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The effect of using ultrasound and microwave pretreatment on the antioxidant and anticancer activity of polysaccharides extracted from the *Chlorella vulgaris* microalgae

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ABSTRACT

For about two decades, researchers have been looking for natural antioxidants to replace with their chemical types in food formulations to reduce the harmful effects of chemical preservatives. Also, in modern cancer treatment strategies, it is very important to use safe and natural compounds that have the ability to destroy cancer cells while not harming healthy cells. Polysaccharides extracted from algae can act as antioxidants and a natural anticancer compound, and the extraction method is one of the determining factors in the manifestation of their numerous properties. The aim of this study was to investigate the effect of using ultrasound and microwave pretreatments on the extraction efficiency, sulfate content, antioxidant and anticancer properties of *Chlorella vulgaris* polysaccharides. For this purpose, power of 300 W and time of 20 minutes, as well as 600 W and 10 minutes (after optimization) were selected for ultrasound and microwave pretreatments, respectively. According to the findings, the use of the aforementioned pretreatments increased ($p<0.05$) the extraction efficiency, sulfate content, antioxidant and anticancer activity of the extracted polysaccharides (compared to the control). The highest extraction efficiency and sulfate content (21.73±0.19% and 34.06±1.07%, respectively), free radical scavenging activity, reducing power, total antioxidant and anticancer properties were related to ultrasound treatment ($p<0.05$). The IC_{50} of this treatment for the inhibition of DPPH and ABTS radicals was calculated to be 1.04±0.03 and 0.99±0.02 mg/ml, respectively. According to the results of the anticancer activity of the treatments, the LC_{50} of the control and ultrasound and microwave treatments for the destruction of T47D breast adenocarcinoma cancer cells was measured as 1033.54±9.27, 412.76±5.02, and 693.05±7.34 µg/mL, respectively. From this study, it can be concluded that the use of ultrasound and microwave pretreatments for extraction, significantly enhances the antioxidant and anticancer properties of *C. vulgaris* polysaccharides. Also, use of these polysaccharides in food formulations as preservatives and in the production of anticancer drugs as active ingredients is proposed.

1- Introduction

Currently, synthetic and chemical antioxidants are used to prevent oxidative spoilage in food products. These antioxidants are very harmful to consumers' health and have raised concerns. In such circumstances, finding natural sources of antioxidants that are not only harmless to health but also offer benefits is of great importance [1]. Among the natural antioxidants that have been produced and extracted from natural sources, especially aquatic organisms, over the past two or three decades, we can mention astaxanthin [2], phycocyanin [3], chitosan [4], gelatin [5], and bioactive peptides [6]. Another category of compounds with natural antioxidant activity are the (sulfated) polysaccharides extracted from seaweeds (green, brown, and red) such as fucoidan, alginate, ulvans, mannans, rhamnans, and galactans [7-10].
One of the common cancers that has become very widespread in most countries during the last two decades is breast cancer [11]. With the advancement of technology, several methods have been considered for treating this disease, including surgery, radiotherapy, and chemotherapy. However, each of these methods has significant disadvantages. The surgical method does not have high efficacy and sometimes, depending on the progression of the disease, can lead to worsening of the disease and the patient's general condition [12]. In the other two methods, because radiation and chemical drugs also damage healthy cells, the patient faces dangerous and irreversible side effects [13]. Furthermore, numerous reports from various countries indicate an increase in the resistance of cancer cells to chemotherapy drugs [14]. Therefore, it seems necessary to use pharmaceutical methods for cancer treatment that are both based on effective and safe natural compounds and do not harm healthy cells (non-toxic to non-cancerous cells). For this reason, in recent years, researchers and specialists have turned to extracting natural anti-cancer compounds from living organisms such as aquatic animals, and through various studies, have produced (extracted) and introduced a range of effective substances for cancer treatment. Among the aquatic organisms with high potential for extracting anti-cancer compounds are algae. Polysaccharides extracted from algae [15 and 16] as well as extracts produced from them [17 and 18] are among the compounds that can fight and destroy cancer cells.
The type of method used for extracting sulfated polysaccharides from algae affects the manifestation of their various properties [19]. Supercritical fluid, subcritical water, high pressure, microwave-assisted extraction, ultrasound, high voltage electrical treatment, pulsed electric field, enzymes, and fermentation-based extraction are among the modern extraction methods, each with their own advantages and disadvantages, producing a product with specific characteristics [20]. In the ultrasound method, waves with a frequency higher than 18 kHz are used to extract active compounds. The extraction mechanism in this method is related to the cavitation phenomenon [21], which means the formation, growth, and collapse of small bubbles due to the creation of large negative pressure in a liquid medium. Not using high temperatures in the ultrasonic method results in the final product having stable bioactive properties and preserving its intrinsic characteristics [22]. Microwaves are electromagnetic waves with frequencies ranging from 300 MHz to 300 GHz and wavelengths between 1 millimeter and 100 centimeters. Microwave heating in dielectric materials occurs due to the polarization of water molecules by electromagnetic radiation. Microwaves, which generate heat very rapidly, transfer energy to materials through three mechanisms. These mechanisms are dipole polarization, ionic conduction, and parallel polarization [23]. In summary, in both techniques (ultrasound and microwave), the waves cause the destruction of the cell wall and the leakage of contents, including polysaccharides, into the solvent medium. Chlorella vulgaris is a unicellular green alga and a pr

otist, having a spherical size with a diameter of 2 to 10 microns. This alga lacks flagella and contains the green photosynthetic pigments chlorophyll a and b in its chloroplast. Chlorella vulgaris reproduces rapidly through photosynthesis and requires carbon dioxide, sunlight, and a small amount of minerals for this purpose [8].

The aim of the present study is to use ultrasound and microwave pretreatments in the process of extracting polysaccharides from Chlorella vulgaris and to evaluate the effect of these pretreatments on the extraction efficiency, sulfate content, antioxidant properties, and anticancer activity of the product.

2-Materials and Methods

2-1-Polysaccharide extraction from Chlorella vulgaris algae

10 grams of this alga powder (National Algae Bank) was mixed in 300 ml of distilled water. It was then stirred for 3 hours at 50°C and subsequently centrifuged at 8000 rpm for 15 minutes. The supernatant was precipitated with 95% ethanol (4:1 V/V) overnight at 4°C, and then centrifuged at 5000 rpm for 15 minutes. The resulting precipitate was redissolved in deionized water, and the protein was removed using a 1000 kDa dialysis bag. Finally, the supernatant was freeze-dried (Vaco 2 Zirbus, Germany) and stored at -20°C [24].

2-2-Pretreatment of microalgae with ultrasound and microwave

The microalgae powder was transferred to a beaker with distilled water in a ratio of 10 g: 300 ml. The resulting suspension was then subjected to ultrasound treatment (El masonic, model P60H, Germany) with an output power of 300 W for 20 minutes. The temperature increase was controlled using an ice-water bath. The power and time for the microwave pretreatment (NN-C2002W, Japan) were set at 600 W and 10 minutes, respectively [25]. It should be noted that in the pre-test, different powers and times were used for the ultrasound and microwave pretreatments, and based on the extraction efficiency and sulfate content, the optimal powers and times (referred to as ultrasound and microwave treatments) were selected. These two treatments, along with the control, were examined for other tests.

2-3 Extraction efficiency of polysaccharides from Chlorella vulgaris algae

For this purpose, first, the total polysaccharide content of the extracted sample was determined using the phenol-sulfuric acid method. Then, the polysaccharide yield was calculated as the percentage of polysaccharide relative to the weight of the microalgae and reported as a percentage [19].

2-4- Evaluation of the chemical composition of extracted polysaccharides

2-4-1- Determination of sulfate content

First, the extracted sample was hydrolyzed using 1 M hydrochloric acid (Merck, Germany) and dried using a rotary evaporator (HS-2005SN, Hahn shin brand, Korea), and the obtained dry residue was dissolved in 1 ml of water. Then, 8% trichloroacetic acid (Sigma, Germany) and 5% barium chloride gelatin solution were mixed and incubated for 20 minutes at room temperature. In the next step, the absorbance of the resulting mixture was read at 360 nm. Finally, sodium sulfate solution (Merck, Germany) was used to plot the standard curve [26].

2-4-2-Carbohydrate content

For this purpose, the extraction solution (0.4 ml) was mixed with 1 ml of phenol solution (5% w/v), and 5 ml of concentrated sulfuric acid (Merck, Germany) was added. This mixture was kept at room temperature for 30 minutes in the dark, and its absorbance was recorded at a wavelength of 490 nm. A standard curve was plotted with different concentrations of glucose [26].

2-4-3-Determination of ash and protein content

0.5 g of dried polysaccharides was incinerated in a porcelain crucible at 600°C for 8 hours in a furnace, and the ash results were reported as the percentage of the incinerated weight of polysaccharides relative to the initial weight. To evaluate the protein content of the samples,

the Kjeldahl method and a factor of 6.25 were used [28].

2-4-4- Measurement of ironic acid content

First, 0.503 g of sodium tetraborate (Sigma, Germany) was dissolved in 100 ml of 98% sulfuric acid to prepare a 0.025 M sodium tetraborate reagent. This solution was stirred for 6 hours. In the next step, 0.125 g of carbazole was mixed with 100 ml of ethanol in a dark glass container and stored at 4°C. 3 ml of the prepared reagent was added to test tubes, and these tubes were cooled in an ice container. In the next step, samples with a concentration of 1 mg/ml in saturated benzoic acid (Merck, Germany) were added to the aforementioned tubes, and after shaking, the tubes were cooled again in the ice container for 5 to 10 seconds. After cooling, the tubes were incubated in a water bath (Memert wnb 29, Germany) at 100°C for 10 minutes. After this time, the tubes were allowed to cool to room temperature. Then, 0.1 ml of the previously prepared carbazole solution was added to the tubes, and incubation was performed in a water bath at 100°C for 15 minutes. After this time and cooling the tubes (25°C), their absorbance was read at 530 nm (Spectrophotometer, CamSpec model M350, England) after zeroing against a blank. D-glucuronic acid (Sigma, Germany) was used as a standard [19].

2-5-Indicators for measuring the antioxidant activity of extracted polysaccharides

2-5-1- 2,2-Diphenyl-1-picrylhydrazyl free radical scavenging activity (DPPH)]

First, 2 ml of different concentrations of the sample were added to 2 ml of 0.1 mM DPPH solution (Sigma, Germany) in ethanol. The sample was incubated in a dark environment (25°C) for 30 minutes, and the absorbance was recorded at 517 nm. Vitamin C was used as a positive control. In the control group, deionized water was replaced with the DPPH solution, and in the blank group, deionized water was replaced with the polysaccharide solution. The DPPH radical scavenging activity of the treatments was calculated using Equation 1 [29]. In this equation, Ac is the absorbance of the control and as is the absorbance of the sample.

(Equation 1) Free radical scavenging percentage = (Ac--As)/Ac × 100

2-5-2- 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity (ABTS)

A 7 mM ABTS solution was prepared in 2.45 mM potassium persulfate (Merck, Germany) and kept in a dark environment (25°C) for 16 hours. This solution was diluted with distilled water until it reached an absorbance of 0.7±0.02 at a wavelength of 734 nm. Then, 20 microliters of the sample (various concentrations) were combined with 980 microliters of the diluted ABTS solution and placed in a dark place (30°C) for 10 minutes. The ABTS radical scavenging activity was calculated based on Equation 2 [30]. Vitamin C was used as a positive control.

(Equation 2) ABTS Free radical scavenging activity (%) = 100 × [(Control absorbance - Sample absorbance)/Control absorbance]

2-5-3- FRAP assay (Ferric ion reducing antioxidant power)

0.5 ml of the sample was combined with 0.5 ml of phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of 1% (w/v) potassium ferricyanide (Sigma, Germany). This mixture was then incubated at 50°C for 20 minutes. In the next step, 0.5 ml of 10% TCA solution was added to the mixture, and the resulting combination was centrifuged at 2500 rpm. 1 ml of the supernatant was mixed with 1 ml of distilled water and 0.2 ml of 0.1% (w/v) ferric chloride (Sigma, Germany), and (after 10 minutes of incubation at 25°C) its absorbance was read at a wavelength of 700 nm. Distilled water was used as the control sample. Higher absorbance of the mixture indicates greater reducing power [31].

2-5-4- Total Antioxidant Capacity (TAC)

0.3 ml of the sample with different concentrations was mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium

phosphate, and 4 mM ammonium molybdate), and the resulting solution was placed in a water bath at 95°C for 90 minutes. After this time and cooling the solution, 100 microliters of the sample were poured into each well of a microplate, and its absorbance was read at a wavelength of 695 nm using an ELISA reader (Hiperion Microplate Reader, MPR4+, Germany). Ascorbic acid solution at concentrations of 12.5, 25, 50, 100, and 200 mg/ml was used to plot the standard curve, and the results for this index were reported as milligrams of ascorbic acid per gram of dried algal powder [32].

2-6- Evaluation of the anticancer activity of treatments against breast cancer cell line by MTT assay

The T47D breast adenocarcinoma cancer cell line and the normal HEK-293 cell line (Pasteur Institute of Iran Cell Bank) were cultured in complete RPMI 1640 culture medium and 10% fetal bovine serum in humidified conditions incubated with 5% CO_2 gas at 37°C. Cultured cells were transferred to 96-well plates at a rate of 10^5 cells per well and incubated for 24 hours under culture conditions. Then, the research treatments at concentrations of 125, 250, 500, and 1000 micrograms per milliliter were added to the wells, and the samples were incubated for 48 hours under culture conditions. Wells containing untreated cells were considered as controls. After the mentioned period, 30 microliters of MTT dye (0.5 mg/ml) were added to the cells, and incubation was performed for 3 hours. The water-insoluble purple formazan crystals were dissolved in 100 microliters of dimethyl sulfoxide solution, and the absorbance of the resulting solution was read using an ELISA reader (Hiperion Microplate Reader, MPR4+, Germany) at a wavelength of 540 nm. Finally, the percentage of viable cells (CL) was calculated as the percentage of treated cells relative to untreated cells using Equation 3. In this equation, AT is the absorbance of cells treated with extracted polysaccharides, and AC is the absorbance of untreated cells (control) [33].

(Equation 3) $CL (\%) = (AT / AC) \times 100$

2-7- Statistical analysis

This research was conducted in a completely randomized design. To analyze the data, one-way analysis of variance (ANOVA) and Duncan's test (for comparing data means) were used at a 95% confidence level. Data analysis was performed using SPSS~22~ statistical software, and figures were drawn using EXCEL software. All experiments were performed in triplicate, and the results were reported as mean ± standard deviation.

3- Results and discussion

3-1- Chemical composition of Chlorella vulgaris algae

Table 1 shows the chemical composition of Chlorella vulgaris algae powder. According to this table, the mentioned powder contains about 48% protein and 21.5% carbohydrates. Additionally, lipids constituted about 14% of the C. vulgaris used.

Table 1- Chemical composition of *Chlorella vulgaris* powder

Chemical composition	Amounts (%)
Protein	47.85±1.52
Lipid	14.06±0.23
Carbohydrates	21.37±1.05
Moisture	2.78±0.11
Ash	9.04±0.36

In another study that used the same alga for polysaccharide extraction, the amounts of protein, lipid, carbohydrate, and ash were reported to be about 47%, 12.5%, 19%, and 7.5%, respectively [9]. The chemical composition of the alga is important because it directly affects the chemical composition of the extracted polysaccharides and subsequently their properties. Prabakaran et al. (2019) reported the chemical composition of Chlorella vulgaris as 45.23% protein, 23.43% carbohydrate, and 18.12% lipid [34].

3-2- Chemical composition and extraction efficiency of research treatments

Table 2 presents the chemical composition and extraction efficiency of the control and research treatments. According to this table, the amounts of protein and ash in the control were significantly higher than in the ultrasound and microwave treatments ($p<0.05$). Also, the ultrasound treatment had the lowest amount of protein and ash ($p<0.05$). The ultrasound treatment showed the highest total carbohydrate (55.27±0.61%) and sulfate (34.06±1.07%) content ($p<0.05$); while the sulfate content in the microwave treatment was measured to be significantly higher than the control ($p<0.05$). These two treatments (microwave and control) did not show significant differences in terms of total carbohydrate content ($p>0.05$). Furthermore, the control and ultrasound treatments showed no significant difference in uronic acid content ($p>0.05$) and were at a lower level in this regard compared to the microwave treatment (15.02±0.93%) ($p<0.05$). According to Table 2, the highest extraction efficiency was related to the ultrasound treatment (21.73±0.19%) ($p<0.05$).

Table 2- Chemical composition and extraction efficiency of treatments

Treatments	Protein	Carbohydrates	Sulfate	Ash	Uronic acid	Extraction efficiency
Control	8.95±0.21^{a}	51.03±0.74^{b}	11.71±0.81^{c}	4.34±0.1^{a}	10.77±1.65^{b}	3.24±0.1^{c}
Ultrasound	2.03±0.18^{c}	55.27±0.61^{a}	34.06±1.07^{a}	0.32±0.07^{c}	9.83±1.24^{b}	21.73±0.19^{a}
Microwave	4.86±0.32^{b}	51.16±1.04^{b}	25.71±1.60^{b}	2.29±0.18^{b}	15.02±0.93^{a}	16.96±0.97^{b}

- Different lowercase letters in each column indicate significant differences between the data ($p<0.05$).

In this study, the use of ultrasound and microwave pretreatments caused a significant increase in the extraction efficiency of polysaccharides from Chlorella vulgaris (to the extent that the yield increased from 3.24±0.1% in the control sample to 21.73±0.19% in the ultrasound treatment and 16.96±0.97% in the microwave treatment). In fact, the effect of the extraction method on the efficiency was confirmed, a finding supported by numerous studies. In a study conducted to extract fucoidan from the brown alga Nizimuddinia Zanardini using microwave treatment, an increase in efficiency was reported for the microwave method compared to the conventional method; the extraction process efficiency was 3.71% in the microwave technique and 2.35% in the conventional method [35]. Alboofetileh et al. (2019) in a study evaluated the effect of different treatments including ultrasound (power 200 W, frequency 20 Hz, temperature 55°C, and two time cycles of 20 minutes), microwave (power 700 W, temperature 90°C, and two time cycles of 20 minutes), supercritical fluid (power 1500 W, temperature 150°C in two time cycles of 20 minutes), and hot water (temperature 65°C and two time cycles of 3 hours) on the extraction efficiency of fucoidan from N. Zanardini algae and reported that the highest efficiency was related to the supercritical fluid

method (13.5%) and the lowest was related to the ultrasound treatment (3.6%). Also, the extraction efficiency of the microwave treatment was measured at 6.17% [19]. In another study investigating the effect of extraction using acidic, enzymatic, and ultrasound methods on the characteristics of fucoidan from the brown alga Sargassum ilicifolium, the extraction efficiency in the enzymatic method (11.37%) was reported to be higher than the acidic (7.87%) and ultrasound (8.07%) methods [36]. These studies, like the present research, confirmed the effect of the extraction method on the process yield.

The reason for the increased extraction efficiency when using microwave pretreatment is that electromagnetic waves create changes in the cellular structure. More clearly, it can be stated that the increase in extraction efficiency (in the microwave technique) results from the combination of two transport phenomena including temperature and mass gradients working in the same direction. In methods where pretreatments are not used (simple and conventional techniques), mass transfer occurs from the inside to the outside and heat transfer from the outside to the inside of the substrate. Moreover, although in the conventional extraction method (without pretreatment) heat is transferred from the heating medium to the inside of the sample, in the microwave technique, heat is dispersed volumetrically within the radiation range. Also, the solvent used enters the microalgae cellular matrix through diffusion with a strong and intense effect, and then the solutes dissolve until reaching a specific concentration (a concentration limited by various characteristics of the solid material). In the next stage of this process, the solution carrying the solutes diffuses to the surface through effective diffusion. Finally, with convective currents, which can be natural or forced, the solution is transferred from the surface to the solvent [23]. These interactions in the microwave method continuously (and ultimately) lead to increased process efficiency. Generally, the mechanisms of microwaves for volumetric h

eat generation in the solvent and also in the algal cellular environment are the two phenomena of dipole polarization and ionic conduction. Since water has a potential ability to dissolve carbohydrates and also absorb microwaves, it is considered the most efficient solvent in this method. In the microwave technique, waves are absorbed by water molecules, and electromagnetic energy is converted into thermal energy. This heat increases the pressure inside the algal cell, and subsequently, the cell disintegrates, and its contents are released into the solvent. The main mechanism of extraction with ultrasound waves is related to the cavitation phenomenon [21]. As a sound wave passes through an elastic medium, it causes longitudinal displacement of particles and acts as a piston on the surface of the medium, resulting in a sequence of compression and rarefaction stages. During these processes, molecules temporarily move away from their original positions as the sound wave passes, and they may collide with surrounding molecules. Then, during the compression stage, the first group of molecules is pulled back towards their original position, and kinetic energy pulls them further back. Therefore, rarefaction areas are created in the medium, and since every medium has a critical molecular distance, when this distance is exceeded, molecular interactions break, and cavities are formed in the liquid [33]. The created cavities in the medium are cavitation bubbles resulting from ultrasound, which can grow during the rarefaction stages and decrease in size during the compression cycles. When the size of these bubbles reaches a critical point, they collapse during the compression cycle, releasing a large amount of energy. The temperature and pressure at the moment of collapse are estimated to be up to 5000 Kelvin and 5000 atmospheres, respectively, in an ultrasonic bath at room temperature. The creation of these hot spots can significantly accelerate chemical reactions in the medium. When these bubbles collapse on the surface of solid

materials, the released high pressure and temperature directly generate microjets and shock waves on the solid surface. The impact of these microjets on the surface causes abrasion, fracturing, and destruction. Ultrasonic waves improve the stages of the extraction process of plant compounds, namely tissue swelling for solvent absorption and the release of compounds from the tissue into the solvent, by creating porosity and pores in the cell wall, facilitating and accelerating mass transfer [22]. Unlike conventional methods, sound waves cause the destruction of the cell wall in a short period, and the cellular contents are released across the cell wall [22]. In the present study, the use of ultrasound and microwave pretreatments increased the sulfate content in the extracted polysaccharides. Specifically, the sulfate content in the control sample was measured at 11.71±0.81%, but in the aforementioned treatments, it increased to 34.06±1.07% (ultrasound) and 25.71±1.60% (microwave). Alboofetileh et al. (2019), who used microwaves with a power of 700 W at 90°C to extract fucoidan from N. zanardini algae, reported a sulfate content of 24.09%, higher than the control sample extracted by the conventional method (18.44%) [19], a finding consistent with the present study. In research by Torabi Dastgerdouei et al. (2021), the sulfate content in the microwave treatment (22.55%) and the polysaccharide extracted from N. zanardini algae by the conventional method (22.46%) did not differ significantly [35]. However, in some studies, the sulfate content in microwave treatments decreased compared to conventional treatments, attributed to the sensitivity of sulfate to high temperatures and subsequent thermal degradation. Okolie et al. (2019) extracted fucoidan from Ascophyllum nodosum algae using ultrasound, microwave, enzyme, and conventional methods and measured the sulfate content in the treatments as 17.3%, 18.8%, 15.4%, and 21.7%, respectively. In their study, the highest sulfate content was related to the conventionalmethod, followed by the microwave technique [37]. Yuan and Macquarrie (2015) evaluated the effect of temperatures of 90, 120, and 150°C and times of 5, 15, and 20 minutes under microwaves on the sulfate content of fucoidan extracted from A. nodosum algae. Their results showed that with increasing temperature at a constant time and also increasing time at a constant temperature, the sulfate content decreased. In their study, the sulfate content under optimal microwave conditions was measured at 27.83%, but in the conventional method based on 6 hours of acid treatment at 70°C, this value was reported as 29.33% [38]. This decrease in sulfate content occurs due to thermal sensitivity (of sulfate), and this point should be considered when using microwaves [37]. In the present study, the reason for the lower sulfate content in the microwave treatment compared to ultrasound is probably related to thermal sensitivity and degradation. The extracted polysaccharides in this study contained about 2 to 9% protein, 51 to 55.5% carbohydrate, and 9 to 15% uronic acid, depending on the type of treatment. Sulfated polysaccharides extracted from the green alga Monostroma nitidum contained 4% protein, 61.1% carbohydrate, and 16.8% uronic acid [39]. The amount of uronic acid in the polysaccharide extracted from N. Zanardini algae by conventional and microwave methods was reported as 14.57% and 16.26%, respectively [35]. In the present study, the amount of uronic acid in the microwave treatment was higher than in the ultrasound treatment and the control. In the microwave method, when waves are absorbed by the algal mass and electromagnetic energy is converted into thermal energy, the temperature inside the cell increases, resulting in cell rupture and release of polysaccharides into the solvent. Under such conditions, uronic acid is released along with the polysaccharide. The presence of uronic acid in polysaccharides contributes to increasing their anticoagulant property due to improving and enhancing the flexibility of the polymer chain [40].

3-3- Antioxidant activity of research treatments

3-3-1- DPPH free radical scavenging activity

Figure 1 shows the DPPH free radical scavenging activity of the control, ultrasound, and microwave treatments at different concentrations. As can be seen in this figure, at all four concentrations examined, the scavenging activity of the ultrasound treatment is significantly higher than that of the control and microwave ($p<0.05$). Also, this index showed higher values in the microwave treatment at all concentrations except 0.5 mg/ml compared to the control ($p<0.05$). At the mentioned concentration, the control and microwave treatment did not show a significant difference in DPPH free radical scavenging activity ($p>0.05$). According to Figure 1, at concentrations of 1, 1.5, and 2 mg/ml, the antioxidant activity of ascorbic acid and the ultrasound treatment did not have a significant difference ($p>0.05$). With a 0.5-unit increase in concentration, the DPPH free radical scavenging activity of the research treatments increased significantly ($p<0.05$). Of course, there was an exception in this case; the scavenging activity of the ultrasound treatment (and also ascorbic acid) did not show a significant change with increasing concentration from 1.5 to 2 mg/ml ($p>0.05$). The scavenging activity of the ultrasound treatment ranged from 27.36±0.59% at a concentration of 0.5 mg/ml to 68.18±1.31% at a concentration of 2 mg/ml. These values ranged from 19.68±0.27% to 55.89±0.92% for the microwave treatment and from 19.35±0.48% to 47.83±1.42% for the control.

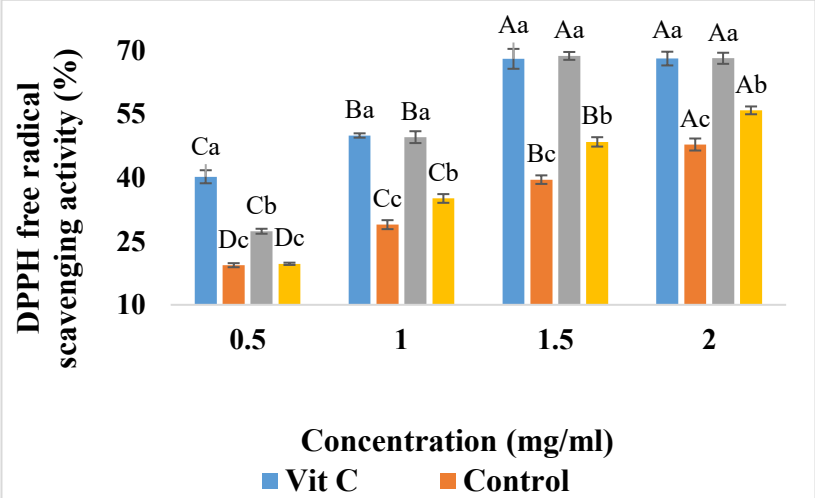

Figure 1-DPPH Free radical scavenging activity of treatments. Different capital letters indicate significant differences between data from a treatment at different concentrations ($p<0.05$). Also, different lowercase letters indicate a significant difference between the data of different treatments at the same concentration ($p<0.05$).

Table 3 shows the IC_{50} of DPPH radical scavenging for the positive control, control, ultrasound, and microwave treatments. As observed in this table, the ultrasound treatment showed the lowest IC_{50} value (1.04±0.03 mg/ml) ($p<0.05$) and in this regard, did not have a significant difference from ascorbic acid (positive control) ($p>0.05$). Also, the IC_{50} of the microwave treatment (1.61±0.05 mg/ml) was significantly lower than that of the control (2.12±0.07 mg/ml) ($p<0.05$).

Table 3- IC_{50} of DPPH free radical scavenging of treatments

Treatments	IC_{50} (mg/ml)
Ascorbic acid	1.03±0.04[a]
Control	2.12±0.07[c]
Ultrasound	1.04±0.03[a]
Microwave	1.61±0.05[b]

- Different letters indicate significant differences between data of different treatments ($p<0.05$).

3-3-2- ABTS free radical scavenging activity

Figure 2 shows the ABTS radical scavenging activity of the positive control, control, ultrasound, and microwave treatments. According to this figure, at the examined concentrations, except for 0.5 mg/ml, the ultrasound treatment had stronger scavenging activity than the control and microwave treatment ($p<0.05$). At the mentioned concentration, the two microwave and ultrasound treatments did not show a significant difference in scavenging activity ($p>0.05$). Also, at all studied concentrations, the scavenging activity of the microwave treatment was significantly higher than the control ($p<0.05$). According to Figure 2, with a 0.5-unit increase in concentration, the scavenging activity of all treatments increased significantly ($p<0.05$). Also, the minimum values were related to the concentration of 0.5 mg/ml, and the maximum was recorded at the concentration of 2 mg/ml ($p<0.05$). The ABTS radical scavenging activity values for the control, ultrasound, and microwave at the two aforementioned concentrations were 20.54±1.46% and 50.19±2.47%, 29.97±1.16% and 77.98±2.02%, and 29.45±1.01% and 62.35±1.84%, respectively.

3-3-2- ABTS free radical scavenging activity

Figure 2 shows the ABTS radical scavenging activity of the positive control, control, ultrasound, and microwave treatments. According to this figure, at the examined concentrations, except for 0.5 mg/ml, the ultrasound treatment had stronger scavenging activity than the control and microwave treatment ($p<0.05$). At the mentioned concentration, the two microwave and ultrasound treatments did not show a significant difference in scavenging activity ($p>0.05$). Also, at all studied concentrations, the scavenging activity of the microwave treatment was significantly higher than the control ($p<0.05$). According to Figure 2, with a 0.5-unit increase in concentration, the scavenging activity of all treatments increased significantly ($p<0.05$). Also, the minimum values were related to the concentration of 0.5 mg/ml, and the maximum was recorded at the concentration of 2 mg/ml ($p<0.05$). The ABTS radical scavenging activity values for the control, ultrasound, and microwave at the two aforementioned concentrations were 20.54±1.46% and 50.19±2.47%, 29.97±1.16% and 77.98±2.02%, and 29.45±1.01% and 62.35±1.84%, respectively.

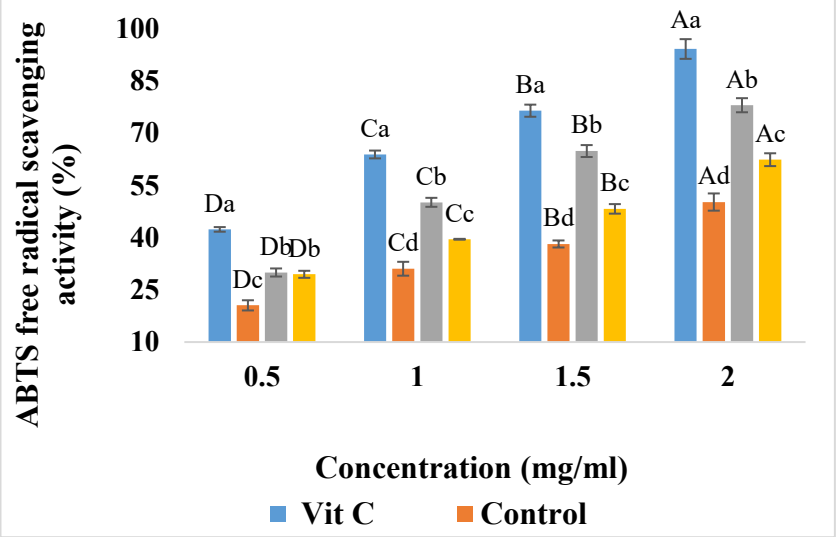

Figure 2-ABTS free radical scavenging activity of treatments. Different capital letters indicate significant differences between data from a treatment at different concentrations ($p<0.05$). Also, different lowercase letters indicate a significant difference between the data of different treatments at the same concentration ($p<0.05$).

Table 4 shows the IC_{50} of ABTS free radical scavenging for the positive control and treatments. As can be seen in this table, among the research treatments, the lowest IC_{50} was related to the ultrasound treatment (0.99±0.02 mg/ml) ($p<0.05$). Also, the microwave treatment (1.56±0.03 mg/ml) had a significantly lower IC_{50} than the control (1.97±0.04 mg/ml) ($p<0.05$).

Table 4- IC_{50} of ABTS free radical scavenging of treatments

Treatments	IC_{50} (mg/ml)
Ascorbic acid	$0.68±0.02^{a}$
Control	$1.97±0.04^{d}$
Ultrasound	$0.99±0.02^{b}$
Microwave	$1.56±0.03^{c}$

- Different letters indicate significant differences between data of different treatments ($p<0.05$)

3-3-3- Ferric ion reducing power

Figure 3 shows the ferric ion reducing power of the control and research treatments alongside the positive control (ascorbic acid). According to this figure, among the control and the two ultrasound and microwave treatments, at all studied concentrations, the highest reducing activity was related to the ultrasound treatment ($p<0.05$). At the two concentrations of 0.5 and 1 mg/ml, the reducing power of the mentioned treatment and ascorbic acid did not differ significantly ($p>0.05$), but at higher concentrations, ascorbic acid showed higher values ($p<0.05$). Furthermore, the reducing power of the microwave treatment was measured to be higher than the control at all concentrations except 1.5 mg/ml ($p<0.05$). At this concentration, the reducing power of the control and microwave treatment did not show a significant difference ($p>0.05$). With a 0.5-unit increase in concentration, the reducing power of all treatments increased significantly ($p<0.05$). However, in the case of the control, increasing the concentration from 1.5 to 2

mg/ml did not result in a significant increase in reducing power ($p>0.05$), which was an exception. The reducing power of the ultrasound treatment ranged from an absorbance of 0.353±0.023 at a concentration of 0.5 mg/ml to an absorbance of 0.756±0.031 (at 700 nm wavelength) at a concentration of 2 mg/ml. These values for the microwave treatment and control ranged from an absorbance of 0.259±0.015 to 0.598±0.022 and from an absorbance of 0.161±0.015 to 0.479±0.034 at 700 nm wavelength, respectively.

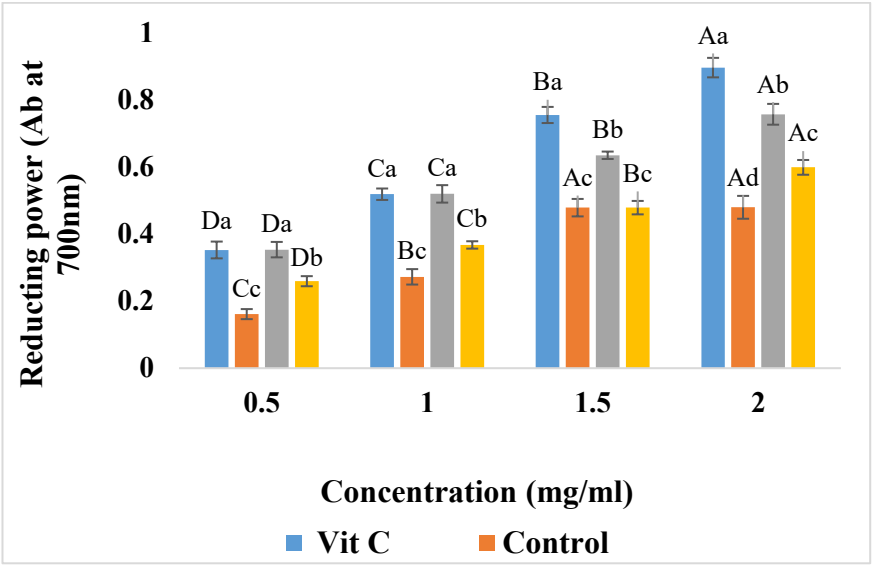

Figure 3- Reducting power of treatments. Different capital letters indicate significant differences between data from a treatment at different concentrations ($p<0.05$). Also, different lowercase letters indicate a significant difference between the data of different treatments at the same concentration ($p<0.05$).

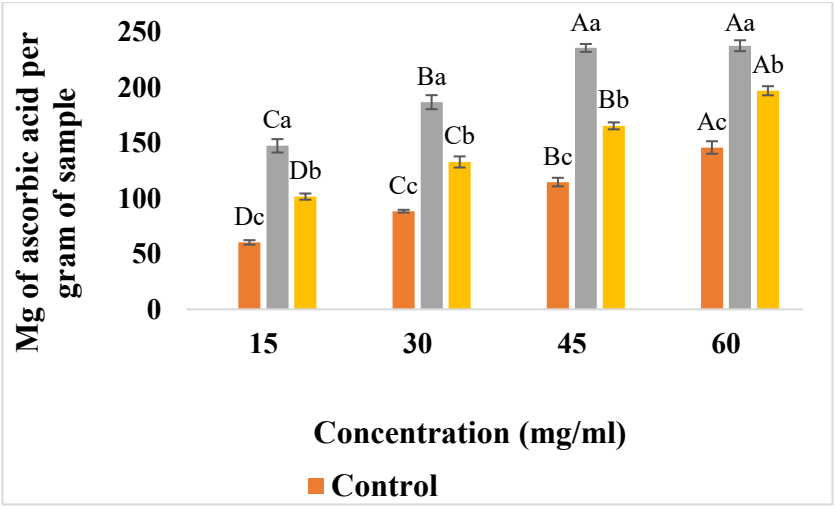

Figure 4-Total antioxidant activity of treatments. Different capital letters indicate significant differences between data from a treatment at different concentrations ($p<0.05$). Also, different lowercase letters indicate a significant difference between the data of different treatments at the same concentration ($p<0.05$).

3-3-4-Total antioxidant activity of research treatments

Figure 4 shows the total antioxidant activity of the control, ultrasound, and microwave treatments at concentrations of 15, 30, 45, and 60 mg/ml. According to this figure, at all four investigated concentrations, the total antioxidant activity of the ultrasound treatment was significantly higher than that of the microwave treatment and the control ($p<0.05$). This superiority was also recorded for the microwave treatment compared to the control ($p<0.05$). With a 0.5-unit increase in the concentration of polysaccharide treatments, the total antioxidant activity of all of them increased significantly ($p<0.05$), except that for the ultrasound treatment, increasing the concentration from 45 to 60 mg/ml did not cause a significant increase in total antioxidant activity ($p>0.05$). The total antioxidant activity values for the control, ultrasound, and microwave at concentrations from 15 to 60 mg/ml ranged from 60.26±2.04 to 145.74±5.63, 147.23±6.04 to 237.39±4.91, and 101.52±2.79 to 196.84±4.11 mg ascorbic acid/g polysaccharide, respectively.

The polysaccharides extracted from Chlorella vulgaris in the present study exhibited significant antioxidant activity. To the extent that in the DPPH radical scavenging test, the IC_{50} of the ultrasound treatment was comparable to that of ascorbic acid. According to the findings of this section, the use of ultrasound and microwave pretreatments increased the antioxidant activity of the extracted polysaccharides. It was further determined that the ultrasound treatment had a significant superiority over microwave in the antioxidant activity assays. The antioxidant activity of sulfated polysaccharides depends on various factors such as sugar type, molecular weight, degree of sulfation, acetylation position, and glycosidic branching, and has a direct and positive relationship with reducing potential [41]. Ultrasound and microwave waves, when interacting with the polysaccharide structure, cause them to break into smaller pieces and subsequently reduce their molecular weight [42]. This reduction in molecular weight increases the ability of these biopolymers to scavenge free radicals (and donate protons) [41 and 43]. Wang et al. (2008) in a study extracted fucoidan from Laminaria japonica algae at different molecular weights. Their research showed that the ability of the extracted polysaccharide to inhibit the oxidation of low-density lipoproteins (LDL) increases with decreasing molecular weight and increasing sulfate content [41]. Numerous studies have confirmed the role of ultrasound and microwave waves in improving and enhancing the antioxidant activity of sulfated polysaccharides. Yuan and Macquarrie (2015) conducted research on extracting fucoidan using microwaves from A. nodosum algae and reported the effective role of this method in increasing the antioxidant activity of fucoidan [38]. In a study where microwaves were used to extract fucoidan from the brown alga N. Zanardini, it was reported that the microwave treatment, due to having shorter chains (lower molecular weight), had a greater ability than the control to scavenge DPPH free radicals [35]. In the present study, one of the reasons for the higher antioxidant activity of the ultrasound treatment compared to microwave, and also the microwave treatment compared to the control, is the higher sulfate content. This is because the sulfate content in polysaccharides extracted from seaweeds plays a special role in manifesting antioxidant activity [41]. Since the sulfate group has the ability to activate the hydrogen atom of the anomeric carbon, it can help the polysaccharide bind hydrogen [44].

3-4- Anticancer activity of research treatments

Figure 5 shows the percentage of survival of T47D breast adenocarcinoma cancer cells against different concentrations of the research treatments. As can be seen in this figure, at all studied concentrations of the treatments, the highest cytotoxicity, leading to the lowest percentage of cancer cell survival,

was related to the ultrasound treatment ($p<0.05$). Also, in terms of cytotoxicity, the microwave treatment was at a higher level than the control at all concentrations ($p<0.05$). With increasing concentration of the polysaccharide treatments, their power to destroy cancer cells increased significantly, and consequently, the survival percentage of these cells decreased significantly ($p<0.05$). However, there was one exception in this regard, related to the control sample. Specifically, by increasing the concentration of this sample from 125 to 250 µg/ml, the survival percentage of cancer cells did not change significantly ($p>0.05$). At concentrations of 125, 250, 500, and 1000 µg/ml of the ultrasound treatment, the survival of the studied cancer cells was calculated as 71.15±2.24%, 60.28±1.12%, 45.33±1.05%, and 23.54±0.97%, respectively. These values for the microwave treatment were measured as 82.96±2.13%, 72.45±1.32%, 58.74±2.19%, and 37.69±1.28%, respectively.

Table 5- LC_{50} of research treatments for killing T47D breast adenocarcinoma cancer cells

Treatments	LC_{50} (µg/ml)
Control	1033.54 ± 9.27^{c}
Ultrasound	412.76 ± 5.02^{a}
Microwave	693.05 ± 7.34^{b}

- Different letters indicate significant differences between data of different treatments ($p<0.05$).

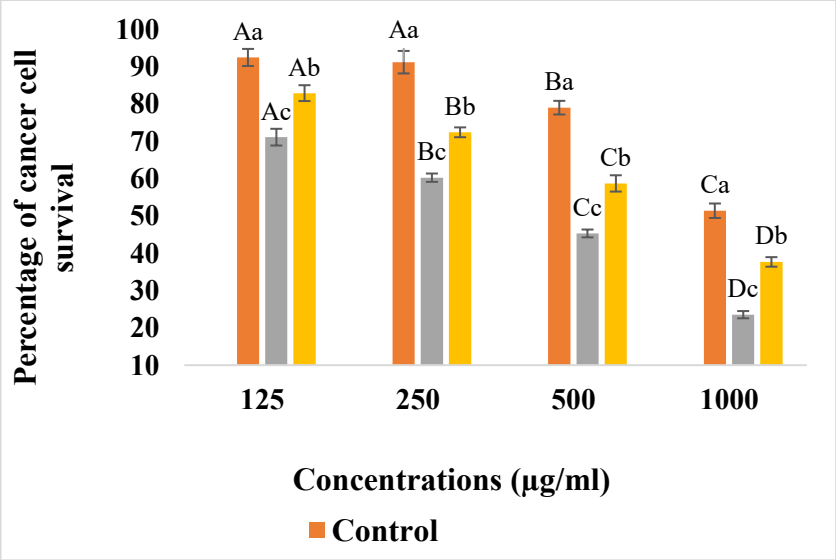

Figure 5- Percentage of survival of T47D breast adenocarcinoma cancer cells against different concentrations of research treatments. Different capital letters indicate significant differences between data from a treatment at different concentrations ($p<0.05$). Also, different lowercase letters indicate a significant difference between the data of different treatments at the same concentration ($p<0.05$).

Polysaccharides present in algae can cause toxicity to cancer cells and destroy them through multiple functional mechanisms including inhibition of cancer cell growth, invasion and metastasis, and induction of cell death [16]. Tran et al. (2023) conducted a study extracting ulvan polysaccharide from the green alga U. papenfussii and investigated its effects on cancer cells. Their findings showed that the IC_{50} of ulvan for destroying liver, breast, and cervical cancer cells was

89.78±6.55, 85.48±5.75, and 66.95±2.45 µg/ml, respectively [15]. In a study examining the anticancer activity of polysaccharides extracted from the brown alga Nemalion caricariense, this polysaccharide at a dose of 1000 µg/ml showed 82.63% anticancer activity against the A549 lung cancer cell line [16]. In addition to the polysaccharides present in algae, their extracts also possess anticancer properties. A study found that aqueous extracts produced from two algae, Hydroclathrus clathratus and Padina arborescens, were effective in destroying MCF-7 and HL-60 breast cancer cells; moreover, these extracts did not harm healthy cells [41]. The results of a study by Ganesan et al. (2008) on the cytotoxicity of extracts from the red alga Polysiphonia lanosa against DLD1 and HTC116 cells (human colorectal cancer) showed that chloroform and methanol extracts performed better than other extracts [45]. Findings from a study evaluating the effect of aqueous, methanolic, chloroform, n-hexane, and ethyl acetate extracts of the brown alga Cystoseira indica on the survival percentage of colorectal cancer cells indicated that the methanolic extract had the strongest effect and the lowest LC_{50} (723.16±14.47 µg/ml). Also, the n-hexane extract did not show a significant effect on destroying the studied cancer cells [46]. In the present study, the LC_{50} of the control, ultrasound, and microwave treatments for the T47D breast adenocarcinoma cancer cell line was measured as 1033.54±9.27, 412.76±5.02, and 693.05±7.34 µg/ml, respectively. This means that the ability of the ultrasound treatment to combat and destroy (the studied) cancer cells is significantly higher than that of the microwave treatment. This greater capability also exists for the microwave treatment compared to the control. Bavi et al. (2017) in a study investigated the cytotoxic effect of organic extracts of the alga Gelidiella acerosa harvested from the Chabahar coasts on breast (MCF-7) and colorectal (HT-29) cancer cell lines using MTT assays. The results of that study showed that the concentration of 1000 µg/ml of the methanolic extract of the alga exhibited the highest cytotoxic effect compared to the control and lower extract concentrations (125, 250, and 500 µg/ml). Also, the LC_{50} of the methanolic extract for colorectal and breast cancer cells was measured as 724.48±25.52 and 845.36±41.05 µg/ml, respectively [47].

4-Conclusion

The use of ultrasound and microwave pretreatments for extracting polysaccharides from the microalga Chlorella vulgaris increases the efficiency of the extraction process and the sulfate content of the extracted polysaccharides. Moreover, using these pretreatments enhances various properties of the sulfated polysaccharides, including antioxidant and anticancer activities. The sulfated polysaccharides extracted in the present study, in addition to their application in the pharmaceutical industry (for use in the formulation of anticancer drugs as well as antioxidant supplements), can also play a significant role in the food industry for use in food formulations as a preservative (antioxidant) compound.

Data Availability

The data used to support the finding of this study are available from the corresponding author upon request.

Conflict Of Interest

The authors have no conflicts interest to report.

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مقاله علمی-پژوهشی

اثر استفاده از پیش‌تیمارهای اولتراسوند و مایکروویو بر فعالیت آنتی‌اکسیدانی و ضد سرطانی پلی‌ساکاریدهای استخراج‌شده از میکروجلبک *کلرلا ولگاریس* (*Chlorella vulgaris*)

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چکیده

حدود دو دهه است که محققین به دنبال یافتن آنتی‌اکسیدان‌های طبیعی به منظور جایگزینی با انواع شیمیایی آن‌ها در فرمولاسیون مواد غذایی هستند تا از مضرات نگهدارنده‌های شیمیایی بکاهند. همچنین در راهکارهای نوین درمان سرطان، استفاده از ترکیبات ایمن طبیعی که هم توانایی نابودی سلول‌های سرطانی را داشته باشند و هم به سلول‌های سالم آسیب نرسانند، بسیار حائز اهمیت است. پلی‌ساکاریدهای استخراج‌شده از جلبک‌ها می‌توانند به عنوان آنتی‌اکسیدان و یک ترکیب ضد سرطان طبیعی عمل کنند و روش استخراج از عوامل تعیین‌کننده در بروز خواص متعدد آن‌ها می‌باشد. هدف این تحقیق بررسی اثر استفاده از پیش‌تیمارهای اولتراسوند و مایکروویو بر بازده استخراج، میزان سولفات، خواص آنتی‌اکسیدانی و ضد سرطانی پلی‌ساکاریدهای *کلرلا ولگاریس* بود. به این منظور، توان ۳۰۰ وات و زمان ۲۰ دقیقه و همچنین ۶۰۰ وات و ۱۰ دقیقه (پس از بهینه‌یابی) به ترتیب برای پیش‌تیمارهای اولتراسوند و مایکروویو انتخاب شدند. مطابق یافته‌ها، استفاده از پیش‌تیمارهای مذکور موجب افزایش بازده استخراج، محتوی سولفات، فعالیت آنتی‌اکسیدانی و ضد سرطانی پلی‌ساکاریدهای مستخرج (نسبت به شاهد) گردید ($p<0/05$). بیشترین حد بازده استخراج و محتوی سولفات (به ترتیب ۰/۱۹±۲۱/۷۳٪ و ۰/۰۷±۳۴/۱٪)، فعالیت مهار رادیکال‌های آزاد، قدرت کاهندگی، آنتی‌اکسیدانی کل و خاصیت ضد سرطانی مربوط به تیمار اولتراسوند بود ($p<0/05$). IC_{50} این تیمار برای مهار رادیکال‌های DPPH و ABTS به ترتیب ۰/۰۳±۱/۰۴ و ۰/۰۲±۰/۹۹ میلی‌گرم بر میلی‌لیتر محاسبه گردید. مطابق نتایج فعالیت ضد سرطانی تیمارها، LC_{50} شاهد و تیمارهای اولتراسوند و مایکروویو برای نابودی سلول‌های سرطانی آدنوکارسینومای پستان T47D به ترتیب ۹/۲۷±۱۰۳۳/۵۴، ۵/۰۲±۴۱۲/۷۶ و ۰۵/۳۴±۶۹۳/۷ میکروگرم بر میلی‌لیتر اندازه‌گیری شد. از این تحقیق می‌توان نتیجه گرفت که استفاده از پیش‌تیمارهای اولتراسوند و مایکروویو جهت استخراج، تا حد قابل توجهی خواص آنتی‌اکسیدانی و ضد سرطانی پلی‌ساکاریدهای *کلرلا ولگاریس* را ارتقاء می‌دهد. همچنین استفاده از این پلی‌ساکاریدها در فرمولاسیون مواد غذایی به عنوان نگهدارنده و در تولید داروهای ضد سرطان به عنوان ماده فعال پیشهاد می‌گردد.