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Research Article

A comparative study on the nutritional and cytotoxic potentials of the polysaccharides extracted from *Chlorella vulgaris*

Yaghoubzadeh Z.[1*], Safari R.[1]

1Caspian Sea Ecology Research Center (CSERC), Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research, Education and Extension Organization (AREEO), Sari, Iran
*Correspondence: Za_yaghoub@yahoo.com

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Abstract

Chlorella, a green microalga, is valued in food and medicine for its high nutritional and functional properties. Reactive oxygen species can cause oxidation reactions linked to cardiovascular diseases, degenerative conditions, and cancer. However, bioactive compounds and carotenoids in microalgae can help to alleviate certain disease symptoms by protecting tissues. Polysaccharides from *C. vulgaris* were extracted using a hot water method and their antioxidant activity was evaluated through the DPPH radical scavenging method and reducing power. The impact on the death rate of mouse colon cancer cells (CT-26) was also assessed using the 2, 5-diphenyl-2H-tetrazolium bromide (MTT) method. The analysis showed that *C. vulgaris* contained 19.08±0.09 w/v % polysaccharides and yielded about 5% of its dry weight. The extracted polysaccharides demonstrated antioxidant activity with the highest DPPH inhibition and reducing power, measuring 91.15% and 0.781% at 6 mg/mL, respectively. BHA at 0.031 mg/mL exhibited DPPH scavenging (39.78%) and reduced the power (3.65%). The polysaccharides also showed cytotoxic effects, specifically against CT-26 cells with an IC50 value of 481.53µg/mL after 48 h. These findings suggest that polysaccharides from *C. vulgaris* may possess potential cytotoxic properties against tumor cell lines.

Introduction

Natural molecules are increasingly recognized in the pharmaceutical and cosmetic industries for their beneficial properties. These molecules include essential fatty acids, proteins, vitamins, and phenolic compounds, which are important because of their specific characteristics, including anticancer, antiviral, and antioxidant properties. Oxidative stress, resulting from an increasing reactive oxygen species (ROS is a collective term that includes oxygen radicals and certain oxidizing agents easily converted into radicals) or a decrease in antioxidants, can lead to serious damage, including heart diseases, inflammatory conditions, and cancer. Therefore, maintaining adequate antioxidant compounds is essential for preserving cell health (Gürlek *et al*., 2020).

Microalgae have attracted high commercial interest due to their potential to produce valuable products and large amounts of biomass (Silva *et al.,* 2019). Microalgae produce a variety of beneficial biological molecules such as polysaccharides that offer a wide range of biochemical structures and functions as antioxidants, antifungals, anticancers, etc (Deamici *et al*., 2021).

One such microalga, *Chlorella*, is single-celled green algae found in fresh and saltwater, and it is widely used as a nutritional supplement. Recently, there has been significant interest in using microalgae to produce functional food materials, as microalgae produce nutrients essential for human health (Sheng *et al.,* 2007). Liu *et al.* (2023) concluded that the extraction of polysaccharides from *Chlorella sp*. using hot water and alkaline methods requires careful consideration of the physicochemical properties and antioxidant activity in determining the extraction conditions for *Chlorella* polysaccharides (Liu *et al*., 2023). The hot water method is cost-effective and straightforward, making it ideal for large-scale applications since it doesn't require complex equipment or harsh chemicals. This accessibility makes it suitable for both laboratory and industrial settings.

Among the various species, *Chlorella vulgaris* stands out as a superfood, boasting an impressive nutritional profile that includes 60% protein, 20 vitamins, 18 amino acids, and essential minerals such as calcium, iron, magnesium, phosphorus, and potassium (Jayshree *et al.,* 2016). Consequently, *Chlorella* is highly valued for its nutritional benefits and effectiveness in food and medicine, including boosting the immune system, acting as an antioxidant, lowering cholesterol levels, fighting tumors, protecting the brain, and reducing asthma symptoms.

Algae, as photosynthetic organisms, produce free radicals and other oxidative agents when exposed to high levels of oxygen and light. Due to their structural integrity, these organisms appear capable of synthesizing the necessary compounds to protect themselves against oxidation. Consequently, algae are regarded as a potent antioxidant source that may be beneficial for safeguarding our bodies against the deleterious effects of oxidative molecules generated as a result of the body's natural metabolism (Soleimani *et al*., 2022). Chen *et al.* (2018) in their research regarding the polysaccharides from *Chlorella pyrenoidosa* and their effects on anti-aging in *Drosophila*

melanogaster, discovered that the isolated polysaccharides were highly effective in neutralizing hydroxyl radicals, 1,1-diphenyl-2-picrylhydrazyl, and superoxide experiments of *in vitro*. Zhang *et al*. (2019a) examined the characteristics of purified exopolysaccharides from *Chlorella zofingiensis* and *Chlorella vulgaris*, including their structure, physical and chemical properties, antioxidant, as well as their abilities as antioxidants and antitumor agents. The Analysis of the monosaccharide compositions indicated that these exopolysaccharides comprise 10 or 11 distinct types of sugars and their derivatives. Their antitumor properties were evaluated using colorectal cancer cell lines HCT8, demonstrating significant inhibitory effects on cell viability.

Among cancer-related deaths worldwide, colorectal cancer (CRC) is one of the most common types, with its prevalence rapidly increasing and recognized as a serious threat to human life and health. Chemotherapy is considered the most effective treatment for colon cancer; however, due to its severe side effects, satisfactory therapeutic outcomes are not achieved, and the quality of life of patients is significantly undermined. Therefore, developing new and potent materials with low toxicity for treating colon cancer, particularly from natural sources, has become an attractive goal. Polysaccharides are important components of plants, fungi, yeasts, algae, and mosses, and have garnered increasing attention in biochemistry and medicine due to their therapeutic efficacy and low toxicity. There is growing evidence of the anticancer effects of polysaccharides on colon cancer cells. *Chlorella* is a nutrient-dense alga rich in proteins, vitamins (like B12), minerals, and antioxidants, supporting overall health, particularly during chemotherapy. It contains high levels of antioxidants such as chlorophyll and beta-carotene, which may protect healthy cells from oxidative stress. Also, *Chlorella* might boost immune function, benefiting cancer patients with weakened immune systems due to treatment. However, research on the anticancer mechanisms of naturally derived polysaccharides has not been adequately conducted so far. The current study aims to extract polysaccharides of *Chlorella vulgaris* algae and their functional properties such as antioxidant and cytotoxic activities were evaluated.

Materials and methods

Samples

In this study, the process of extracting water-soluble polysaccharides from the dry biomass of *Chlorella vulgaris* microalgae was carried out, and the algae cultivated with the appropriate and optimal culture medium was used to do this work.

Preparation of Chlorella vulgaris powder

In this study, *Chlorella vulgaris* (UTEX 580) alga was cultured using the BG11 growth medium. The algae were dried using an oven-drying method, where the cultured algae were first centrifuged and then the concentrated sediment was spread thinly in glass petri dishes. The Petri dishes were placed in an electric oven at 60°C for 12 h until they reached a stable weight after drying (Razi *et al*., 2019).

Composition analysis of Chlorella vulgaris powder

In this study, the process of extracting water-soluble polysaccharides from the dried biomass of *Chlorella vulgaris* microalga was carried out, and the algae cultured in a suitable and optimal growth medium were used for this purpose. For the approximate composition analysis of the samples, the AOAC method (2002) was used. For moisture measurement, an oven at 105°C was used to stabilize the sample weight. For ash determination, the dried sample was poured into a crucible and incinerated in a furnace (Coldale unit made in Germany, Nabertherm company, model FX 118-30) at 550°C for 5 h. The amount of protein was quantified utilizing the Kjeldahl method with a nitrogen coefficient of 6.25 (made in Germany, Behrcompany, model S3). Total fat was also determined using the Soxhlet extractor with hexane solvent (Model: R254S, Behr Company, Germany).

Extraction of water-soluble polysaccharides from Chlorella vulgaris

The hot water method was used for extracting polysaccharides from *C. vulgaris*. First, a 2.5% suspension of algae in water was prepared and shaken vigorously at 80°C for 8 h in a water bath. The suspension was spun at 5000 rpm for 20 min and the supernatant was concentrated (by concentrating the polysaccharide sample in a water bath at 40°C until it reached one-third of the Initial volume). Five times the amount of 95% ethanol was added to the concentrated liquid, and after freezing overnight in a freezer, the sample was centrifuged once more (at 5000 rpm for 10 min). The precipitate obtained was then rinsed with acetone and the polysaccharide content was calculated after solvent dispersion (suction-filtered) (Wang *et al*., 2018):

$$The\ crude\ polysaccharide\ yield\% = \frac{Crude\ poly\ saccharide\ weight(mg)}{Dride\ microalgae\ powder(mg)} \times 100$$

Antioxidant properties of Chlorella vulgaris polysaccharide

To examine the antioxidant characteristics of extracted metabolites, two indicators of free radical scavenging DPPH and Ferric Reducing Antioxidant Power (FRAP), various levels of polysaccharides from the *Chlorella* algae (0.375, 0.75, 1.5, 3, and 6 mg/mL) were prepared for each experiment to evaluate their antioxidant effects. The antioxidant properties of the polysaccharides were compared to the synthetic antioxidant BHA (0.031mg/mL) (Butylated hydroxyl anisole).

DPPH free radical scavenging power

The experiment by Xiao *et al.* (2020) was used to determine the activity of DPPH radicals. First, 89.7 mg of DPPH was dissolved in 100 mL of 99.5% ethanol and kept in darkness for 2 h. The amount of 1000 µL DPPH solution was combined with 800 µL of Tris-HCl buffer at pH 7.4 in a test tube, followed by the rapid addition and mixing of 200 µL of the sample solution (chlorophyll seaweed polysaccharide). The solution was subsequently placed in a dark room at ambient temperature for 30 min, while absorbance readings were recorded at 517

nm with a microplate reader (Tecan sunrise). A blank was created using a mixed solution of 1200 µL ethanol with 800 µL buffer (pH Tris-HCl 7.4), the calculation of inhibition percentage was established through the utilization of this formula:

Inhibition (%) = (A1 - A2) × 100 / A1

A1 represents the absorbance measured when ethanol is used instead of the polysaccharide sample, while A2 indicates the absorbance of the solution containing the polysaccharide sample.

Reducing power

A mixture of 180 µL of the FRAP working solution and 5 µL of the *Chlorella* algae polysaccharide sample was combined in a 96-well plate. This mixture was thoroughly mixed and subsequently incubated in the dark at 37°C for 15 min. Next, absorbance was measured at a wavelength of 593 nm. The standard used was Trolox, with distilled water employed as the control. A range of Trolox concentrations from 0.2 to 0.8 was employed to construct the standard curve under the specified absorption conditions (Athukorala *et al*., 2006).

Calculate IC50

The half-maximal inhibitory concentration (IC50) value for every sample was determined by plotting inhibition ratios (y) against the concentration of polysaccharides in the sample (x) at six different points and drawing a regression line (y=ax+b). The origin was not necessary for the regression line, due to the slight curvature seen in the inhibition curve instead of being completely linear. The IC50 value was calculated using an interpolation method that involved drawing a straight line between two points corresponding to 50% inhibition. To find the IC50, two points indicating the 50% inhibition level were sorted out to create a regression line (Y=X+ B). As with the earlier approach, the regression line did not need to intersect the start. The sample concentration (X) was then determined by replacing with 50 into the regression equation Y=AX+B (Xiao *et al*., 2020).

Cell culture

The CT-26 murine colorectal carcinoma cell line, sourced from the North Research Center at the Pasteur Institute of Iran, was grown in RPMI 1640 medium enriched with 1% penicillin-streptomycin and 10% FBS at 37°C, 5% CO2, and 95% humidity Upon reaching 80-90% conflux, the cells were detached using trypsin, centrifuged, and counted with a Neubauer chamber to prepare a murine model. To investigate the impact of fluid resistance, the cells were transferred to microplates and stored in the refrigerator.

MTT assay

In this research, the viability of cancer cells was determined using the MTT method. For this purpose, 100 µL of each cell group with an initial number of 1.2×10^4 cells/mL were plated in the wells of 96-well plates, using RPMI medium with 10% FBS as the control. Wells were treated with *Chlorella vulgaris* polysaccharides in 62.5 to 1000 µg/mL concentrations. The cells were then incubated for another 48 h at 37°C. The culture medium in each well was then changed, and 50 µL of MTT substance (Promega) was added to each well and allowed to incubate for 4 h in a 5% CO2

humid atmosphere at 37°C. The control-only culture medium is without suspension. Next, the plate underwent centrifugation at 800 rpm for 5 min, after which the supernatant was extracted to remove the residual MTT. The formazan crystals in each well were dissolved using 150 µL of DMSO. The quantity of purple formazan was assessed by measuring absorbance at a wavelength of 540 nm. When treating cells, cell survival was indicated as a percentage of control cells. The cell survival percentage and inhibition ratio are computed using the provided formula. Cisplatin (Cis-Pt) was used as a positive control. All experiments were performed in triplicate (Xiao *et al*., 2020):

Cell survival percentage = A540 treated cells / A540 control cells × 100

Morphological analysis

May-Grünwald-Giemsa staining was utilized to assess alterations in cell morphology. On Lab-Tek Chambers, CT-26 cells were placed (450 µL/well) at 5×10^4 cells/mL density. The next day, the culture medium was removed and cells were exposed to peptide fractions tested at concentrations ranging from 62.5 to 1000 µg/mL, simultaneously (at a 1:1 ratio) or separately. Then 10 µM of 5-fluorouracil (FU-5) and 10 µM of Cis-Pt were used. The peptide fraction and cytostatic solution were prepared in a medium containing 10% FBS. After 48 h, the cells were stained using the May-Grünwald-Giemsa method. The stained cells were observed using an Olympus BX51 System Microscope (Olympus Optical Co., Japan) (Lemieszek and Rzeski, 2020).

Statistical analysis

The distribution of quantitative variable data at each level of the qualitative variable was normal. SPSS version 18 was used for data analysis and Excel software for plotting graphs. The data were presented as means (n≥3)±the standard deviation and One-way analysis of variance (ANOVA) was applied for the analysis of multiple comparisons among all the groups. *P*-values less than 0.05 were described as statistically significant.

Results

Proximate composition of biochemical compounds

The approximate determination results of biochemical compounds in *Chlorella vulgaris* algae are shown in Table 1. Extraction of crude polysaccharides from *Chlorella vulgaris* was carried out using the hot water method. The yield of crude polysaccharides was approximately 5% of the dry weight.

Table 1: Biochemical compounds of *Chlorella vulgaris* algae.

	Biochemical compounds	Amounts (%)
1	Crude protein (%)	0.05±47.02
2	Carbohydrates (%)	0.09±19.08
3	Lipid (%)	0.07±12.52
4	Humidity (%)	0.03±4.67
5	Ash (%)	0.04±7.45

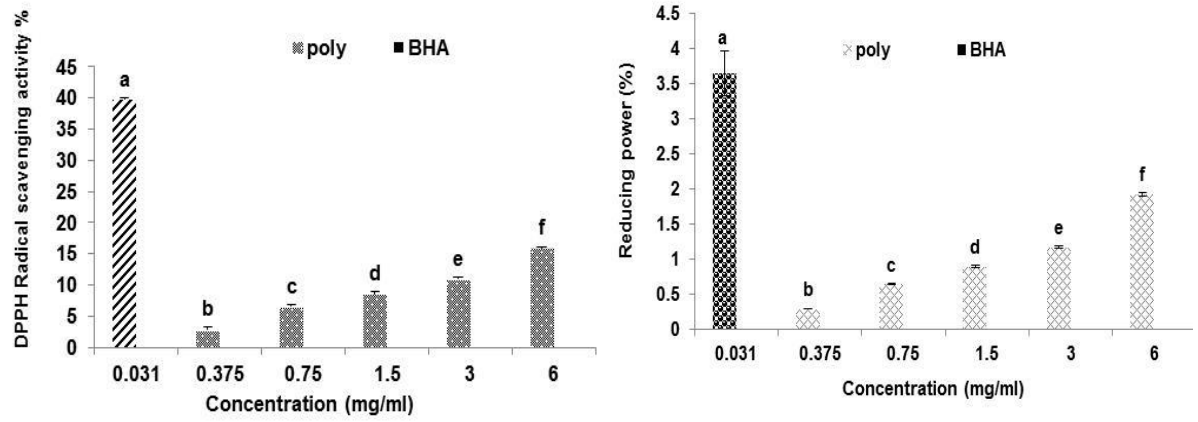
Data are based on mean ± standard deviation (n=3).

The antioxidant properties

DPPH free radical scavenging power

Polysaccharides extracted from *Chlorella vulgaris* exhibited DPPH radical scavenging activity at five concentrations

of 0.375, 0.75, 1.5, 3, and 6 mg/ml, yielding inhibition values of 2.84±0.5, 6.59±0.367, 8.59±0.344, 10.98±0.327, and 15.91±0.091, respectively (p<0.05). The synthetic antioxidant BHA, at 0.031 mg/mL concentration, demonstrated a DPPH radical scavenging activity of 39.78±0.277 (p<0.05). The IC50 value was 22.342 mg/mL (Fig. 1A).



A B

Figure 1: Antioxidant activities of polysaccharides from *Chlorella vulgaris* A: DPPH radicals scavenging activities B: reducing power. BHA was used as standard. (The values with different superscripts are significantly different at p<0.05).

Reducing power of iron

Polysaccharides extracted from *Chlorella vulgaris* algae at concentrations of 0.375, 0.75, 1.5, 3, and 6 mg/mL had reducing power values of 1.928±0.027, 1.177±0.0177, 0.903±0.013, 0.645±0.009, and 0.781±0.054 respectively (p<0.05). BHA at 0.031 mg/mL concentration had an iron chelating capacity of 3.65±0.324 (p>0.05). The IC50 value was 187.408 mg/mL (Fig. 1B and Fig. 2).

Cytotoxicity activity of the extracted polysaccharide of Chlorella vulgaris

The cytotoxicity of the polysaccharide extracted from *Chlorella vulgaris* was evaluated at five concentrations of 62.5, 125, 250, 500, and 1000 µg/mL, yielding respective values of 78.95±0.06, 73.39±0.16, 46.08±0.18, 36.52±0.42, and 35.65±0.26 (p<0.05) (Figs. 3 and 4). Cis-Pt exhibited a cytotoxicity of 22.86±0.01 at 125 µg/mL concentration (p>0.05). The IC50 value of the polysaccharide was determined to be 305.08µg/mL.

Morphological changes in cells

Polysaccharides extracted from *Chlorella vulgaris* demonstrated notable cytotoxicity against CT-26 cell lines. This study revealed significant cytotoxic activity, reflected by a correlation coefficient of R^2=0.6473. Morphological changes were observed in cells treated with varying concentrations of *Chlorella vulgaris* polysaccharides (5.62, 125, 250, 500, and 1000 µg/mL). In contrast to the control cells, which exhibited a shiny and adherent morphology, the treated cells displayed disruption of the monolayer, as well as rounding and shrinking which is one of the characteristic cell deaths in treated cells.

These results indicate that polysaccharides extracted from *Chlorella vulgaris* not only have antibacterial and antioxidant properties but also exhibit cytotoxicity properties (Fig. 5).

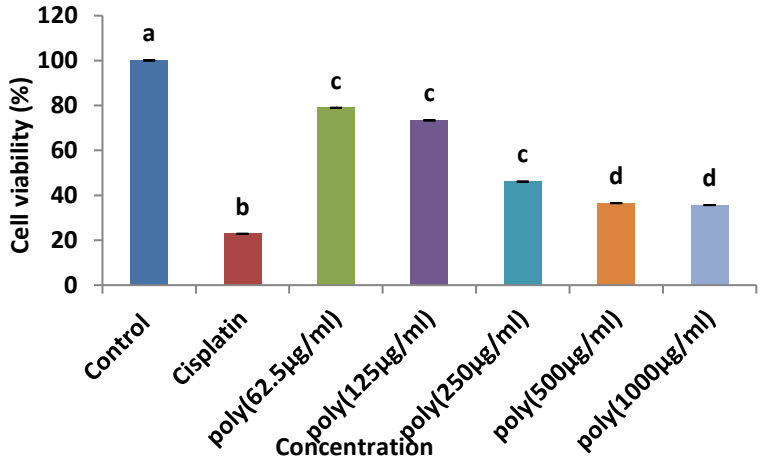
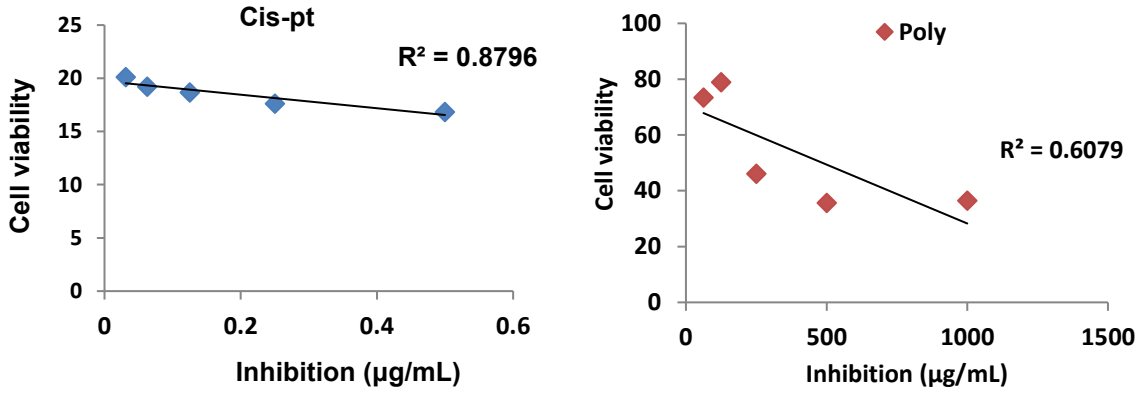


Figure 3: Effect of polysaccharides from *Chlorella vulgaris* on the growth of Mouse colon cancer cell lines CT-26. CT-26 cells were seeded in 96-well plates at a density of 1.2 × 10^4 cells/mL and incubated at 37 °C. After treatment with various concentrations of polysaccharide for 48 h, cell viabilities were estimated by the proliferation assay. (The values with different superscripts are significantly different at p<0.05).



A **B**

Figure 4: Correlation diagram cell viability of *Chlorella vulgaris* polysaccharides and Cis-Pt by regression analysis.

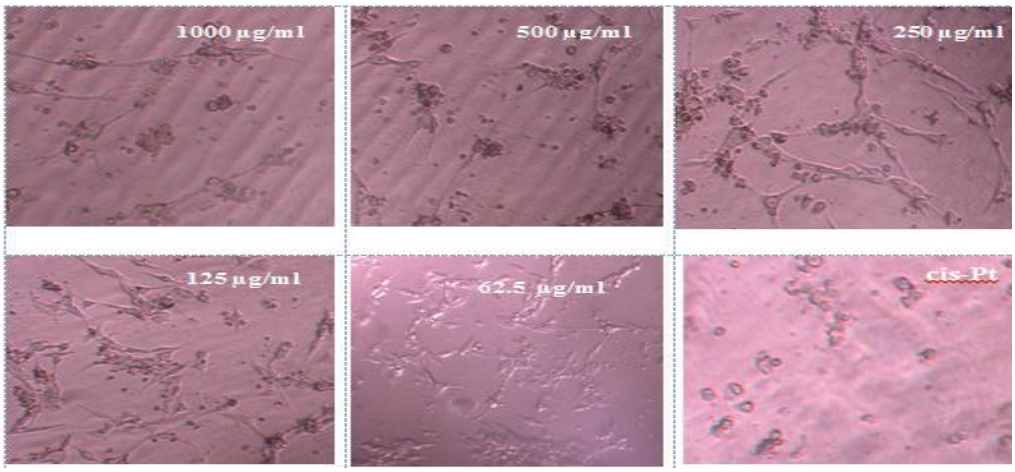


Figure 5: Cell images showing the cell death viability of CT-26 cells at different mg/mL concentrations of polysaccharide compared to Cis-Pt-treated cells.

Discussion

To evaluate the *in vitro* antioxidant activities of *C. vulgaris* polysaccharides, DPPH and FRAP radical scavenging assays were performed to assess their radical scavenging capacities at various concentrations ranging from 0.375 to 6 mg/mL. The results indicated that the scavenging activity for DPPH was maximized at a concentration of 6 mg/mL, yielding a value of 15.91±0.091. The recorded IC50 value for DPPH was 22.342 mg/mL. In comparison, BHA positive control exhibited a scavenging activity for DPPH at a concentration of 0.031 mg/mL, yielding a value of 39.78±0.277. The IC50 value for DPPH was also recorded at 0.028 mg/mL (the lowest IC50, highest antioxidant activity).

In similar research reported by Zhang *et al*. (2019a), the exopolysaccharides displayed notable antioxidant properties, with radical scavenging activities against 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radicals ranging from 6% to 71.5% and 44.5% to 70.4% respectively *in vitro.* El-fayoumy *et al*. (2021) reported that chloroform extracts from unicellular green algae (*Chlorella sp*) and filamentous microalgae (*Spirulina sp*) may yield new anticancer natural products. Recent findings indicate that algae-derived resources can modulate various cellular mechanisms, enhancing apoptosis in cancer cells and reducing tumor cell invasion.

The reducing power of polysaccharides extracted from *Chlorella vulgaris* was evaluated at 600 nm, revealing an increase in reducing power with increasing concentration. In this study, the maximum reducing power of *Chlorella vulgaris* polysaccharides recorded was 1.928 ± 0.027 at a concentration of 6 mg/mL. The recorded IC50 value for FRAP was 45.79 mg/mL, in comparison to BHA 3.65±0.324. The IC50 value for DPPH was also recorded at 3.816 mg/mL, as shown in Table 2. Regression analysis of DPPH assay and reducing power activity for BHA and *Chlorella vulgaris* polysaccharides is shown in Figure. 4. A positive slope in the regression line may indicate that higher concentrations of *Chlorella* correlate with increased antioxidant activity.

Table 2: IC50 (mg/mL) value of free radical scavenging activities of *Chlorella vulgaris* polysaccharide.

Parameters	BHA	*Chlorella vulgaris*
DPPH free radical inhibition power	0.028	22.342
The reducing power	3.816	45.79

Wang *et al.* (2008) showed that antioxidant activity correlates positively with reducing potential, affected by factors like sugar type, molecular weight, sulfation degree, and glycosidic branching. Thus, the antioxidant potential of soluble polysaccharides from *Chlorella vulgaris* is linked to their sulfated fraction (El-Naggar *et al*., 2020).

To assess the in vitro cytotoxicity activity of *C. vulgaris* polysaccharides, an MTT assay was employed to determine the inhibitory rates of polysaccharides on CT-26 cells. Figure 3, *Chlorella vulgaris* polysaccharides showed ordinary results with inhibition on the proliferation of CT-26 cells. In particular, the minimum cell viability rate was 35.65±0.26 in a concentration of 1000 µg/mL. As shown in Figure 3, after treatment with different

concentrations (62.5, 125, 250, 500, and 1000 µg/mL), polysaccharides exhibited inhibitory effects on the proliferation of CT-26 cells in a dose-dependent fashion. The 50 % inhibitory concentration (IC50 value) against CT-26 cells was 305.08µg/mL. It is important to note that a high IC50 is acceptable as *Chlorella vulgaris* is classified as food, not medicine (Saad *et al*., 2006). Polysaccharides may not directly induce cytotoxicity in certain cell types, suggesting they can have beneficial effects like immunomodulation or antioxidant properties without being toxic at lower concentrations (Kiddane and Kim, 2021). Regression analysis of DPPH assay and reducing power activity for Cis-pt and *Chlorella vulgaris* polysaccharides is shown in Figure 2. A negative slope could indicate cytotoxic effects at certain concentrations. Zhang *et al.* (2019b) showed that the extracted exopolysaccharides from *Chlorella pyrenoidosa*, *Chlorella zofingiensis*, and *Chlorella vulgaris* at a concentration of 0.6 mg/mL exhibited significant inhibitory effects of 28.3% and 18.0% against human colon cancer cell lines HCT8, respectively. Microalgal carotenoids, particularly from *Chlorella ellipsoidea* and *Chlorella vulgaris*, have been shown to enhance recovery in colorectal cancer treatment alongside 5-fluorouracil. Carotenoids from *Chlorella ellipsoidea* demonstrated 2.5 times greater cytotoxicity and apoptosis induction in human colon cancer cells (HCT116) compared to those from *Chlorella vulgaris* (Cha *et al.,* 2008). Studies have shown that the *Chlorella vulgaris* extract induced dose-dependent cytotoxic impacts on MCF7 cells, resulting in 84% cell survival following a 48-hour exposure to 100µg/mL (Balaji *et al*., 2017). Prakash and Vedanayaki (2019) documented the cytotoxic effects of *Chlorella vulgaris* on the MCF-7 cell line, a human breast adenocarcinoma cell line. They also highlighted the presence of bioactive components, including curcumin-A, within *Chlorella vulgaris*, which exhibited inhibitory effects on colon and breast cell lines.

Polysaccharides can induce apoptosis in cancer cells through pathways involving caspase activation and modulation of apoptosis-related proteins (Gan *et al.,* 2020). Polysaccharides from algal sources may disrupt the cancer cell cycle and induce oxidative stress, damaging cells and potentially inhibiting tumor migration and invasion (Ferdous and Yusof, 2021). The mechanisms vary by cancer type and polysaccharide concentration, highlighting the need for further research on their clinical implications. Structural modifications such as sulfating and variations in molecular weight can expand the bioactivities of polysaccharides, making them valuable in various fields, including medicine, nutrition, and biotechnology. Understanding these structural features allows for the design of polysaccharides with tailored properties for specific applications (Li *et al.,* 2016).

Conclusions

Due to their wide range of bioactive compounds, the polysaccharides derived from the microalga *Chlorella vulgaris* are likely to serve as effective mediators in eliminating free radicals in dietary supplements. Moreover, they possess the

potential to function as natural antioxidants for use in food and pharmaceutical products. In this study, the yield of crude polysaccharides was approximately 5% of the dry weight. Extraction polysaccharides exhibited good scavenging activities on DPPH radicals scavenging and reducing power. Furthermore, the antitumor effects of the obtained polysaccharides were studied in vitro on mouse colon cancer cell lines CT-26. Polysaccharides had obvious inhibitory effects on cell viability (35.65 on CT-26, at a concentration of 1000 µg/mL). Our study showed that polysaccharides from *Chlorella vulgaris* may be worth further investigating as alternative potential antitumor agents.

Acknowledgments

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Conflicts of interest

The authors declare no conflict of interest.

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