Int. J. Aquat. Biol. (2023) 11(6): 563-570
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Effect of *Tetraselmis suecica* microalgae isolated from the Persian Gulf on the expression level of AKT/mTOR genes in the liver cancer cell line, Huh7

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Abstract: The medicinal and therapeutic properties of microalgae, especially their anti-cancer properties in modulating cellular mechanisms such as cytotoxicity, reducing tumor cell invasion, and increasing apoptosis, have drawn attention to their therapeutic application in cancer. However, there are few reports on the effects of *Tetraselmis suecica* microalgae on liver cancer. As the aim of this study, after ethanolic extracting from *T. suecica*, and IC50 determination, the effect of *T. suecica* microalgae extract on cytotoxicity and apoptosis of Huh7 cells was investigated by MTT and Annexin V/PI staining, respectively. In addition, the expression of AKT/mTOR genes was measured by real-time PCR test. Based on the results, ethanolic extract of *T. suecica* to the Huh7 cell line significantly decreased the expression of AKT and mTOR genes compared to the control group. The vitality of cancer cells in concentrations of 500 and 1000 µg/ml of *T. suecica* extract in a 48-hour culture, decreased significantly. In addition, in the 72-hour treatment, a significant decrease in cell viability was observed in the concentrations of 250, 500, and 1000 µg/ml. According to the results, *T. suecica* microalgae can offer good benefits as valuable natural materials for the pharmaceutical industry, especially anticancer drugs.

Article history:
Received 13 August 2023
Accepted 4 December 2023
Available online 25 December 2023

Keywords:
PI3K/AKT/mTOR
Microalgae
Liver cancer
Huh7 cell line

Introduction

In the 21st century, cancer is one of the main causes of death worldwide (Bray et al., 2018). Cancer encompasses a spectrum of diseases characterized by the loss of regulated cell growth and division, leading to the growth of a cell mass that can invade surrounding tissues and migrate to new sites through a process called metastasis (Todd et al., 2018). Liver cancer is a type of cancer that originates from the liver and the resulting tumor is aggressive (Liu et al., 2015). Hepatocellular carcinoma (HCC) is the most common type of liver cancer, but there are other types of this cancer, such as angiosarcoma, hemangiosarcoma, and intrahepatic cholangiocarcinoma, which are less common. Among the risk factors involved in developing liver cancer are gender, race, chronic viral hepatitis, cirrhosis, hereditary metabolic diseases, drinking alcohol, smoking, obesity, type 2 diabetes, and exposure to carcinogens such as aflatoxins can be mentioned (Chuang et al., 2009; Huang et al., 2021).

Protein kinase B (Akt) is a type of serine/threonine kinase, is a vital regulator, and plays a key role in cellular mechanisms such as apoptosis, proliferation, and differentiation. Perturbations in the pathways regulated by Akt can lead to cancer, diabetes, and cardiovascular diseases. Due to this important regulatory role of the Akt signaling pathway, Akt becomes a valuable therapeutic target (Nicholson and Anderson, 2002; Nitulescu et al., 2018). The mammalian target of rapamycin (mTOR), by participating in multiple signaling pathways in the body, can target and regulate cell proliferation and apoptosis. The mTOR signaling pathway is also associated with cancer, arthritis, insulin resistance, osteoporosis, and other diseases. In addition to the classical role of mTOR signaling at the level of protein transcription and translation, today the role of this signaling in tumor metabolism is of great interest and therefore it is a potential therapeutic target (Zou et al., 2020). Several signaling pathways are observed in liver cancer. One of these pathways is the PI3K/AKT/mTOR signaling pathway, which

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DOI: https://doi.org/10.22034/ijab.v11i6.1986

regulates signal transmission and biological processes such as cell proliferation, apoptosis, metabolism, and angiogenesis. However, compared to other signaling pathways, the components of the PI3K/AKT/mTOR signaling pathway are complex. The regulatory mechanisms and biological functions of the PI3K/AKT/mTOR signaling pathway are important in many human diseases, including ischemic brain injury, neurodegenerative diseases, and tumors. In addition, the PI3K/AKT/mTOR signaling pathway is the most active in about 50% of liver cancers (Whittaker et al., 2010; Chen and Wang, 2015; Xu et al., 2020).

Due to microalgae's medicinal and therapeutic properties, attention has been drawn toward their therapeutic application. Recently, it has been shown in some studies that some compounds derived from algae with anticancer properties play a role in modulating cellular mechanisms such as cytotoxicity, reducing tumor cell invasion, and increasing cancer cell apoptosis (Farooqi et al., 2012; Lee et al., 2013; Abd El-Hack et al., 2019). *Tetraselmis suecica* is a marine green microalga of the Chlorophyceae and is a good source of protein, carbohydrates, and fatty acids essential for cultivating organisms. In addition, it has a rich source of bioactive compounds such as vitamin E, carotenoid, phenolic, flavonoid, and terpene compounds that have antioxidant, anticancer, and antimicrobial activities (Sansone et al., 2017; Sharawy et al., 2020). The extract of *T. suecica* in autotrophic and heterotrophic cultures has a high cytotoxic effect on tumor cells, including human leukemia cell line HL-60, breast cancer cell line MCF-7, and lung cancer cell line NCI-H460 (Parra-Riofrío et al., 2020). Based on the above-mentioned background, this study aims to investigate the effect of the ethanolic extract of *T. suecica* microalgae isolated from the Persian Gulf on the expression level of AKT/mTOR genes in the Huh7 liver cancer cell line.

Materials and Methods

***Tetraselmis suecica* culture:** A sterile stock of *T. suecica* was obtained from the Science and Technology Park, Shiraz Province. It was cultured in 2000 ml Erlenmeyer flasks containing sterilized distilled water (including 1900 ml distilled water and 100 ml stock). The salinity of the environment was measured with sea salt using a digital scale, and the salinity level was adjusted to 40 ppt using a salinity meter (ATAGO, Japan). To culture the cells of these microalgae, a TMRL culture medium was used and it was checked daily until it reached its highest growth i.e. 10^6 cells per ml. *Tetraselmis suecica* microalgae cells during the entire period, under the same biological conditions, temperature 25°C, pH 7 (by Hach-sension1 pH meter, USA), light intensity 2500 lux (PHYWE, Germany) and photoperiod of 18L/6D and continuous aeration using an aquarium pump, was cultured in laboratory conditions.

Ethanol extraction of *T. suecica* and condensation of ethanolic extract: At first, the desired microalgae precipitated by a centrifuge at 3500 rpm for 10 minutes. The sediment was obtained with a dry temperature of 50℃ and biomass was kept at a temperature of 4℃ until extraction. Then 50 grams of biomass was extracted in 400 ml of ethanol with a Soxhlet machine and the ethanolic extract was obtained. Afterward, this extract was centrifuged at 30°C with 3500 rpm for 10 minutes and the supernatant was removed as the final extract. A freeze dryer was used to condense the ethanolic extract; The OD of the ethanolic extract was 8.7 μg/ml.

Huh7 cell line culture : For proper cultivation and growth of the desired cell line in the flask, combinations of DMEM culture, penicillin G and streptomycin (Pen/Strep), and FBS were provided and the cells were transferred into the flask and then incubated at 37°C, 95% humidity, and 5% CO_2. After the initial passage, when the density of cells in the flask reached 70%, the passage repeated and the cells were counted.

Cell viability test (MTT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide): Briefly, 1×10^4 of Huh7 cells were seeded in triplicate on 96-well plates. After 24 hours of treatment, the medium was substituted with fresh medium containing different concentrations (0-1000 μg/ml) of ethanolic extract of *T. suecica* in complete DMEM culture.

Table 1. Specific primers of the studied genes.

Gene	Primers
AKT	Forward: 5'- GAAGGACGGGAGCAGGCGGC -3´ Revers: 5'- CCTCCTCCAGGCAGCCCCTT -3'
mTOR	Forward:5'- GCTTGATTTGGTTCCCAGGACAGT-3´ Revers: 5'- GTGCTGAGTTTGCTGTACCCATGT-3'
β-actin	Forward: 5'- TCCTCCTGAGCGCAAGTAC-3´ Revers: 5'- CCTGCTTGCTGATCCACATCT-3'

After 48 to 72 hours of treatment, media were carefully removed, the cells were washed with PBS, 4 mg of tetrazolium powder diluted in 2 ml of PBS, and 200 µl added to each well. After 3 hours, the supernatant solution was removed and then isopropanol was transferred into each well. 15 minutes later, the plate was removed from the incubator and read with an ELISA reader. the survival percentage of cancer cells was calculated using the formula of % cell viability = (Average absorbance of cells treated with extract / Average absorbance of negative control) × 100. To determine the dose of 50% lethality, the cell viability percentage from the samples was calculated, and using the Pharm-PCS statistical package software, the exact amount of IC50 was determined.

RNA extraction and cDNA production :48 and 72 hours after the effect of *T. suecica*, cells were lysed using RNX solution and incubated for 5 minutes at room temperature. RNA extraction was done according to the protocol of the RNA extraction kit of CinnaGen Company (CinnaGen Co, Iran) and cDNA was made from the extracted RNA according to the protocol of the Revert AidTM First Strand cDNA Synthesis Kit (Fermentas).

Examining the expression of mTOR, and AKT genes using Real-Time PCR: After receiving the sequence of mTOR, AKT, and β-actin genes from the NCBI database, we design specific primers by GeneRunner software (Table 1) .Then, Real-Time PCR was performed using the specific primers of the target genes, cDNA, and SYBR Green PCR Master Mix. The β-actin gene was used as an internal control and the relative change in expression of the mentioned genes was calculated by ΔΔCt method. All of the reactions were performed in duplicate.

Investigating apoptosis by flow cytometry : To investigate the induction of apoptosis in Huh7 cells treated with *T. suecica*, and compare it with the control group, the cells were stained with Annexin-FITC and propidium iodide (PI), according to the instructions of the Annexin V-FITC kit (Affymetrix, eBioscience, USA). We achieved analysis of the results using the division made by the device software into four regions from Q1 to Q4. Q1 are necrotic cells with Annexin-FITC (--) and PI (+), Q2 represents old apoptotic cells with Annexin-FITC (+) and PI (+), Q3 represents young apoptotic cells with Annexin-FITC (+) and P (--) and finally, Q4 represents healthy cells with both Annexin-FITC and PI negative.

Huh7 cells were treated with IC50 (a concentration that kills 50% of Huh7 cells) of *T. suecica* ethanolic extract, for 24 hours. Huh7 cancer cells were treated with different concentrations of 0, 125, 250, 500, and 1000 µg/ml of *T. suecica* extract. The cells then were washed with phosphate-buffered saline (PBS), and 200 µl of binding buffer was added to the sediment resulting from the centrifugation of the cells. Then 5 µl of Annexin V dye was added and incubated for 10 minutes at room temperature. The cells were washed with the binding solution, 10 µl of PI dye was added to the cells, and the analysis was done by flow cytometry device and Flow Jo software (ver. 10).

Statistical analysis: The data analysis was done using SPSS software (ver. 16). One-way ANOVA and Tukey's HSD post hoc test were used to investigate the difference in target gene expression between control and treatment samples. Data are presented as mean ± standard deviation (SD) and $P<0.05$ was considered significant.

Results

Effect of *T. suecica* extracts on survival of Huh7 cells :After 48 and 72 hours, cell viability was measured by MTT assay. In 48 h treatment, in

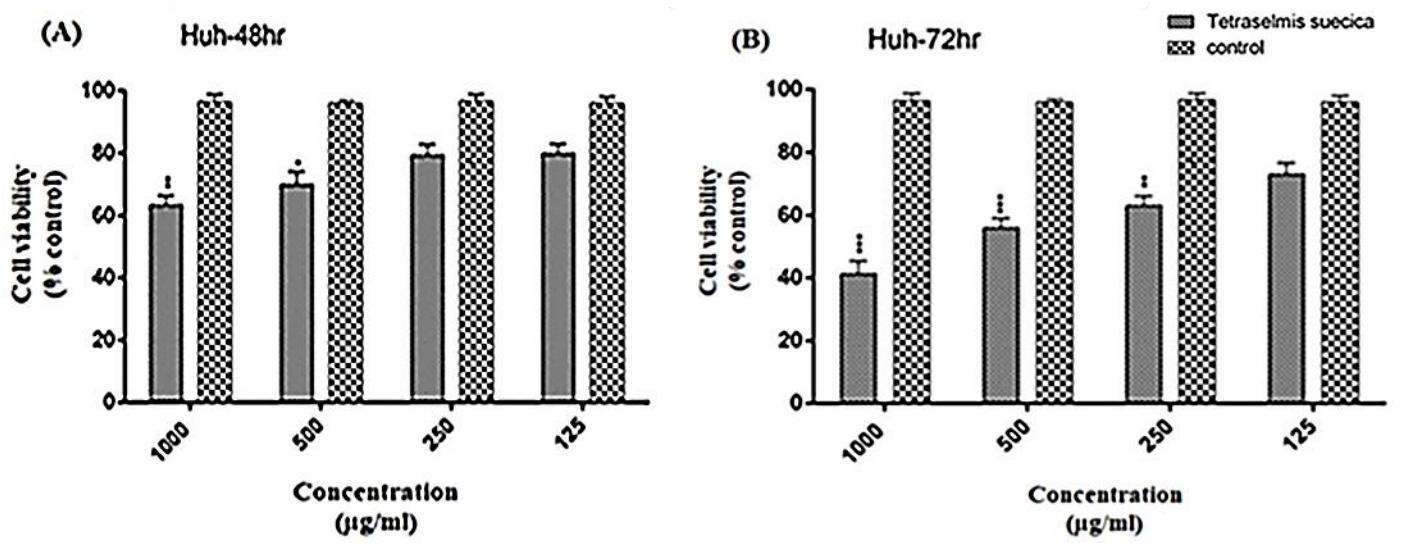

Figure 1. (A) The 48-hour effect of *Tetraselmis suecica* extracts on the vital activity of Huh7 cancer cells. Concentrations of 500 and 1000 µg/ml of *T. suecica* extract led to a significant reduction in the vital activity of the cells, and (B) The 72-hour effect of *T. suecica* extracts on the vital activity of Huh7 cancer cells. Concentrations of 250, 500, and 1000 µg/ml of *T. suecica* extract significantly reduced cell viability activity (**P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

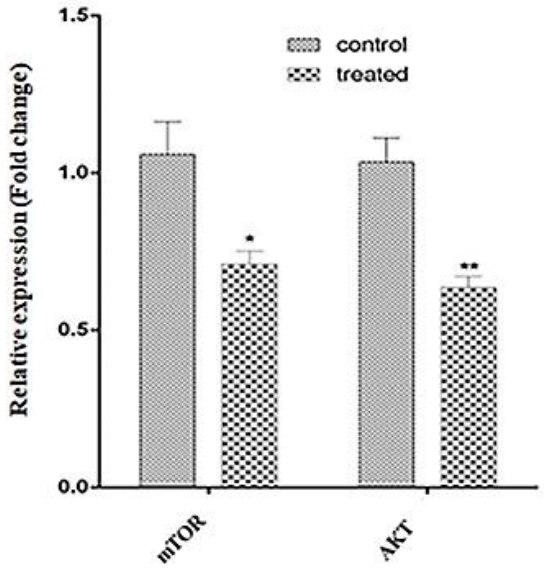

Figure 2. Analysis of the expression of mTOR, and AKT genes in Huh7 cell line treated with *Tetraselmis suecica* microalgae compared to the control group in RT-PCR reaction. *Tetraselmis suecica* extract led to a significant decrease in the expression of mTOR, and AKT genes compared to the control group (**P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

concentrations of 500 and 1000 µg/ml, there was a significant decrease in cell viability compared to the control group (*P*<0.05 and *P*<0.01, respectively) (Fig. 1A). In 72 h treatment, a significant reduction was observed in cell viability at concentrations of 250, 500, and 1000 µg/ml of *T. suecica* extract compared to the control group (*P*<0.01, *P*<0.001 and *P*<0.001, respectively) (Fig. 1B). Based on MTT test results, the amount of IC50 of *T. suecica* microalgae extract at 48 h and 72 hours was 1289 and 786 µg/ml, respectively.

Effect of *T. suecica* microalgae extract on mTOR and AKT gene expression: After synthesizing cDNA from RNA samples extracted from the culture of Huh7 cells, an RT-PCR test was performed by specific primers of mTOR, AKT, and β-actin genes in two repetitions. The expression of AKT and mTOR genes in Huh7 cells treated with *T. suecica* microalgae extract significantly decreased compared to the control group (*P*<0.01 and *P*<0.05, respectively) (Fig. 2). Therefore, it can be concluded that *T. suecica* extract can inhibit the growth pathway of AKT/mTOR by reducing the expression of these genes.

Effect of *T. suecica* microalgae extracts on cell death of Huh7 cells: The percentage of apoptotic and necrotic cells was evaluated by flow cytometry. In this test, cells were stained with Annexin, FITC, and PI. In the control group, most of the cells survived. However, comparing the 48-hour and 72-hour treatment of cells with *T. suecica* extract, in the group treated for 72 hours, fewer cells survived than in the 48-hour treatment, and more cells underwent apoptosis (*P*<0.001). Therefore, it can be concluded

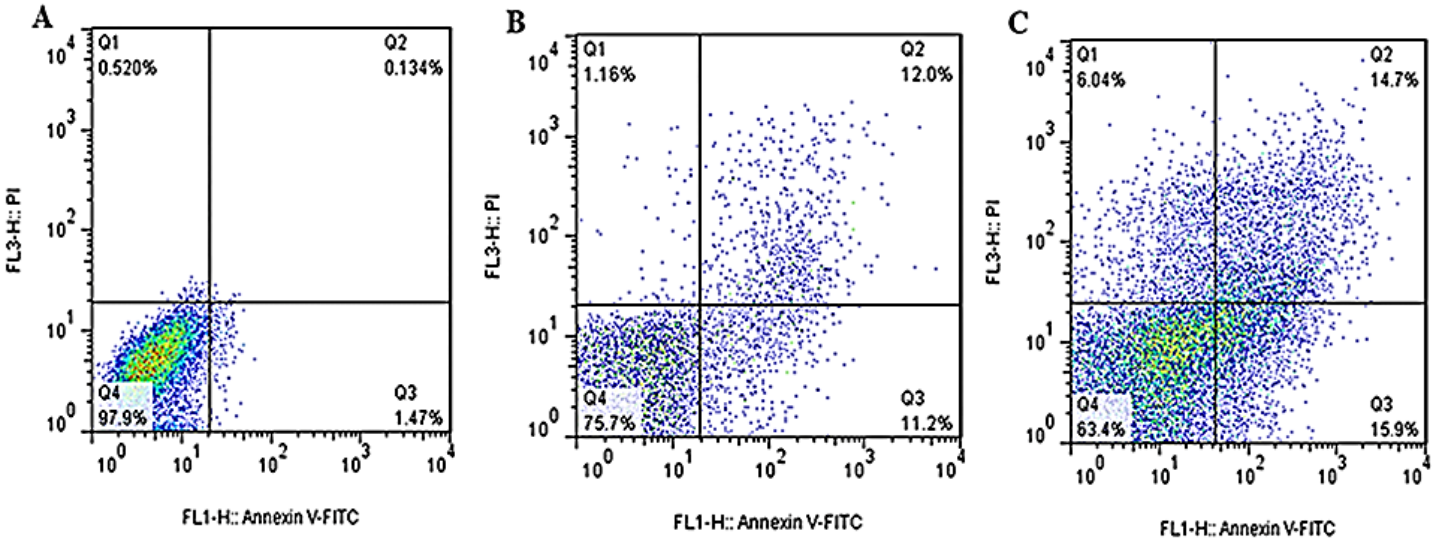

Figure 3. Dot plot view of the induction of apoptosis in Huh7 cells by *Tetraselmis suecica* microalgae extract. (A) In the control group, more than 96% of cells survived in this group, and the percentage of necrotic cells is very small and negligible, (B) In the 48 hours-treated groups, 75.7% of the cells survived and a lower percentage than in the 72-hour treatment group were apoptotic, and (C) In the 72-hours-treated group, 63.4% of the cells survived and a higher percentage underwent apoptosis than the previous two groups. Q1 indicates necrotic cells, Q2 indicates cells with late apoptosis, Q3 indicates cells with early apoptosis, and finally, Q4 indicates live cells.

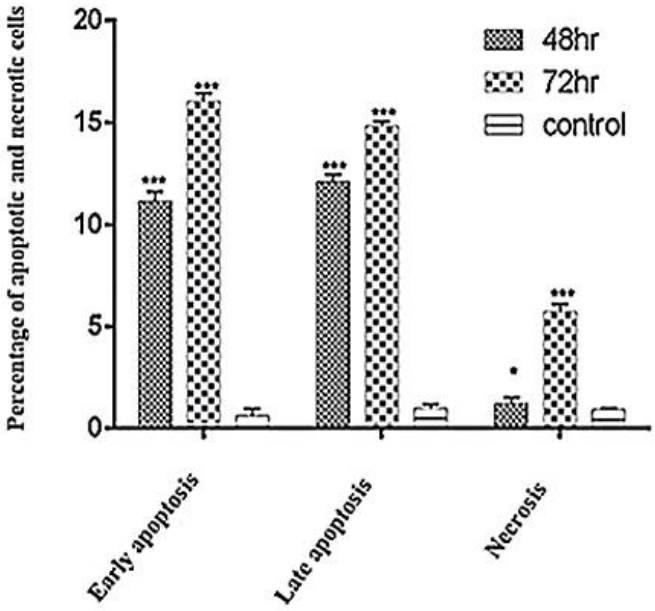

Figure 4. Analysis of the rate of cell death (early apoptosis, late apoptosis, and necrosis) in Huh7 cells treated with *Tetraselmis suecica* microalgae extract in 48- and 72-hour cultures compared to the control group. The results show an increase in the rate of apoptosis due to the treatment of Huh7 cells with *T. Suecica* microalgae extract. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, **** $P \leq 0.0001$).

that the 72-hour culture with *T. suecica* microalgae extract has a better effect on the death of Huh7 cancer cells (Figs. 3, 4).

Discussions

The PI3K/AKT/mTOR signal transduction pathway is abnormally activated in many tumorigenic processes and plays a key role in tumorigenesis and tumor progression. In addition, the PI3K/AKT/mTOR pathway is involved in the regulation of cancer cell survival, proliferation, invasion, and migration. For this reason, inhibition of this pathway has become an attractive research topic in cancer treatment (Jafari et al., 2019; Li et al., 2019; Xu et al., 2020; Galicia-Moreno et al., 2021).

Extensive studies have focused on the biological actions of phytochemicals derived from plants, but very few have been on phytochemicals derived from microalgae (Cha et al., 2008). Phytochemicals derived from microalgae have more biological activities than those of terrestrial origin (plant chemicals) (Holst and Williamson, 2008; Prabakaran et al., 2018). Due to this advantage of microalgae, worldwide attention has been focused on the valuable therapeutic and medicinal properties of microalgae, and many studies are being conducted on the beneficial role of microalgae metabolites in the treatment of various human diseases. In this regard, several studies of cellular and molecular research have suggested the

natural and strong anti-malignant activity of algae-derived compounds (Kumar et al., 2013; Talero et al., 2015; Abd El-Hack et al., 2019). The present study investigated the effect of the ethanolic extract of *T. suecica* microalgae isolated from the Persian Gulf on the expression level of AKT/mTOR genes in the Huh7 liver cancer cell line. The results of the present study showed that in the 48-hour treatment, at concentrations of 500 and 1000 µg/ml of the ethanolic extract of *T. suecica* microalgae, a significant decrease in cell viability. In addition, in the 72-hour treatment, a significant decrease in cell viability was observed in the concentrations of 250, 500, and 1000 µg/ml.

In a study conducted by Geovanna Parra-Riofrío et al. (2020) on exopolysaccharides obtained from *T. suecica* in autotrophic and heterotrophic cultures, this microalga had cytotoxic effects on HL-60, MCF-7, and NCI- H460 tumor cells. Also, in another study that was conducted on the combination of *T. suecica* extract and silver nanoparticles in 24, 48, and 72-hour cultures of MCF-7 and 4T1 cell lines, the simultaneous treatment with these two compounds led to cell cycle arrest and increased apoptosis in cancer cells (Hussein et al., 2020). Gardeva et al. (2012) also showed by studying the microalgae *Porphyridium cruentum* and *Dixoniella grisea* and the MCF-7 and HeLa cell lines that these microalgae have high cytotoxic and apoptogenic activities on cancer cells. The results of these studies are consistent with the findings of the present study. The results of the Real-Time PCR test in the current study showed that the expression of AKT and mTOR genes in Huh7 cells treated with *T. suecica* microalgae extract significantly reduced compared to the control group. Therefore, *T. suecica* extract can inhibit the AKT/mTOR growth pathway by reducing the expression of these genes. Some studies showed that compounds such as β-carotene by suppressing the PI3K-Akt signaling pathway, which is involved in the process of cell proliferation and cell death, lead to the induction of apoptosis and cell cycle arrest in MCF-7 cancer cells and other human breast adenocarcinoma cell lines (Gloria et al., 2014; Sowmya Shree et al., 2017).

By examining the amount of apoptosis in the present study, more than 96% of the cells survived in the control group. In the groups treated with *T. suecica* microalgae extract, the number of live cells decreased significantly and the percentage of apoptotic cells increased significantly. However, the percentage of necrotic cells was very small. In the comparison of the 48- and 72-hour treatment of cells with *T. suecica* extract, fewer cells survived than in the 48-hour treatment and more cells underwent apoptosis in the group treated for 72 hours. Therefore, the 72-hour treatment of *T. suecica* microalgae extract has a better result in the death of Huh7 cancer cells, which is consistent with previous studies (Hussein et al., 2020). In line with the current study, the use of *Phaeodactylum tricornutum* on the apoptosis rate of the HepG2 cell line showed that the rate of apoptotic cells increased significantly (Yang et al., 2019).

Conclusion

we studied the effect of ethanolic extract of *T. suecica* microalgae on liver cancer cell line Huh7 and the expression level of AKT/mTOR genes in this cell line for the first time. The extract of *T. suecica* led to a decrease in the percentage of survival, an increase in apoptosis, and a decrease in the expression of AKT/mTOR genes in the Huh7 cancer cell line. In addition, *T. suecica* can probably provide good benefits as a valuable natural material for the pharmaceutical industry, especially anticancer drugs.

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