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# Dose-dependent cytotoxic and proliferative effects of *Microcystis aeruginosa* extract and its fractions on human endothelial cells

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## ABSTRACT

*Microcystis aeruginosa,* which spreads in five continents in the world and reported in drinking water resources in 257 countries, is a dangerous microalgae for human and animal health due to its toxins. The aim of current study was to evaluate the effects of *M. aeruginosa* extract and its chromatographically separated fractions on human endothelial cells. In this context, crude extract was prepared from *M. aeruginosa* cultured in BG-11 medium, and it was fractionated by an optimized HPLC method. Algae extract and its six fractions were then analyzed for their cytotoxic effects on ECV304 using MTT assay. The results revealed that algae extract inhibited ECV304 cells by around 72%, a higher percentage than all fractions. The most toxic fraction was the first fraction, which inhibited the cells by 55%. Other fractions, except the third one, were also toxic with 35-40% inhibition percentages. Third fraction and certain doses of some fractions showed proliferative activity on ECV304 cells. These results showed that the activities of the total extract and its fractions in promoting or inhibiting cell proliferation varied depending on not only the content but also the treatment dose.

**Keywords:** *Microcystis aeruginosa*, Human endothelial cells, ECV304, Cytotoxicity, Cell proliferation, Algae

## Introduction

Cyanobacteria are organisms that are also called blue-green algae because of their photosynthetic pigments and have a wide habitat from water habitats that can freeze temporarily to hot water sources (Pearson et al., 2010; Harke et al., 2016). Due to their ability to perform photosynthesis, they increase the ratio of nutrients and $O_2$ in the water environment. Since cyanobacteria do not have nucleus and organelle membranes, their genetic material and pigmentous substances are free in the cytosol. They have a cell wall containing a small amount of peptidoglycans and 80S ribosomal RNA, similar to the cell wall of Gram (-) bacteria (Paiva et al., 2017). As the most primitive photosynthetic organism, they are described as "bacteria" because they do not contain a nucleus membrane, and as "algae" because they able to do photosynthesis. Cyanobacteria can form single-celled or multi-celled colonies. They can reproduce by vegetative division or spores, and they produce a large number of toxins (cyanotoxins) (Bryant, 1994).

Due to the increase in worlds' population, especially safety and quality of drinking water resources have become very important in recent years worldwide. The entity of cyanobacteria in water, and identification of their toxic components have become primary research subjects, since these data must be achieved to avoid their toxic or fatal effects on human and all living organisms. Furthermore, toxic substances and their mechanisms of action must be fully elucidated to develop efficient strategies for the prevention or treatment of pathological processes arising from cyanobacterial contamination (Carmichael, 1994; Campos and Vasconcelos, 2010).

It has been determined that at least 46 cyanobacterial strains are toxic to vertebrates worldwide. The most common cyanobacteria species in fresh waters are *Microcystis, Anabaena, Oscillatoria, Planktothrix, Chroococcus* and *Nostoc*. They synthesize a stable hepatotoxin molecule called microcystin (Kurmayer, 2011).

Studies with *Microcystis aeruginosa*, a microalgae living in almost all fresh water sources in all over the world, have revealed that this species has higher toxicity than other algae species. This toxicity threatens the lives of all living beings, especially humans and animals (Karjalainen et al., 2007). The toxic components participate to the plant circulation system through the absorption by the plants during the irrigation, and accordingly take part to food chain by not only the use of contaminated water, but also the consumption of the plants irrigated with this water (Lawton et al., 1994; Pearson et al., 2010).

Many peptides with high hepatotoxic activity have been described in *M. aeruginosa*. While these toxic peptides are generally retained in the cell, they are also released from the cell due to cell lysis, or by active transport systems (Babica et al., 2006). Dietary toxic peptides are transported to the liver by organic anion transport proteins and inhibit protein phosphatase 1 and protein phosphatase 2A enzymes, resulting in an increase of intracellular phosphoproteins, and associated intrahepatic bleeding, cell necrosis and tumor development in the liver (Lawton et al., 1994; Bagu et al., 1997; Tonk et al., 2005; Welker and von Dohren, 2006; Pearson et al., 2010).

*M. aeruginosa* contamination that has been reported in water resources in different parts of the world possess a vital threat to all living things in the region, especially humans, who come into contact with these waters. Reviews reporting the studies on the geographic distribution, toxins and genome of *M. aeruginosa* (Pearson et al., 2010; Harke et al., 2016), exert the seriousness of the subject, and draw attention to the importance of toxicity studies on *M. aeruginosa*. Those studies often appear to be a reference to the major toxin, microcystin (-leucine-arginine or -arginine-arginine forms) in total algae extract (Chong et al., 2000; Alverca et al., 2009; Dias et al., 2009; Piyathilaka, et al., 2015; Ramos et al., 2015; Herrera et al., 2018; Gutiérrez-Praena et al., 2019). However reports on the other toxins of *M. aeruginosa* are very limited in the literature (Kotak et al., 1995, Welker and von Dohren, 2006, Karjalainen et al., 2007, Yu et al., 2015, Entfellner et al., 2017).

**Table 1.** Some cellular peptides and proteins of *M. aeruginosa*.

| | Peptide/protein | Molecular weight | Reference |
|---|---|---|---|
| TOXIC PEPTIDES | Microcystin -LR | 995 Da | Chen et al., 2018 |
| | Microcystin -RR | 1038 Da | Zhong et al., 2017 |
| | Microcystin -YR | 1045 Da | Moreno et al., 2004 |
| | Microcystin -LA | 910 Da | Ramanan et al., 2000 |
| | Microcystin -LY | 1002 Da | Birungi and Li, 2009 |
| | Microcystin -LW | 1025 Da | Faassen and Lürling, 2013 |
| | Microcystin -LF | 986 Da | Faassen and Lürling, 2013 |
| | Cyanopeptolin | 957 Da | Kotak et al., 1995 |
| | Anabaenopeptide | 836 Da | Kotak et al., 1995 |
| OTHER PEPTIDES/PROTEINS | Microcystin synthetase | 116-205-402 kDa | Tillett et al., 2000 |
| | Phosphoribulokinase | 38.036 kDa | Wei et al., 2016 |
| | Acetyl-Coa acetyltransferase family protein | 41.396 kDa | Wei et al., 2016 |
| | Phosphoglycerate kinase | 42.811 kDa | Wei et al., 2016 |
| | Fructose-bisphosphate aldolase, class II, Calvin Cycle subtype | 39.156 kDa | Wei et al., 2016 |
| | Glyceraldehyde-3-phosphate dehydrogenase | 37.128 kDa | Wei et al., 2016 |
| | 60 kDa chaperonin | 57.701 kDa | Wei et al., 2016 |
| | ATP synthase subunit alpha | 54.116 kDa | Wei et al., 2016 |
| | ThiF family protein | 42.979 kDa | Wei et al., 2016 |
| | Oligo-ulvans | 50-60 kDa | Kim and Chojnacka, 2015 |
| | Akt substrate | 160 kDa | Kim and Chojnacka, 2015 |
| | Phlorogluquinol | 162-650 kDa | Kim and Chojnacka, 2015 |
| | Ulvan | 189-8200 kDa | Kim and Chojnacka, 2015 |

*M. aeruginosa* contains several peptides and proteins, including toxic microcystins (Table 1). Among the microcystin derivatives, microcystin leucine-arginine (MC-LR) is the metabolite with the highest toxicity (Karan et al., 2015). For this reason, cytotoxicity studies in the literature have focused on this toxin. Studies on various cancer cells, such as kidney cancer, colon cancer hepatocellular carcinoma, breast cancer have shown that cell viability decreases depending on the MC-LR concentration (Dias et al., 2009; Ramos et al., 2015; Abdel-Rahman et al., 2020; Bittner et al., 2021). In addition to its cytotoxic properties, MC-LR is known to increase the effect of some inhibitors that block DNA repair, and intracellular reactive oxygen species. Besides, it damages the enzymes responsible for protecting DNA from oxidative stress, and causes DNA breaks (Zegura et al., 2003).

Apart from *M. aeruginosa*, the toxins belonging to other cyanobacteria also have various effects on endothelial cells. It is reported that cylindrospermopsin (CYN), produced by the *Anabaena* species, has a cytotoxic effect depending on the treatment dose, and 48-hour exposure, especially with 40 μg $mL^{-1}$ CYN, reduces endothelial cell viability by 95% (Gutiérrez-Praena et al., 2012). In addition, another study in the literature shows that this cyanotoxin initiates apoptosis in endothelial cells (Wang et al., 2020). Despite its cytotoxic effects, it is reported that polysaccharides isolated from another cyanobacteria, *Nostoc* species, found in freshwaters, induce endothelial cell proliferation at some concentrations and may be used as a natural product for vascular repair in the future. (Foroh and Mahrouz, 2016).

In the present study, effects of crude algae extract and its chromatographic fractions on the cell viability of human endothelial cells were investigated in a dose-dependent manner, as human may be exposed to them by swallowing contaminated water or eating seafood contaminated with toxins. Main purpose was to make a prediction the effects of different constituents of *M. aeruginosa* on the veins, and on other tissues containing endothelial cells in general when they are taken into the body and transported to the organs/tissues through the veins.

## Material and Methods

### *Preparation of Algae Culture, Algal Extraction and Measurement of Protein Concentration of Algal Lysate*

Starting culture of *M. aeruginosa* (PCC7806) was obtained from Professor Reyhan Akçaalan Albay (Istanbul University, Faculty of Aquatic Sciences) as a gift, and cultivated in BG-11 medium in a shaking incubator under the conditions of 28°C, 110 rpm and continuous light (Stanier et al., 1971) for 28 days as determined by UTEX. The culture was centrifuged at 3901 x*g* for 50 min, the pellet was dried and suspended in PBS. The cell suspension was homogenized in a homogenizer at 5000 x*g* for 1 min, repeated 8 times. Cell disruption was confirmed by microscopic observations.

The protein concentration of the algae extract was determined by the SMART™ BCA Protein Assay Kit (iNtRON Biotechnology), according to manufacturer's instructions.

### *HPLC Analysis*

Chromatographic fractionation of algae extract was carried out according to the method described by Lawton et al. (1994) previously, with some modifications.

Shimadzu Prominence UFLC System (Shimadzu Corporation, Kyoto, Japan) equipped with LC-20AD pumps, SPD-20A photodiode-array (PDA) detector, DGU-20A degasser, Inertsil® ODS-3 column (5 μm, 4.6 x 250 mm). The signal was recorded using Shimadzu LC Solution Software. The column temperature was maintained at 40°C and injection volume was 50 μL. The flow rate of the mobile phase was kept as 1 mL/min. Mobile phase A was composed of ultrapure water and 10% acetonitrile mixture containing 0.05% (v/v) trifluoroacetic acid (TFA) while mobile phase B was composed of acetonitrile containing 0.05% (v/v) TFA. The gradient conditions were as follows: 0-10 min (20→25% B), 10-40 min (25→80% B), 40-44 min (80→100% B), 44-46 min (100→20% B), 46-50 min (20% B). The chromatograms were monitored at 240 nm.

The algae extract was diluted with PBS to a protein concentration of 1 mg/mL before HPLC. Fractionation was maintained until no peak was observed, and repeated 13 times. Six fractions were collected separately by this process. Each fraction was lyophilized using a freeze drier (CHRIST/ALPHA 1-4 LD Plus). Lyophilized samples were dissolved in 100 μL of PBS and kept at -80°C until the cytotoxicity assays.

### *Mammalian Cell Culture and Cytotoxicity Assay*

Cytotoxic activity of different concentrations of the algae extract and its fractions were assessed on human umbilical vein endothelial cell line (ECV304). DMEM/High Glucose medium (Gibco, 41966) supplemented with 10% fetal bovine serum (HyClone, SH3007003HI), 1% penicillin-streptomycin and 1% L-glutamine was used as growth medium. Cells were cultivated in 25 $cm^2$ polystyrene cell culture flasks, and incubated in a humidified atmosphere containing 5% $CO_2$ at 37°C. Adhesive ECV304 cells were detached by 0.5% trypsin–EDTA solution (HyClone™, SH30236.01), washed once with PBS and resuspended in DMEM at density of $1\times10^5$

cells/mL (Atasever-Arslan et al., 2016). The cytotoxic activity of *M. aeruginosa* extract and HPLC fractions on ECV304 cells was measured by using MTT (Sigma, M-5655) assay, as previously described (Pırıldar et al., 2010; Svobodova et al., 2012). The cell culture was incubated 24 h before each treatment.

Stock solution of the microalgae extract was prepared in PBS at a protein concentration of 13.06 mg/mL. Serial dilutions of the stock solution (6.53, 3.27, 1.63, 0.82, 0.41, 0.205, 0.102, 0.05 and 0.025 mg/mL) were prepared in PBS. Six fractions (No.1-6) obtained from HPLC having a dry weight of 9.6, 4.8, 4.9, 6.3, 6.2 and 6.1 mg, respectively, were diluted with PBS as 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 ratios.

On the mid-log phase of ECV304 cell growth (24$^{th}$ hour of the culture), 10 µL of each sample (algae extract, 6 HPLC fractions or their serial dilutions) was dispensed into 96-well round-bottom plates containing ECV304 cells. As a negative control, only 10 µL of sterile phosphate buffer saline (PBS) was used instead of algal extract and HPLC fractions, and cell viability for this sample was regarded as 100%.

After 48 h of incubation with samples, 10 µL MTT solution (5 mg/mL) in PBS was added to each well and the plates were incubated in a $CO_2$ incubator at 37°C for 3 h. Subsequently, 80 µL of supernatant was removed from each well and 100 µL of freshly prepared isopropanol-DMSO solution [1:1 (v/v)] was added. The microplates were stored at room temperature in the dark for 45 min, in order to dissolve the formazan crystals formed by reduction of MTT in living cells. Optical densities of the samples were measured at 570 nm wavelength in microplate reader (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer). The cell viability was calculated as percentage of viable cells in experimental group (exp.) versus untreated (negative) control group (cont.) using the following formula, where A=absorbance of related groups:

Cell viability (%) = [$A_{exp.}/A_{cont.}$] x 100

Two independent experiments with at least three repeats were carried out, and the results were evaluated using GraphPad Prism® 7 program. One-way ANOVA with Dunnett's test was used in order to determine the differences between the groups. The limit of significance was accepted as $P<0.05$. Nonlinear regression analysis was also performed for calculating the half-maximal inhibitory concentration ($IC_{50}$ in mg/mL) of algae extract.

## Results and Discussion

Apart from the studies in the literature, here we separated *M. aeruginosa* total extract into 6 fractions by optimizing a RP-HPLC method. The effect of total extract and each fraction on the growth of endothelial cells (ECV304) was investigated. Different concentrations of total extract and fractions introduced to cells on mid-log phase for 48 hours, and their dose-dependent effects on cell viability were statistically evaluated.

### *HPLC Analysis of Cell Extract*

According to the appearance of the peaks on the chromatogram, six fractions were collected, consisting of Fr.1-6 (Figure 1).

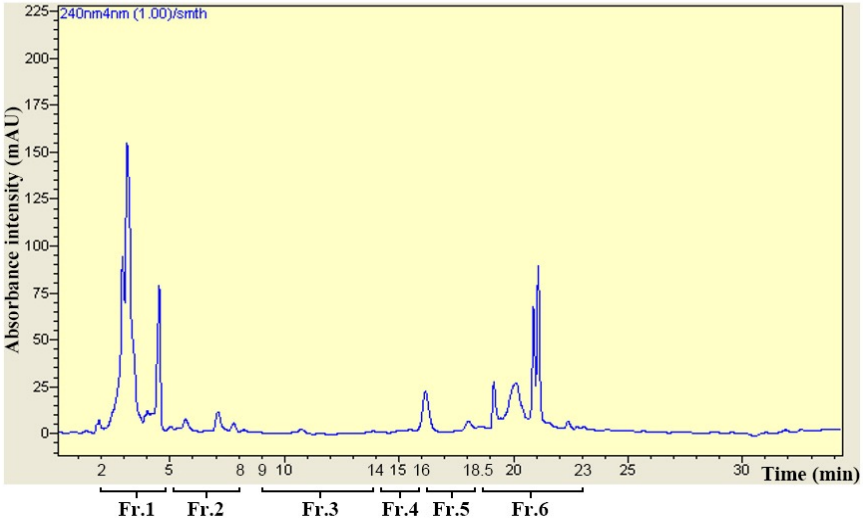



**Figure 1.** HPLC chromatogram of *M. aeruginosa* extract.

Dry weights of Fr.1, Fr.2, Fr.3, Fr.4, Fr.5 and Fr.6 collected at the end of 13 run were 9.6, 4.8, 4.9, 6.3, 6.2 and 6.1 mg, respectively, following the lyophilization.

### *Effects of Algae Extract and HPLC Fractions on ECV304 Cells*

One-way ANOVA test was used to analyze the consistency between the data obtained from MTT tests to determine the effects of algae extract and its fractions on ECV304 cell viability.

The algae extract inhibited ECV304 cells in a dose-dependent manner. The highest inhibition percentage (72±12.99%) was detected in stock solution of the algae extract containing 13.06 milligram protein per milliliter (Figure 2). There was a correlation between the cytotoxic effect and protein concentration, up to 32 fold dilution (0.41 mg/mL), and statistically significant cytotoxic avtivity was detected in the samples containing 0.41-13.06 mg protein per milliliter compared to control (***P<0.001). However, dilutions with a protein concentration less than 0.41 mg/mL had no effect on cell viability (P>0.05). The $IC_{50}$ value of algae extract on ECV304 cells was estimated as 2.737 mg/mL from nonlinear regression analysis.

The effects of different concentrations of the fractions (Fr.1-6) on ECV304 cells were presented comparatively in Figure 3. The cell viability was 55±5.04% when the cells were treated with the highest Fr.1 concentration obtained (96 µg/µL) (Figure 3). Very low inhibition percentages were detected for two dilutions of Fr.1 (20±4.98% for 48 µg/µL and 10±5.04% for 24 µg/µL) (***P<0.001). Neither cytotoxic nor proliferative activity was observed in other dilutions (P>0.05). This result showed that Fr.1 contains only moderately toxic substances (Figure 3a).

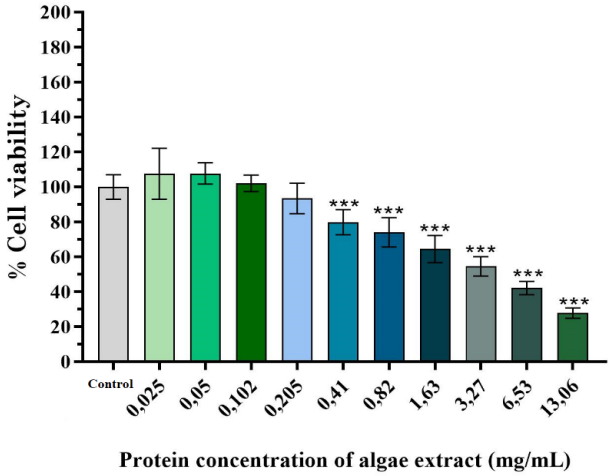
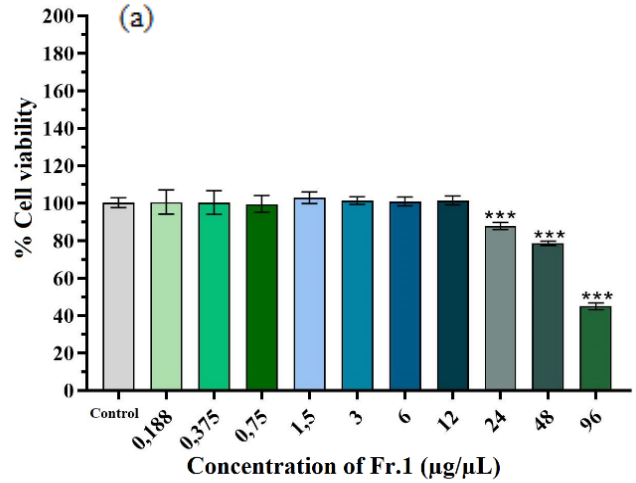
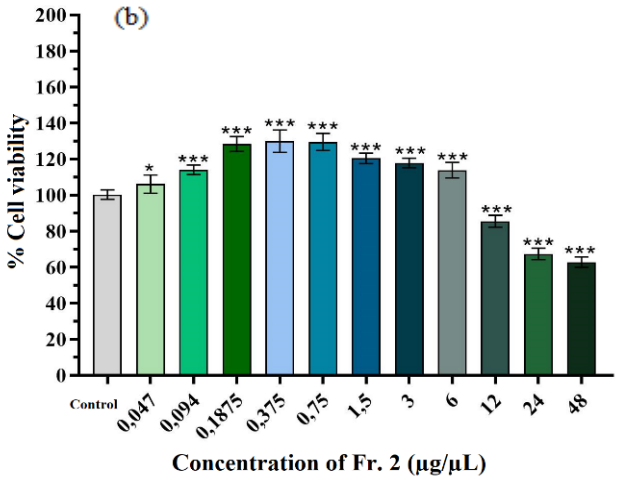



**Figure 2.** The effect of algae extract on the viability of ECV304 cells (***P<0.001, vertical bars show standard deviations)



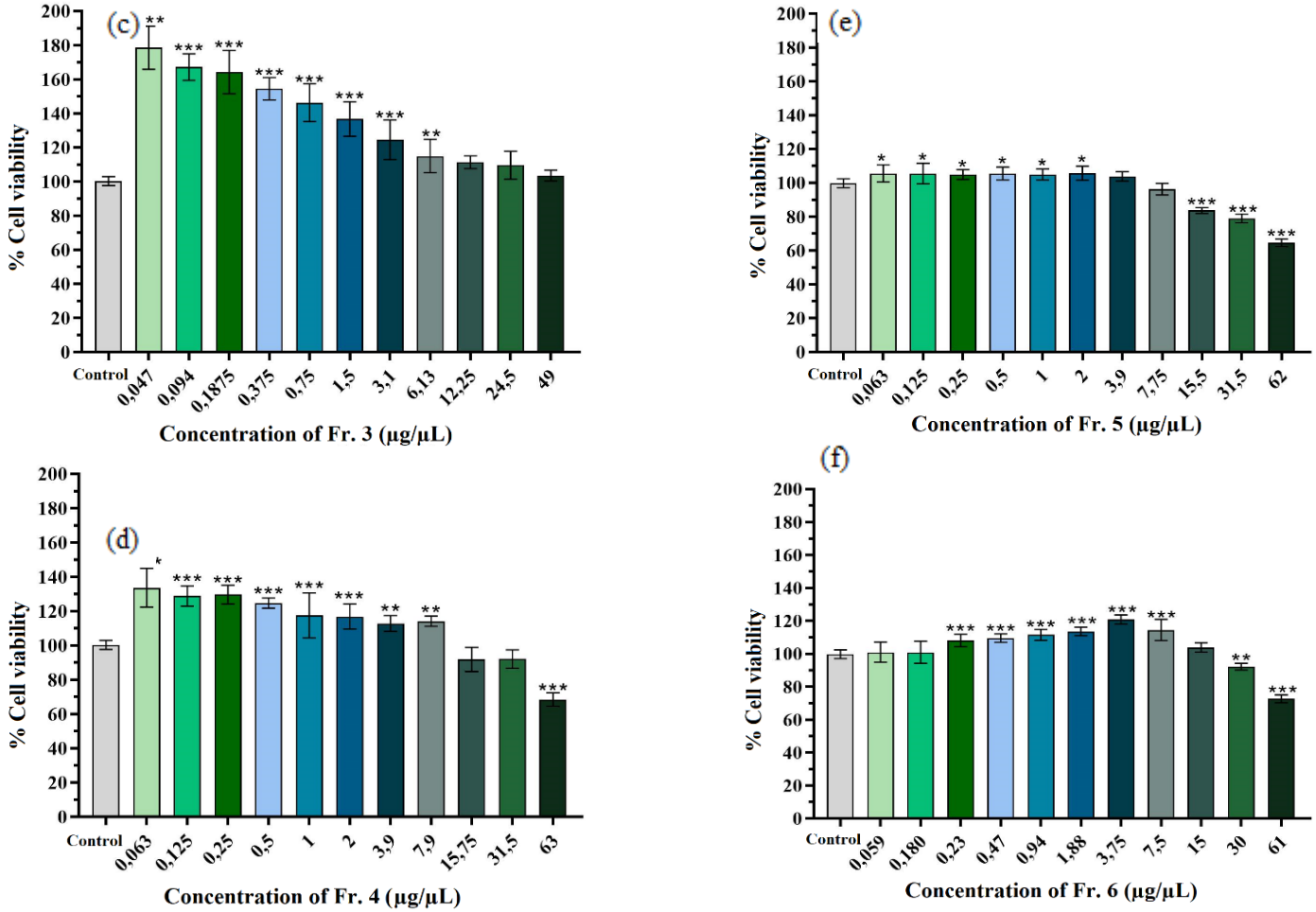



**Figure 3.** The effects of the fractions on the viability of ECV304 cells. (a) Fr.1, (b) Fr.2, (c) Fr.3, (d) Fr.4, (e) Fr.5, (f) Fr.6. (*P<0.05, **P <0.01, ***P<0.001, vertical bars show standard deviation values.

The highest test concentration was 48 µg/µL for Fr.2, and its inhibition percentage was 38±4.85% (Figure 3b). The cytotoxic effect of two dilutions (24 µg/µL and 12 µg/µL) were determined as 33±5.28% and 14±5.27%, respectively. Other concentrations (6, 3, 1.5 and 0.094 µg/µL) were observed to have a significant proliferative effect on ECV304; they induced the cell proliferation by 13±5.28%, 17±5.28%, 20±5.28%, respectively. Certain concentrations (0.75, 0.375 and 0.187 µg/µL) were more effective, with 29±5.62%, 29±6.09%, 28±6.09% proliferation, respectively. However, proliferative effect was not higher than approx. 29% (Figure 3b).

The highest application concentration (49 µg/µL) and subsequent two dilutions (24.5 and 12.25 µg/µL) of Fr.3 had no effect on the cell viability (Figure 3c). However, proliferative effect ranging from 14±12.36% to 78±13.16% was observed for lower concentrations. Interestingly, proliferative effect increased as the concentration decreased. The lowest concentration (0.047 µg/µL) exerted the highest proliferative activity (Figure 3c). This result showed that Fr.3 contains only proliferative substances.

The highest application concentration (63 µg/µL) of Fr.4 slightly (32±9.22%) inhibited the cell viability (Figure 3d). As detected in the lower doses of Fr.3, proliferative effect was also detected for two doses of Fr.4. The lowest dose (0.063 µg/µL) induced the cell proliferation by 33±10.64%.

The highest application concentration of Fr.5 (62 µg/µL) was found to inhibit the cell viability by 35±5.04% (Figure 3e).

Its two dilutions (31.5 and 15.55 µg/µL) also showed cytotoxic activity to a lesser extent while some dilutions (2-0.063 µg/µL) induced the cell viability by around 5%.

The highest application concentration of Fr.6 (61 µg/µL) and its 1:1 dilution (30 µg/µL) inhibited the cell viability by 27±5.68% and 8±5.67%, respectively (Figure 3f). In contrast, lower doses between 7.5 and 0.23 µg/µL had proliferative effect, and one dose (3.75 µg/µL), which causes proliferation by 21±5.67%, was the most effective one. Other concentrations less than 0.23 µg/µL were found to have no effect on cell growth.

The most interesting finding of the study was the variation of cell viability upon different treatment doses of the samples. There were several concentrations among all fractions, except Fr.1 and Fr.3, inducing or inhibiting the cell growth dose-dependently (Figure 3). Some concentrations of the Fr.1 exerted only inhibitory or no effect on cell growth, while Fr.3 induced the proliferation, or had no effect on cell growth. Especially lower concentrations of Fr.3 were very active. For example, 0.047 µg/µL of Fr.3 exerted significant proliferative effect (78%). However, the total extract containing all these fractions inhibited cell proliferation by 72 ±12.99%, the highest inhibition percentage within the all samples. Thus it seems that toxic constituents in total extract have a synergistic effect against the action of proliferative ones.

As a result, it was confirmed that proliferative substances are present aside from cytotoxic peptides/proteins in algae extract. Proliferation of endothelial cells is important in many aspects. First of all, endothelial cells form a single-cell layer called endothelium that lines all of blood vessels, and is critical for both vascular biology and endocrine system (Krüger-Genge et al., 2019). Endothelial cells originated from various tissues possess different functions under different microenvironments (Cines et al., 1998). Proliferation and survival of endothelial cells are of prime importance, since dysfunction of endothelial cells is associated with several diseases such as diabetes, pulmonary diseases, inflammatory diseases, cardiovascular diseases, immune diseases, cancer and currently COVID-19 (Rajendran et al., 2013; Fosse et al., 2021). Especially, prevention of coronary endothelial damage observed after ischemia and reperfusion is vital (Laude et al., 2001; Singhal et al. 2010). Today, various chemicals are tried to prohibit endothelial damage or accelerate healing. It is thought that the components detected in Fr.3 that cause the proliferative effect can be tested in future studies as a natural product as an alternative to the chemicals studied for vascular regeneration. However, it should be considered that this activity give rise to risk since endothelial cell proliferation is closely related to pathological angiogenesis in several diseases such as proliferative retinopathy, rheumatoid arthritis, psoriasis, and tumor angiogenesis (Plate et al., 1994).

On the other hand, some peaks in the HPLC chromatogram may refer various substances other than polypeptides. Thus it was concluded that total proteins precipitated from algae extract should be examined in order to identify toxic peptides in *M. aeruginosa* more accurately. Water-soluble organic substances other than proteins in algae extract should also be taken into consideration as bioactive constituents, and other biological activities of all constituents should be evaluated in the future, as in the previous reports (Singh et al. 2005; Khalid et al. 2010; Silva-Stenico et al., 2013). Studies on the exhibition of cytotoxic/proliferative peptides/metabolites in the separated fractions are in progress.

## Conclusion

This study deals with the effects of *M. aeruginosa* total extract and its fractions separated by an optimized HPLC procedure on the viability of endothelial cells. Cell proliferation promoting or inhibiting activities of total extract and the fractions vary depending on the treatment dose. It is figured out that one fraction contains cytotoxic constituents while another contains only proliferative ones, at least for the test concentrations. Accordingly, *Microcystis aeruginosa* that is a famous organism with its toxic peptides, produces not only harmful but also potentially helpful constituents, which can be used as natural products in the future. Current study is expected to contribute fractionation of *M. aeruginosa* extract as well as evaluation of *in vitro* effects of total algae extract, and its fractions on the viability of healthy cells, and to provide a basis for related studies in the future.

**Compliance with Ethical Standard**

**Conflict of interests:** The authors declare that for this article they have no actual, potential or perceived conflict of interests.

**Ethics committee approval:** There is no need ethics committee approval.

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**Disclosure:** -

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