Int. J. Aquat. Biol. (2024) 12(5): 495-502
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

The effect of the ethanolic extract of *Tetraselmis suecica* microalgae isolated from the Persian Gulf on the expression of *BAX/BCL-2* genes in the Hela cervical cancer cell line

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Abstract: Marine organisms produce a variety of compounds with medicinal activities, including anticancer effects. This study aimed to investigate the effect of the ethanolic extract of *Tetraselmis suecica* algae isolated from the Persian Gulf on the expression level of *BAX/BCL-2* genes in the Hela cervical cancer cell line. The ethanolic extract was obtained from T. *suecica*, and then, using the IC50 formula, the best extract concentration was obtained. MTT, Annexin V-FITC, and Real-time PCR tests were performed to investigate the effect of *T. suecica* microalgae extract on cytotoxicity, apoptosis, and expression of *BAX/BCL-2* genes. *Tetraselmis suecica* extract significantly decreased the survival rate, increased apoptosis, increased the expression of the pro-apoptotic *BAX* gene, and reduced the expression of the anti-apoptotic *BCL-2* gene in the Hela cell line. The results showed that the ethanolic extract of *T. suecica* microalgae may be helpful in the management of cervical cancer.

Article history:
Received 7 September 2024
Accepted 15 October 2024
Available online 25 October 2024

Keywords:
BAX-BCL2
Microalgae
Cervix cancer
Hela cell line

Introduction

Cancer is the second leading cause of death worldwide. Overall, the prevalence of cancer is increasing (Siegel et al., 2013). In women, the highest prevalence of cancer is in the breast, lung, colon and rectum, uterus, and thyroid, respectively (Siegel et al., 2014). Cervical cancer is one of the main causes of cancer deaths among women. It has been established that persistent infection with the high-risk human papillomavirus (HPV) is an essential cause of cervical cancer (Permatasari et al., 2022). There are several other risk factors for cervical cancer, including having sex at a young age (less than 16 years), multiple sexual partners, smoking, and low socioeconomic status (Roura et al., 2014; Ghebre et al., 2017).

Cells have an innate mechanism to control tissue homeostasis associated with apoptosis. Defects in apoptosis-inducing pathways can ultimately expand the neoplastic cell population (Pfeffer and Singh, 2018). This process is a vital component of healthy cell circulation and tissue homeostasis. It acts as one of the vital barriers against cancer development, and resistance to cell death is one of the symptoms of cancer. Apoptosis is a programmed cell death process that includes two main pathways: the extrinsic and intrinsic pathways. External stimuli trigger the extrinsic pathway, specifically involving death receptors (DRs). However, in the intrinsic pathway, when *Bax*/Bak enters the mitochondrial membrane, the released cytochrome c combines with Apaf-1 and procaspase-9 and produces the apoptosome, followed by the activation of the caspase 3 apoptotic cascades. In this pathway, *Bcl-2* is an anti-apoptotic protein that prevents apoptosis.

Early attention to anti-apoptotic proteins in cervical cancer progression focused on *Bcl-2*, because it was the best-known member of the *Bcl-2* family of proteins. *BCL-2* expression in different grades of cervical intraepithelial neoplasia showed that *BCL-2* expression increases with increasing grade of CIN (cervical intraepithelial neoplasia – there are three CIN grades, namely CIN 1, CIN 2, and CIN 3) (Jan, 2019). These studies showed that advanced-stage tumors show higher expression of *BCL-2* compared to

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DOI: https://doi.org/10.22034/ijab.v12i5.2384

early-stage tumors (Shukla et al., 2014). In addition, *BCL-2* expression was increased in cervical cancer tissues compared to the normal cervix, chronic cervicitis, and CIN (Zhou and Wang, 2015), i.e., inactivation of pro-apoptotic proteins (*Bax* and Bak) is a crucial feature of carcinogenesis (Jan, 2019). In cancer cells, the *Bcl-2* protein is often overexpressed to inhibit *Bax* (Brahmbhatt et al., 2015), and *Bax* pro-apoptotic protein is a new therapeutic strategy to overcome apoptosis resistance mechanisms in a wide range of tumors (Lopez et al., 2022).

Today, three surgical methods, radiotherapy and chemotherapy or their combination, are used in cancer treatment (WHO, 2014). The failure rate of chemotherapy in solid tumors has been reported to be 90% in the last six decades (Maeda and Khatami, 2018). Also, toxicity for normal cells, adverse side effects, and drug resistance are the main obstacles to using chemotherapy (Sak, 2012). Studies to find new therapeutic agents are ongoing (Hadisaputri et al., 2021; Raju and George, 2023; Ramnath et al., 2023; Taştan et al., 2024; Behinska et al., 2024). Marine organisms, such as bacteria, fungi, actinobacteria, and seaweed, have been used to treat cancer (Lakmal et al., 2014; Drugs et al., 2020).

Tetraselmis suecica microalgae is a marine green microalga belonging to the Chlorophyceae class, which is widely used in aquaculture to feed mollusks and crustacean larvae and as a probiotic in fish. *Tetraselmis suecica* is rich in vitamin E, carotenoids, chlorophyll, and tocopherols, and scientists suggest it as a food supplement in human and animal diets. This extract has a strong antioxidant and cell repair activity in a human lung cancer cell line (A549), a laboratory model often used to study antioxidant effects (Sansone et al., 2017). The carotenoids, retinol, vitamin D, K, and polyphenols in *T. suecica* have been studied for their anti-inflammatory, antitumor, and anti-cancer activities, and their anti-cancer potential has been confirmed against neuroblastoma, non-Hodgkin's lymphoma, prostate, breast, liver, pancreas, colorectal and gastric cancer (Ávila-Fritz et al., 2011; Goiris et al., 2014; Román et al., 2021; Ferdous and Yusof, 2021; Sharma et al., 2023). Despite all these studies, no research has been conducted on the effect of *T. suecica* algae extract on gene expression, affecting the apoptosis process in the Hela cell line and on cervical cancer treatment. Therefore, this study aimed to investigate the effects of this microalgae ethanol extract on *BAX/BCL-2* gene expression in the Hela cervical cancer cell line.

Materials and Methods

***Tetraselmis suecica* microalgae culture:** A sterile stock of microalgae *T. suecica* was obtained from Shiraz Provincial Science and Technology Park. TMRL medium with a salinity of 40 ppm is used for algae cultivation. The inoculation was done in a completely sterile environment with a proportion of 5% algal stock and 90% culture medium at a constant temperature (25°C) and pH=7. The photoperiod was set to 18 h of light (irradiance 40-µmol photons m^{-2} s^{-1}) and 6 h of darkness (18:6). Microalgae breeding tanks were kept under stirring with a constant air bubble system to avoid sedimentation of microalgae and achieve a homogeneous distribution of nutrients and irradiance in each cell and checked daily until it reached its highest growth, 10^6 cells/ml.

Ethanol extraction of *T. suecica*: The cultured microalgae was centrifuged at 3500 rpm for 10 minutes, and the sediment dried at 50°C for 24 h. The obtained biomass was kept at 4°C until extraction. A 12.5% ethanol solution (Merck, Germany) was prepared from the mentioned biomass and the ethanol extract, extracted with a Soxhlet apparatus. This extract was centrifuged again at 30°C with 3500 rpm for 10 min, and the supernatant was used in the next steps of the experiment. A freeze dryer was used to concentrate the ethanolic extract. The final concentration of the ethanolic extract was 8.7 mg/ml.

Huh7 cell line culture: HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen) containing 10% fetal bovine serum (Gibco) in a humidified atmosphere containing 5% CO2 at 37°C.

Cell viability assay (MTT): Cells were seeded into 96-well plates at a density of 10^4 cells/well (HeLa) in 100 µL of the medium. Then, the medium was

Table 1. Specific primers of the studied genes.

Gene	Primers
BAX	Forward: 5'- GAGCTGCAGAGGATGATTGC-3'
	Revers: 5'- AAGTTGCCGTCAGAAAACATG-3'
BCL-2	Forward: 5'- ATTGGGAAGTTTCAAATCAGC-3'
	Revers: 5'- CAGTCTACTTCCTCTGTGATGTTG-3'
β-actin	Forward: 5'- TCCTCCTGAGCGCAAGTAC-3'
	Revers: 5'- CCTGCTTGCTGATCCACATCT-3'

replaced with the fresh medium, and the cells were treated with serial concentrations of *T. suecica* alcoholic extract (0-1000 µg/ml) for 48 and 72 h. After the end of the treatment, cells were washed with PBS, and 100 µl of fresh medium containing 10 µl of MTT (5 mg/ml) was added to each well for 3 h at 37°C. After removing the medium, 100 µl of isopropanol was added. The optical density was measured at 570 nm using a microplate reader (Elx808, Biotek, USA). Each experiment was carried out in triplicate and repeated at least three times.

Apoptosis analysis: Apoptosis in Hela cells treated with *T. suecica* alcoholic extract was determined through flow cytometry measurement Annexin V-fluorescein isothiocyanate (FITC) kit (Affymetrix, eBioscience, USA). Hela cells were treated with a concentration of 1000 µg/ml for 48 and 72 hours. The cells were washed with phosphate-buffered saline. Then 200 microliters of binding buffer and five µl of Annexin V dye were added to the sediment resulting from centrifugation of the cells and incubated for 10 min in a dark place. Cells washed with a binding solution and 10 µl of propidium iodide (PI) dye were added immediately before the analysis by flow cytometry (MACSQuant10, Miltenyi Biotec Germany). The experiments were repeated three times.

Real Time-qPCR analysis: The expression level of *BAX* and *BCL2* genes was assessed using RT-qPCR. First, cDNA was synthesized by Revert AidTM First Strand cDNA Synthesis Kit (Fermentas) according to the abovementioned program. Then, Real Time-qPCR was carried out using AccuPower® 2X GreenStar™ qPCR Master Mix (Bioneer, Korea) in ABI StepOneTM instrument (Applied Biosystems) according to the following program: 95°C for 1 min, followed by 45 cycles at 95°C for 20 s, 52°C for 20 s and 72°C for 20 s. The applied primers are listed in Table 1. The β-actin gene was used as the internal control gene, and the relative expression of potential targets was measured by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis: The results were expressed as mean±SD. Statistical analyses were performed using Prism®6 software (GraphPad Software Inc., La Jolla, CA). Data analyzed using Student's t-test or one-way analysis of variance, followed by Tukey's post-test. Data displayed as mean±standard deviation (SD) and $P<0.05$ considered significant. Each experiment was repeated independently at least three times.

Results

Cell viability study (MTT Assay): Cytotoxic effect of ethanol extract from *T. suecica* against Hela cells tested using colorimetric method MTT assay. The bioactivity of *T. suecica* alcoholic extract was determined based on the concentration that induced 50% inhibition (IC50) on the growth of the treated cells as compared to the controls in triplicate. Hela cells were exposed to various concentrations of *T. suecica* alcoholic extract (0-1000 µg /mL) for 48 and 72 hours. The IC50 value was 1379.5 for 48-hour exposure. A further reduction in IC50 values was observed after treatment at 72 h (Fig. 1).

Annexin V/Propidium Iodide study: In the 48-hour analysis of flow cytometry results, 76.8% of cells survived, and the percentage of early, delayed apoptosis and necrosis was 11.2, 9.85, and 2.20%, respectively. In the 72-hour analysis of flow cytometry, these percentages were 61.7, 5.21, 25.4, and 7.68%, respectively. In the comparison, in the group treated for 72 hours, cell viability decreased (61.7 versus 76.8%), and apoptosis (30.61 versus

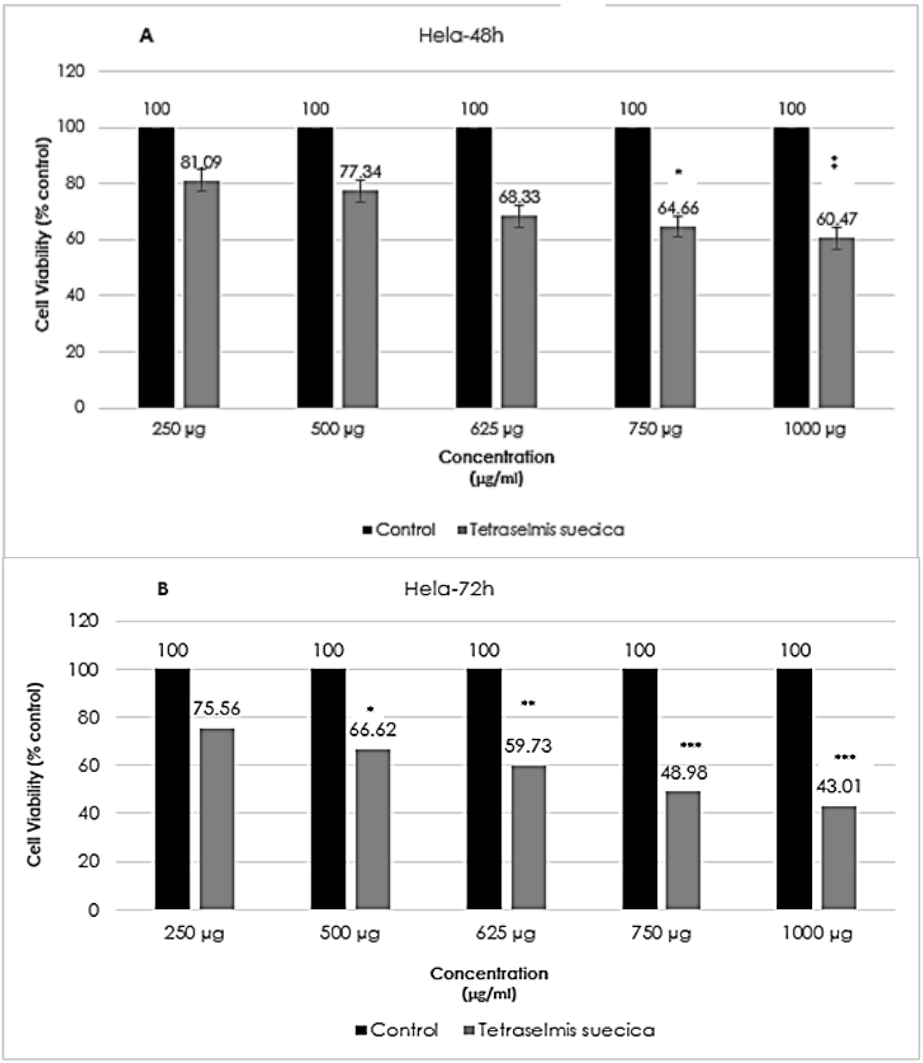

Figure 1. (A) The 48-hour effect of *Tetraselmis suecica* extract on the vital activity of Hela cancer cells. Concentrations of 750 and 1000 µg/ml of *T. suecica* extract led to a significant reduction in the vital activity of the cells, and (B) The 72-hour effect of *T. suecica* extract on the vital activity of Hela cancer cells. Concentrations of 500 to 1000 µg/ml of *T. suecica* extract significantly reduced cell viability activity (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$).

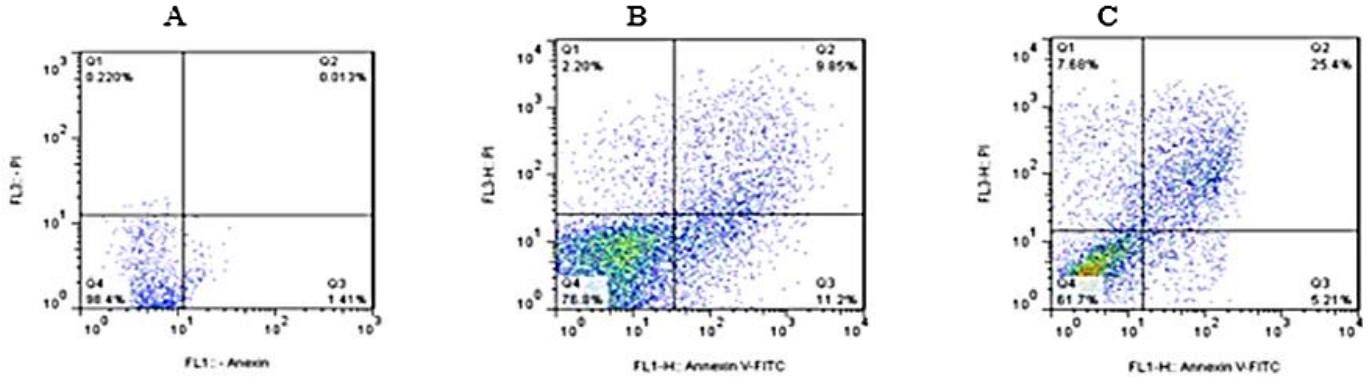

Figure 2. Dot plot view of the induction of apoptosis in Hela cells by *Tetraselmis suecica* microalgae extract. (A) In the control group, more than 98.4% of cells survived, and the percentage of necrotic cells is very small and negligible, (B) In the 48 hours-treated groups, 76.8% of the cells survived and a lower percentage than in the 72-hour treatment group were apoptotic, and (C) In the 72-hours-treated group, 61.7% 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.

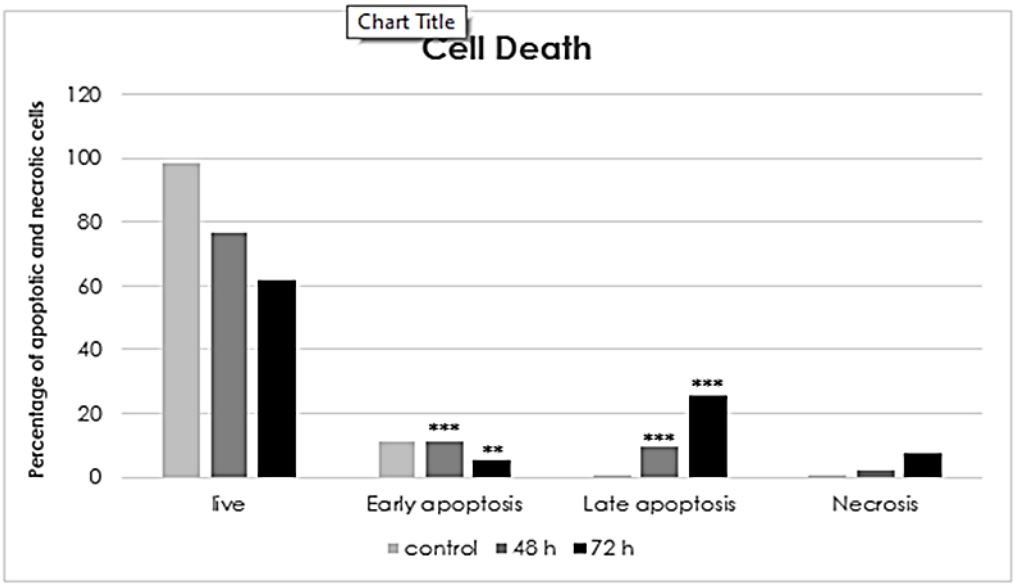

Figure 3. Analysis of the rate of cell death (early apoptosis, late apoptosis, and necrosis) in Hela cells treated with *Tetraselmis suecica* microalgae extract in 48- and 72-hour cultures compared to the control group. The results show an increase in the rate of apoptosis due to the treatment of Hela cells with *T. suecica* microalgae extract (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$).

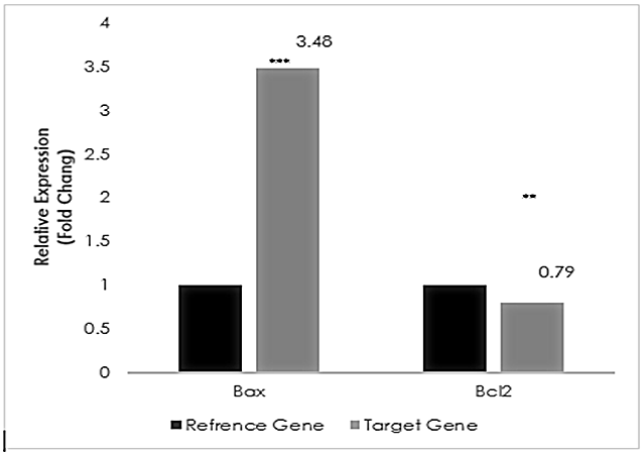

Figure 4. Analysis of the expression of *BAX*, and *BCL*-2 genes in Hela cell line treated with *Tetraselmis suecica* microalgae compared to the control group in RT-PCR reaction. *Tetraselmis suecica* extract led to a significant increase in the expression of BAX, and decrease of BCL-2 gene compared to the control group (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$).

21%), increased than 48-hour treatment (Figs. 2, 3). **Gene expression study:** The differential expressions of genes in Hela cells after treatment compared with the controls (untreated Hela cells) were expressed as fold change. As shown in Figure 4, the fold change of the antiapoptotic gene, *BCL2*, in Hela cells decreased while the apoptotic gene, BAX, increased in the control group at 24 hours (Fig. 4).

Discussions

In the 20th century, the use of secondary metabolites of bacterial and plant origin has received special attention worldwide. About 30% of commercial drugs used to treat diseases are derived from plants. The marine environment covers about 75% of the earth's surface, which accounts for about half of the world's biodiversity. Marine microalgae and macroalgae contain various new bioactive compounds with antimicrobial, anti-neoplastic, and antiviral properties. Macro and micro seaweeds have different chemicals to protect themselves against various adversities (Majumder et al., 2020). Algae extract has anticancer activity and promising therapeutic properties that can be developed to prevent side effects associated with chemotherapy drugs and radiation therapy in conventional cancer treatment (Hussein et al., 2020).

Based on the present study result of the MTT test, in the 48-hour treatment, at concentrations of 750 and 1000 µg/ml of *T. suecica*, there was a significant decrease in cell viability. Also, in the 72-hour treatment, a substantial reduction in cell viability was observed in the concentrations of 500, 625, 750, and 1000 µg/ml extract. Therefore, it can be concluded that the desired result can be achieved by increasing the treatment time, even at lower concentrations of

Tetraselmis algae extract. The vitality of the Huh7 cancer cell line in concentrations of 500 and 1000 µg/ml of *T. suecica* extract decreased significantly in previous work (Roshan Cheragh and Mabudi, 2023). In the same way, Riofrío et al. (2020), in investigating the antioxidant and cytotoxic effects of *T. suecica* polysaccharides, showed highly lethal effects on tumor cells, and they suggested that marine microalgae can be used as functional ingredients in foods or possible nutrients to reduce the possibility of tumor formation and development in the human body. Also, the new anti-cancer agent based on *T. suecica* microalgal extract and silver nanoparticles formulation showed strong cytotoxicity on MCF-7 and 4 T1 cancer cells (Hussein et al., 2020).

In cancer treatment, the appearance of apoptotic cells is a useful marker for better survival. Studies have shown that among the various genes involved in regulating cell apoptosis, *BCL-2* is particularly important because it is considered one of the last common pathways of apoptosis regulation. *BCL-2* is overexpressed in several tumors and can inhibit the natural apoptosis of cells, suppress the apoptosis induced by many antitumor drugs, and reduce their cytotoxicity. In contrast, *BAX* does not directly block apoptosis but inhibits the anti-apoptotic role of *Bcl-2* (Jiang et al., 2016). The results of the present study showed that the expression of the *BCL-2* gene in the cells treated with Tetraselmis algae extract had a significant decrease, while the expression of the *BAX* gene had a significant increase compared to the control group. Therefore, the anticancer effects of *Tetracelmis* algae on Hela cells can be related to the *Bcl-2/BAX* signaling pathway. In line with these results, Gupta et al. (2022) showed the upregulation of pro-apoptotic proteins (*Bax*) and the downregulation of anti-apoptotic proteins (*Bcl-2*) in Dalton's lymphoma (DL) cells treated with *Euglena tuba* extract through western blot technique. Murad et al. (2015) showed that red algae *Laurencia papillosa* induced apoptosis through apoptosis signaling pathway elements like caspase-3, caspase-9, p53, Bax, and *Bcl- 2*. In the study of the anticancer effects of zeaxanthin in algae by Sheng et al. (2020) on HT-29 cells and several human gastric cancer cells, apoptosis was associated with increased expression of *BAX* and decreased expression of *BCL-2*. In the study of Sowmya et al. (2017) on colon cancer cells, astaxanthin in algae increased the expression of *BAX* and decreased the expression of *BCL-2*. Also, in skin cancer cell lines treated with astaxanthin by Brotosudarmo et al. (2020), the activity of the *BCL-2* proto-oncogene was stopped.

Apoptosis is one of the most commonly studied pathways in cancer, which directly affects the growth and proliferation of cells. In the 48-hour study of the effects of Hela cell line treatment with *Tetracelmis* algae extract in the present study, 76.8% of the cells survived. The number of cells that underwent early and delayed apoptosis was 11.2 and 9.85%, respectively, and 2.20% underwent necrosis. These values were 61.7, 5.21, 25.4 and 7.68%, respectively, in the 72-hour Hela cells treatment results. The comparison of the 48-hour and 72-hour treatment of cells with *Tetracelmis* extract showed that fewer cells survived in the group treated for 72 hours than in the 48-hour treatment, and more cells underwent apoptosis. Therefore, the 72-hour effectiveness of *Tetraslamis* algae extract has a better result in the death of Hela cancer cells. In line with the present study, Hussein et al. (2020) revealed that the cells treated with *Tetracelmis* algae extract showed the characteristic features of apoptotic cells, including round shape, shrinking in size, separation from the surface of the monolayer of the wells, the number of cells less than the control, membrane bubble, and formation Apoptotic bodies in the form of round or oval masses of cytoplasm, much smaller than the original cells. The apoptotic effects of extracts of other types of algae have also been reported in other studies. Bernardini et al. (2018) showed that the acetic extract of *Padina pavonica* (EPP), a brown seaweed, has a strong anti-apoptotic effect on human osteosarcoma cells.

Conclusion

In the present study, the ethanolic extract of *T. susica* algae decreased the survival rate, increased apoptosis,

increased the expression of the pro-apoptotic *BAX* gene, and reduced the expression of the anti-apoptotic *BCL-2* gene in the Hela cancer cell line. We conclude that the extract of *T. susica* can be a potential candidate for treating cervical cancer by regulating the *Bax/Bcl-2* signaling pathway. With the limited pharmaceutical options and toxicity of this natural extract, this alga appears to be a strong option and deserves further research.

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