12.5	2.84
C18:4 octadecatetraenoic	5.8	1.4	None	None
C20:0 arachidic	None	0.6	None	0.35
C20:1 gadoleic	0.5	0.3	None	1.3
C20:2 eicosadienoic	None	1.2	2.21	0.87

Table 1. Comparison of the fatty acid compositions (%) of different H. pluvialis strains.

Astaxanthin is a red xanthophyll carotenoid and, thanks to its powerful antioxidant properties, has the potential to protect against diseases associated with oxidative stress. Its structure consists of two β-ionone rings linked by a polyene chain. This structure enables it to neutralize free radicals, and as a result, it has positive biological effects against a variety of diseases such as cardiovascular diseases, diabetes, and cancer. Found in high concentrations in nature, particularly in Haematococcus pluvialis, astaxanthin accumulates within the cell during the red phase. To extract astaxanthin, the cell wall must first be broken down. Innovative techniques such as solvent extraction or various enzyme-assisted methods are then applied. These new methods stand out for using fewer solvents and achieving higher yields.

The biological properties of astaxanthin can be summarized as antioxidant, anti-inflammatory, and antidiabetic effects. Astaxanthin protects cell membranes by neutralizing free radicals, reduces lipid peroxidation, and prevents oxidative damage (Pashkow et al., 2008; Hussein et al., 2005). It has been reported that astaxanthin reduces the levels of pro-inflammatory cytokines produced by immune cells during inflammatory processes and may exhibit a protective effect in diseases related to the nervous system and the immune system (Chew & Park, 2004; Jyonouchi et al., 2000). Furthermore, various studies have demonstrated that astaxanthin regulates glucose metabolism by enhancing insulin sensitivity, protects pancreatic β-cells against oxidative stress, and may thereby slow the progression of diabetes (Nakano et al., 2008; Bhuvaneswari et al., 2012). Due to these properties, astaxanthin is considered a beneficial and versatile natural bioactive compound for human health. Astaxanthin is an important bioactive carotenoid that, thanks to its potent antioxidant properties, suppresses oxidative stress mechanisms involved in cancer development.Reducing oxidative stress exerts a protective effect on carcinogenesis by limiting both DNA damage and mutagenic processes. Studies have shown that astaxanthin can slow the proliferation of cancer cells and even trigger cell death by activating apoptosis mechanisms (Palozza et al., 2009). For example, it has been reported that astaxanthin-rich extracts derived from Haematococcus pluvialis halt the cell cycle and promote apoptosis in human colon cancer cells (Palozza et al., 2009). The anticancer effects of astaxanthin are not limited to in vitro studies. Animal studies have reported that this compound strengthens the immune system, particularly by increasing levels of gamma interferon, a key marker of natural killer cells and the immune response (Chew & Park, 2004; Jyonouchi et al., 2000). It has also been noted that astaxanthin exhibits superior activity in suppressing cancer development compared to other carotenoids such as β-carotene and canthaxanthin (Chew et al., 1999). It has been demonstrated that astaxanthin exerts a dose-dependent antiproliferative effect in breast cancer, hepatoma, and other tumor cell lines, thereby holding high potential for therapeutic research (Song et al., 2011).

The therapeutic potential of astaxanthin is not limited to cancer. When considering cardiovascular diseases, astaxanthin's protective mechanisms are particularly noteworthy, given that oxidative stress and inflammation are key risk factors for cardiovascular diseases. Studies on the antioxidant and anti-inflammatory effects of astaxanthin in cardiovascular diseases suggest that this compound may improve endothelial function by reducing oxidative damage and, consequently, contribute to a reduction in cardiac risks (Pashkow et al., 2008). Experimental animal studies have demonstrated that astaxanthin lowers blood pressure, regulates nitric oxide metabolism, and can reduce blood pressure (Hussein et al., 2005).

The effects of astaxanthin on the visual system are also being increasingly investigated. Astaxanthin can cross the blood-retinal barrier and, by incorporating into the cell membrane, forms a protective barrier against oxidative damage. In studies using retinal ischemia-reperfusion models, astaxanthin has been shown to reduce retinal cell death, and an increase in retinal blood flow velocity has also been reported (Saito et al., 2012; Otsuka et al., 2016).From a clinical perspective, it has been suggested that astaxanthin may be helpful in the treatment of age-related macular degeneration, glaucoma, and other ocular diseases (Giannaccare et al., 2020). Another important therapeutic area for astaxanthin is kidney and nervous system diseases. It has been determined that astaxanthin exhibits protective effects against oxidative stress and inflammation mechanisms in kidney tissue and reduces kidney damage in experimental studies (Mosaad et al., 2016). From a neurological perspective, it has been reported that astaxanthin exerts a neuroprotective effect by suppressing neuroinflammation, oxidative stress, and apoptotic processes. Due to its ability to reduce the mechanisms of damage in nervous tissue, it is considered a potential therapeutic agent for Parkinson's disease, Alzheimer's disease, cerebral ischemia, and other neurodegenerative disorders (Hussein et al., 2005).

Overall, astaxanthin is a promising natural compound for the treatment of cancer, cardiovascular diseases, eye diseases, kidney damage, and neurodegenerative diseases, thanks to its potent antioxidant capacity, its role in regulating cell death, its ability to enhance the immune response, its anti-inflammatory effects, and its influence on metabolic processes. For this reason, it is anticipated that astaxanthin may find broader applications in the future in pharmacological applications, nutritional supplementation, and clinical treatment approaches.

Method

During the project, the algae extraction steps were carried out in the school's biology laboratory under the supervision of a biology teacher, while the treatment of the algae extract with cancer cells was conducted at the Proteomics Laboratory of the Faculty of Medicine at Kocaeli University with the support of Prof. Dr. Gürler Akpınar.

Materials Used

- HEK293T cell line
- DMEM culture medium
- Fetal bovine serum (FBS)
- Penicillin–Streptomycin (P/S)
- L-Glutamine
- Trypsin–EDTA
- PBS buffer
- DMSO
- Algal extract
- 6-well culture plates
- Hemocytometer (Neubauer chamber)

The experimental study conducted within the scope of the project was carried out in the following order:

1. Procurement of red Haematococcus pluvialis algae.
2. Preparation of the algal extract.
3. Treatment of cancer cells with the prepared extract.
4. Performance of the MTT cell viability assay.

Haematococcus pluvialis algae were procured through the online sales platform of Trojan Bio Algae and Natural Products Industry and Trade Ltd. Co., located at the Çanakkale Onsekiz Mart University Technology Park campus.

Human embryonic kidney cell line (HEK293T) was used in the experiments.

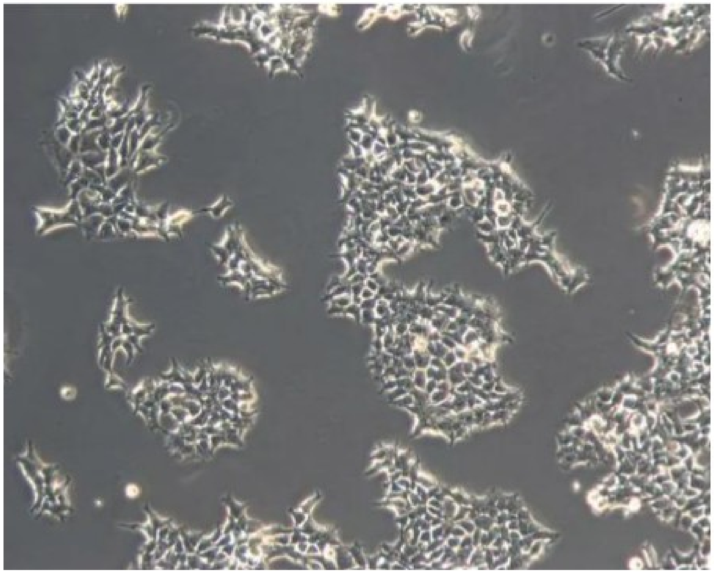

Figure 4. Inverted microscope image of the cells at 40x magnification.

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). The culture medium was prepared to contain 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. The cell culture was maintained at 37°C in a humidified incubator containing 5% CO_2. All procedures were performed under aseptic conditions.

HEK293T cells were seeded into 6-well plates one day before the experiment. A total of 2 mL of culture medium was used in each well, containing 3.0×10^5 cells per well. The cells were incubated for 24 hours to allow attachment to the surface and entry into the logarithmic growth phase.

The alg extract stock solution was prepared in DMSO at a concentration of 3 mg/mL. An equal volume of DMSO was added to the control group (the final DMSO concentration was kept constant across all groups). To the experimental groups, 2, 4, 8, 16, and 20 µL of the stock solution were added, respectively.

The final concentrations were adjusted to range from 3 to 30 µg/mL, with a final volume of 2 mL. All experiments were conducted with two technical replicates (n=2) and three independent biological replicates (n=3).

Final concentration calculations (for a 2 mL volume):

Stock: 3 mg/mL = **3000 µg/mL**

Consequently, the final concentration range in each well was 3–30 µg/mL.

Cells were harvested at 24 and 48 hours post-treatment. The medium was removed from each well, and the cells were washed with PBS. The cells were detached from the surface by incubating with 0.25% trypsin-EDTA for 2–5 minutes. Trypsin activity was stopped by adding an equal volume of DMEM + 10% FBS. The cell suspension was homogenized and transferred to 1.5 mL tubes.

A 10 µL sample was taken from the cell suspension and mixed with 0.4% trypan blue at a 1:5 ratio. The resulting mixture was loaded onto a Neubauer counting chamber. Three different counting fields were evaluated for each sample, and the average value was calculated. The number of live cells was determined by taking the dilution factor into account.

Project Work Schedule

In this study, the effect of an algal extract derived from Haematococcus pluvialis on the viability of HEK293T cells was evaluated. In measurements taken at 0, 24, and 48 hours using the Trypan blue exclusion method, it was observed that cell viability in the control groups (2, 8, and 20 µL DMSO) increased steadily over time, reaching a cell count of approximately 1.9–2.0 × 10^6 by the end of 48 hours. This finding indicates that the volume of DMSO used did not exert a significant toxic effect on cell proliferation. In cells treated with the algal extract, however, significant decreases in cell viability occurred in a dose- and time-dependent manner. At low doses (2–4 µL), the results at 24 hours were similar to those in the control groups; however, by the end of 48 hours, the proliferation rate was found to be lower compared to the control. At medium doses (8–16 µL), a marked slowing in the rate of cell count increase was observed, and particularly with the 16 µL application, a significant decline in cell count was noted by the 48th hour.

It was determined that at high doses (20 µL), cell viability fell below the baseline value and the cell count dropped to 350,000 by the 48th hour. The data obtained demonstrate that the algal extract inhibits proliferation in HEK293T cells and exhibits cytotoxic effects at high concentrations. Overall, it was concluded that cell viability decreased as time and dose increased, and that the toxic effect became particularly evident in measurements taken at the 48-hour mark. These findings suggest that Haematococcus pluvialis extract possesses cell proliferation-inhibiting effects and may increase cell death at high concentrations. It is anticipated that these results will shed light on future mechanistic studies investigating the biological activity of the algal extract.

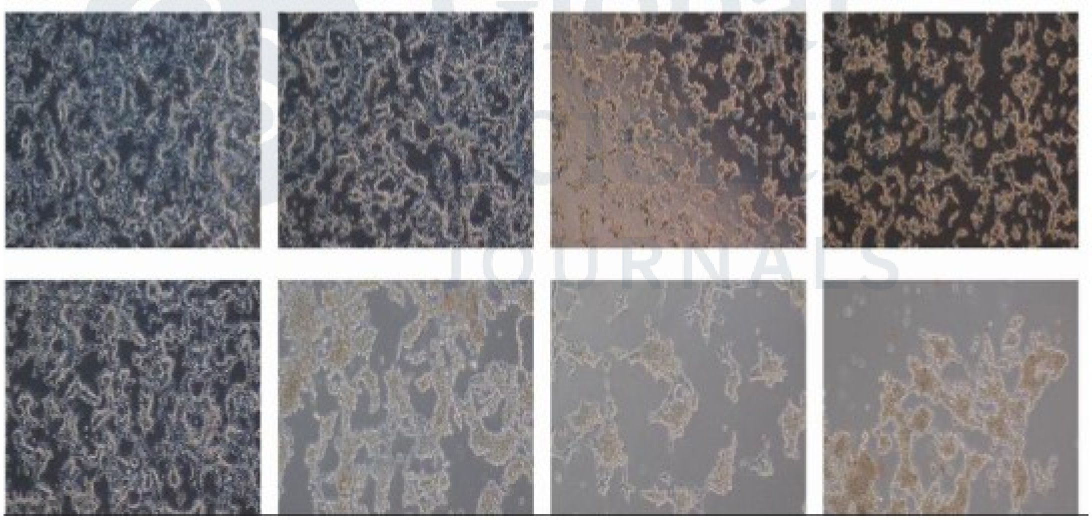

Figure 7. Control – 2 µL – 16 µL – 20 µL: Images of cells treated with algal extract at 24 and 48 hours

In this study, the effects of an algal extract derived from Haematococcus pluvialis on cell viability and proliferation in a human embryonic kidney cell line (HEK293T) were investigated under in vitro conditions. Although the potential of bioactive compounds contained in microalgae in the fields of health biology and biotechnology is increasingly recognized, it is evident that experimental studies demonstrating the direct effects of these compounds on human cells remain limited. In this context, the present study aimed to contribute to the understanding of the cellular-level effects of natural compounds derived from microalgae. The findings revealed that H. pluvialis extract reduced cell viability and proliferation in HEK293T cells in a dose- and time-dependent manner. At low concentrations, cell viability remained close to that of the control groups; however, as concentration and exposure time increased, cell proliferation was significantly inhibited. In particular, the significant decrease in cell count at high doses over a 48-hour treatment period suggests that the extract may possess a strong inhibitory effect on cellular proliferation. The fact that cell growth continued regularly in the control groups supports the conclusion that the observed effect is not solvent-related.

These results suggest that the bioactive compounds contained in Haematococcus pluvialis may influence cell cycle, cell viability, and cellular stress mechanisms. Considering the antioxidant, anti-inflammatory, and metabolism-regulating properties of astaxanthin reported in the literature, it is hypothesized that this effect may be related to mechanisms such as the reduction of oxidative stress, the inhibition of lipid peroxidation, or the arrest of the cell cycle at specific stages. However, this study did not involve a direct analysis of the mechanism, and the data obtained serve as a preliminary assessment at the level of cell viability.

Another important finding of the study is that it demonstrates that safe dosage ranges and biological effects of natural products derived from microalgae can be experimentally determined in cell culture models. This highlights the importance of cell line-based in vitro studies in evaluating natural compounds for biotechnological and biomedical applications. Furthermore, the findings are considered to serve as preliminary data for more detailed studies that may be conducted in the future, such as apoptotic marker analyses, oxidative stress measurements, and cell cycle analyses.

In conclusion, this study has demonstrated that Haematococcus pluvialis extract may have inhibitory effects on cell viability and proliferation in human cells, thereby contributing to the experimental evaluation of the biological potential of microalgae. It is anticipated that the findings will serve as a guide for future research on the use of natural compounds derived from microalgae in the fields of health biology and biotechnology.

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