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RESEARCH ARTICLE

Research on the structuring of water clusters in *Chlorella vulgaris* water suspension

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Abstract

Many bioactive compounds of natural origin have beneficial effects on human health and are used to treat different diseases. *Chlorella* is a genus of green algae with a high potential for producing biologically active substances. Exposure to extreme conditions can enhance its antioxidant activity and the production of concrete metabolites. *C. vulgaris* is cultivated in plantations. It is accessible in pharmacies and drugstores. The Health Act of 2005 in Bulgaria allows the therapeutic and prophylactic use of herbs, both independently by patients and as prescribed by a doctor. This study performed comparative spectral analyses of C. vulgaris using a 1% suspension of *C. vulgaris* in deionized water (v/v) by the methods of Non-equilibrium energy spectrum (NES) and Differential non-equilibrium energy spectrum (DNES). The research was performed in order to make indirect studies of the biological effects of *C. vulgaris*, which are connected with calcium conductivity and anti-inflammatory and anti-tumor effects. The effects of structuring of water clusters by *C. vulgaris* were examined. The data from spectral analyses, connected with a peak at (E =-0.1312 eV)(λ=9.45 μm) ($\tilde{\nu}$=1058 cm^{-1}), revealed anti-inflammatory effects. The anti-oxidant and anti-tumor effects of *C. vulgaris* were shown at (E=-0.1387 eV)(λ=8.95 μm) ($\tilde{\nu}$=1117 cm^{-1}). The results showed effects of improvement of calcium conductivity and anti-inflammatory, antioxidant and antitumor effects of *C. vulgaris* on human health.

Keywords

Chlorella vulgaris, Non-equilibrium energy spectrum analysis, Differential non-equilibrium energy spectrum analysis, cluster structuring

Introduction

Microalgae include unicellular, simple and photosynthetic organisms. Today, it has been found that they can be a valuable source of various biologically active molecules for use in various areas of life, such as the food industry,

4.10^{-5}, $MnSO_4$–3.10^{-4}, $FeSO_4 2H_2O$–0.003, $CaCl_2 6H_2O$–0.065, KH_2PO_4–1.25, $MgSO_4 7H_2O$–2.5; KNO_3–5.0 The Tamya medium was applied to grow *C. vulgaris* (24).

The source of nitrogen in ammonium nitrate (NH_4NO_3) and of the carbon is diamide of carbamic acid or urea $(CO(NH_2))_2$ (25, 26).

Light conditions for research

LED workplace articulated lamp 40W

Spectral methods Non-equilibrium energy spectrum (NES) and Differential non-equilibrium energy spectrum (DNES)

The spectral methods NES and DNES in infrared spectroscopy from 8 to 14 μm were applied. The methods NES and DNES are based on the evaporation of water droplets from a flat hydrophobic surface (27, 28). With the methods, NES and DNES were made in the studies of different types of water and 1% solutions in deionized water of plant juices (29-32).

The method of molecular dynamic simulation was applied for research on the interaction of the components in water droplets on the surface of sandstone under different conditions (33, 34).

A model of hydrogen-bonded liquids of Luck was used for the preparation of spectral methods NES and DNES (35-39).

The research with spectral methods NES and DNES was performed with a device with an optical design to measure wetting angle θ (40, 41). The process of evaporation was in a hermetic chamber.

Transparent polyester film BoPET or mylar was used for water drops (Fig. 2).

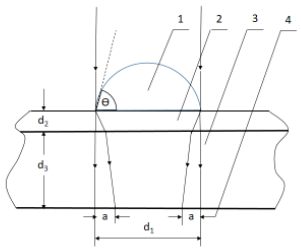

Fig. 2. Design of hard surface for study of wetting angle during the evaporation of water drops. 1 – water drop, 2 – mylar, 3 – glass base, 4 – width of the optical refraction ring.

The parameters **a** and $\mathbf{d_1}$ were included in the function of θ.

The relation between θ and hydrogen bonds' average energy is the following:

$\theta = \arccos(-1+bE)$, where $b = I/(1+\cos\theta_0)/C\gamma o$ (1)

where θ is the wetting angle, E is the average energy of hydrogen bonds and b is a temperature-dependent parameter (43-45).

Let's name with Δf(E) the difference between the energy spectrum NES of a certain water sample and that of a control sample with water. The sample is influenced by a certain physical and chemical method. The measurement of the sample and control sample are done simultaneously. The difference:

$\Delta f(E) = f(sample) - f(control\ sample)$ (2)

is defined as spectrum DNES (46-48).

The biggest local maximums of methods NES and DNES in eV^{-1} of the function of distribution of energy f(-E) are:

1. For E=-0.1112 eV or 897 cm^{-1} is the maximum, connected with the conductivity of calcium ions (29);

2. For E=-0.1212 eV or 978 cm^{-1} is the maximum, indicated with anti-inflammatory effects (29-32);

3. E=-0.1387 eV or 1117 cm^{-1} is the maximum for estimating anti-tumor effects (30-32).

These local maximums were registered with the tested plant solution (30, 32).

Fourier Transform IR Spectral Analysis

The spectral range of Fourier-IR spectrometer Brucker Vertex is in the near and middle infrared range from 1.28 to 27.03 μm or 3700 to 370 cm^{-1}.

The spectral range of Thermo Nicolet Avatar 360 Fourier-transform IR is the near and middle infrared range from 2.5 to 12.5 μm or 4000 to 800 cm^{-1}.

Methods for research in heavy water

For the studies of the effects of heavy water, the following chemicals, growth media and growth conditions were used (20-21).

Chemicals

For the research with heavy water for cultivation, media were used D_2O and DCl. The parameters are D_2O (99.9 atom. %) and DCl (95.5 atom. %). The crystallization of inorganic salts and glucose was in D_2O. The chemical compounds were dried in a vacuum. With ^{1}H-NMR on a Brucker WM-250 device, it was applied to control isotopic enrichment of D_2O distilled over $KMnO_4$.

Statistical analysis

The statistical processing of the results was performed according to the classical method of Student-Fisher with a t-test. Microsoft® Office Professional Plus Excel 2013 (15.0.4569.15060) was used for the calculations, with rights from the institutes and centers of the authors, Sofia, Bulgaria. The average values and their standard deviations were calculated. Student's t-test analysis for independent samples was applied to determine the statistical dependence and reliability of the results. The Student's t-test was counted for 3 results in each group. The significance of the differences was defined at significance level $p<0.01$ and <0.05.

Results and Discussion

The data received in the present research by application of infrared spectral methods–NES, DNES and Infrared Fourier spectral analyses in the middle infrared range (8-14 μm) were as follows.

agriculture, the pharmaceutical sector, food supplements etc. They can also be very successfully used in the cosmetic industry. *Chlorella vulgaris* is a green microalga, spherical, subspherical, or ellipsoid in shape and dimensions 2-10 µm in diameter. Because of the lack of flagella, it is non-motile. Usually, the cells are individually located, but it is able to form colonies of up to 64 cells in freshwater, marine and soil environments. Mixotrophic, heterotrophic and autotrophic conditions stimulate the cell photosynthesis of *C. vulgaris*. The algae division process is from one mother cell into 2-32 daughter cells. This process is called sporulation; the cells are autospores (1, 2). The effects of metabolites of this microalga have been demonstrated in many studies (2, 3). *C. vulgaris* was applied in the practice of receiving different food additives.

C. vulgaris has the following metabolites: lipids, proteins, carbohydrates, pigments, vitamins and minerals (2, 3).

The bio components of the metabolites are:

Lipids – triglycerides, phospholipids, glycolipids;

Carbohydrates – cellulose, sulfonated polysaccharides, β-1-3-glucans, starch;

Proteins – amino acids;

Pigments – chlorophyll, β-carotene, lutein, astaxanthin, canthaxanthin;

Vitamins and minerals – microelements, macroelements, vitamins A, B, C, E

The structuring of water clusters in *C. vulgaris* has yet to be studied.

The hydroxyl groups (-OH) in water molecules are with covalent chemical bonds. There are electromagnetic hydrogen bonds between the water molecules. These bonds are weaker than the covalent ones Clusters of water molecules are the subject of scientific interest by some research groups. Exciting results in this aspect are presented. Theoretical models were created to describe the structuring of water clusters with formula $(H_2O)_n$ with hydrogen bonds. Three theoretical models for n=6-20 were represented by five parameters of water molecules: hydrogen bonding, charge-charge, polarization, intramolecular relaxation and repulsive(4).

Another theoretical model was created with tetrameter (n=4) and pentameter rings (n=5). The clusters with the cubstructure were described for n=8, 12, 16, and 20. The pentamer structures with n=10 and 15 were also shown (5). A model of water clusters for n=2-20 was made method using DFT (6). In 1964, Hohenberg and W. Kohn created the Density functional theory (DFT).

For n=20, approximately 25% of water molecules were combined in clusters (7).

The model with Gaussian distribution explained a maximum number of clusters with n=12 and 13 at (-E=-0.1162 eV)(λ=10.67 µm)($\tilde{V}$= 937 cm^{-1}) (8). Saykally et al. (9, 10) investigated water clusters $(H_2O)_n$ with n=3-50. Infrared spectroscopy studies have detected the following clusters: $H^+(H_2O)_{22}$ (11) and $(H_2O)_n$, n=6-22 (12).

Recently, an investigation was made to study the binary structure of water clusters of *Haberlea rhodopensis* Friv. in a dry state with n=2 (13). The dynamic process of structuring of water clusters in *H. rhodopensis* Friv. was performed. The biggest water cluster with n=15 was measured at (E=-0.1112 eV)(λ=11.15 µm)($\tilde{V}$=897 cm^{-1}) (14).

The following methods were applied to study water clusters in different materials and compounds:

^{1}H-NMR (15, 16), vibration–rotation–tunneling (VRT) (17), neutron diffraction (18) and SCC-DFTB method (19).

The present study aims to examine and illustrate the structuring of water clusters in a 1% solution of *C. vulgaris* in deionized water. Our research was performed with *Chlorella vulgaris* B-8765 (20,21). The present investigations aimed to analyze the parameters of adaptation of *Chlorella* sp. in heavy water. A comparative analysis of water clusters of *Chlorella* sp. in deionized and heavy water was performed. The expected results are in connection with positive biological influences on human health.

The study aims to show with the model of water clustering that *C. vulgaris,* which is growing in the medium of heavy water, has stronger hydrogen bonds with biomedical effects. The results are connected with anti-inflammatory, anti-oxidant and anti-tumor effects.

Materials and Methods

Algae. Pure culture of *C. vulgaris* B-8765 was used in our research (Fig. 1). A sample of 1% suspension of *C. vulgaris* cells with pH=6.49 and ORP=+216 mV was used for the experiments. The control sample was with deionized water with pH=6.30 and ORP=+280.

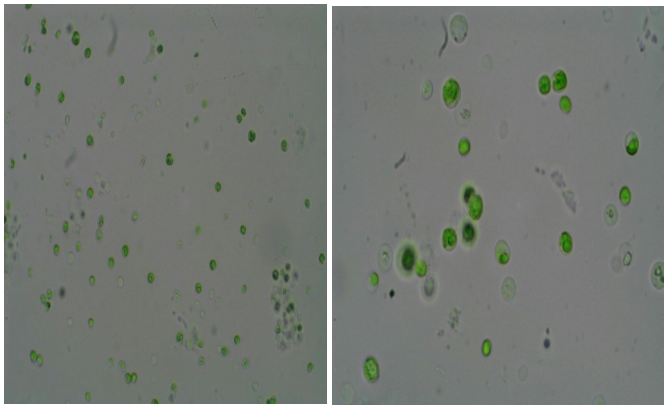

Fig. 1. *Chlorella vulgaris* culture: 400 x (left), 1000 x (right).

The chemical constitution of C. vulgaris B-8765.

The chemical constitution of *C. vulgaris* is the next: Phosphorus (P^{3-})–1680; Potassium (K^+)–732.7; Calcium (Ca^{2+})–564.8; Zinc (Zn^{2+})–77 mg.100 g^{-1}. This alga was used for studies with deionized and heavy water (22, 23).

Cultivation Media

The concentration of D_2O for cell growth and adaptation of green algae *C. vulgaris* in various nutrient media was 0; 24.5; 49.0; 73.5 %.

The composition of nutrient Tamya medium ($g.L^{-1}$) was: $CoCl_2.6H_2O$ – 5.10^{-6}; $CuSO_4.5H_2O$– 5.10^{-5}, $ZnSO_4.7H_2O$–

Results by the spectral methods NES and DNES

Studies with spectral methods NES and DNES were performed with a 1% suspension of *C. vulgaris* in deionized water. A 1% suspension of *C. vulgaris* cells was a sample and deionized was the control sample.

The result of the sample was E=-0.1248 eV and the control sample was E=-0.1142 eV. The result for spectrum DNES was Δ E=-0.0106 eV.

For each example, ten measurements were performed. Student's t-test was applied. A statistically significant difference was proved between the 2 groups of results with the samples and control samples according to the Student's t-test at the p <0.01 level.

Research has been executed on structuring water molecule clusters after altering hydrogen bond energies. The restructuring appears with the rearrangement of water molecules by the energy levels of hydrogen bonds. Local extrema can be detected in the spectrum with the largest water molecules.

Table 1 and Fig. 3 illustrate the number distribution of water molecules in the sample and control sample according to the energies of hydrogen bonds.

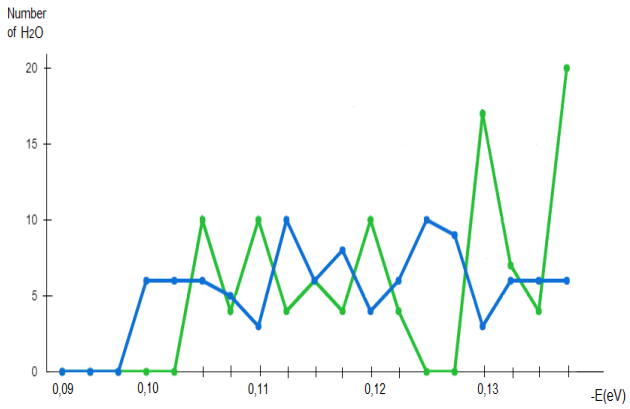

Fig. 3. The number distribution of water molecules in the sample (red color) with a 1% suspension of *C. vulgaris* and a control sample (blue color) with deionized water according to the energies of hydrogen bonds.

The most significant number of clusters of water molecules were provided at E= -1112, -1212, -1387 eV (Table 1 and Fig. 3).

These spectral analyses provide information about the parameters related to the effects on conduction connected with calcium (Ca^{2+}) ions and maximum at (E=-0.1112 eV)(λ=11.15 μm)($\tilde{V}$=897 cm^{-1}). The water cluster is with 10 water molecules. The investigations were also performed to anti-inflammatory maximum (E = -0.1212 eV) (λ=10.23 μm)($\tilde{V}$=978 cm^{-1}). The water cluster is with 10 water molecules. The local maximum for anti-tumor effects is at (E=-0.1387 eV)(λ=8.95 μm)($\tilde{V}$=1117 cm^{-1}). The water cluster is with 20 water molecules (48-50).

The marked difference between the 2 curves in Fig. 3 for the values of (–E) greater than 0.13 eV indicates that the 1% suspension of *C. vulgaris* in deionized water contains more clusters of more than 10 water molecules compared to the control sample of deionized water.

IR Fourier spectral analysis of C. vulgaris

Fig. 4 illustrates the IR Fourier spectral analysis results of a 1% suspension of *C. vulgaris*.

In the range of the NES (-E) methods, 2 local maximums were found corresponding with the local maximums in IR Fourier spectral analysis. There were at E=(-0.1312 eV)(λ=8.95 μm)($\tilde{V}$=1058 cm^{-1}) and (E=-0.1387 eV)(λ=8.95 μm) ($\tilde{V}$=1117 cm^{-1})

The local maximum at (-0.1312 eV)(λ=8.95 μm) ($\tilde{V}$=1058 cm^{-1}) is typical for anti-inflammatory and anti-oxidant effects (47, 51, 52). These obtained results indicate the potential for the anti-inflammatory and anti-oxidant activity of *C. vulgaris* if administered for prophylactic and therapeutic purposes in animals and humans.

Results of C. vulgaris in heavy water

The research of adaptation of *C. vulgaris* was made with content of 24.5, 49.0 and 73.5 up to 98 % (v/v) D_2O. The Tamiya liquid nutrient medium was used. The results are shown in Fig. 5.

Table 1. The number distribution of water molecules in the sample with 1% suspension of *C. vulgaris* and a control sample with deionized water according to the energies of hydrogen bonds.

-E(eV) x-axis	Number of Water Molecules in		-E(eV) x-axis	Number of Water Molecules in	
	1% Suspension of *C. vulgaris* y-axis	Control Sample of Deionized Water y-axis		1% Suspension of *C. vulgaris* y-axis	Control Sample of Deionized Water y-axis
0.0937	0	0	0.1187	4	8
0.0962	0	0	0.1212	10^2	4^2
0.0987	0	0	0.1237	4	6
0.1012	0	6	0.1262	0	10
0.1037	0	6	0.1287	0	9
0.1062	10	6	0.1312	17	3
0.1087	4	5	0.1337	7	6
0.1112	10^1	3^1	0.1362	4	6
0.1137	4	10	0.1387	20^3	6^3
0.1162	6	6	–	–	–

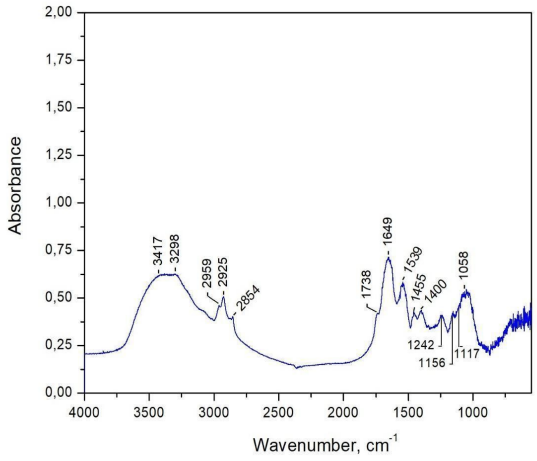

Fig. 4. Results of IR Fourier spectral analysis of *Chlorella vulgaris*.

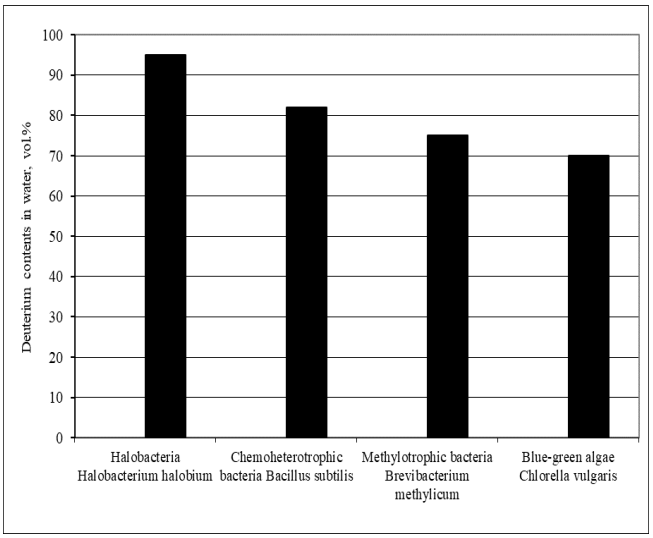

Fig. 5. Survival of different microorganisms in water with different deuterium content (%, v/v).

The parameters of deuterium enrichment in *C. vulgaris* of carotenoids were investigated.

The stimulation of *C. vulgaris* was with the biosynthesis of deuterated carotenoid pigments and chlorophyll (23).

The research shows that *C. vulgaris* multiplies on 70% (v/v) ^2H_2O. Fig. 5 shows the results of other organisms. The result for methylotrophic bacteria is 75% (v/v), chemo-heterotrophic bacteria – 82% (v/v) and photo-organotrophic halobacteria – 95 % (v/v).

Studies with spectral methods NES and DNES were performed for a 1% suspension of *C. vulgaris* in 24.5% heavy and 75.5 % deionized water. This was a sample. The control sample was with 24.5% heavy and 75.5 % deionized water.

The result of the sample was E=-0.1260 eV and the control sample was E=-0.1138 eV. The result for spectrum DNES was Δ E=-0.0122 eV.

For each example, ten measurements were performed. Student's t-test was applied. A statistically significant difference was proved between the two groups of results with the samples and control samples according to the Student's t-test at the p <0.01 level.

Table 2 and Fig. 6 illustrate the number distribution of water molecules in the sample and control sample according to the energies of hydrogen bonds.

The most significant number of clusters of water molecules were provided at E= -1212; 0.1312; -1387 eV (Table 2 and Fig. 6).

These spectral analyses provide information about the parameters related to the anti-inflammatory effects at (E = -0.1212 eV)(λ=10.23 μm)($\tilde{V}$=978 cm^{-1}) and (E = -0.1312 eV)(λ=9.45 μm)($\tilde{V}$=10.58 cm^{-1}). The number of water

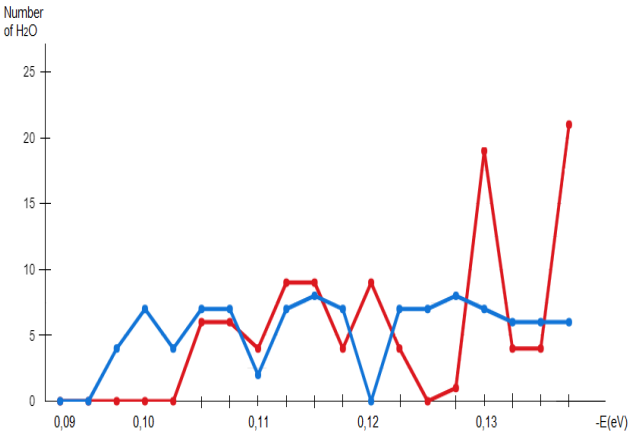

Fig. 6. The number distribution of water molecules in the sample (red color) with 1% suspension of *C. vulgaris* in 24.5% heavy and 75.5 % deionized water and a control sample (blue color) with 24.5% heavy and 75.5 % deionized water according to the energies of hydrogen bonds.

Table 2. The number distribution of water molecules in the sample with 1% suspension of *C. vulgaris* in 24.5% heavy and 75.5 % deionized water and a control sample with 24.5% heavy and 75.5 % deionized water according to the energies of hydrogen bonds.

-E(eV) x-axis	Number of Water Molecules in		-E(eV) x-axis	Number of Water Molecules in	
	1% Suspension of *C. vulgaris* 24.5% Heavy Water y-axis	Control Sample of Deionized Water y-axis		1% Suspension of *C. vulgaris* 24.5% Heavy Water y-axis	Control Sample of Deionized Water y-axis
0.0937	0	0	0.1187	4	7
0.0962	0	0	0.1212	**9^2**	**0^2**
0.0987	0	4	0.1237	4	7
0.1012	0	7	0.1262	0	7
0.1037	0	4	0.1287	1	8
0.1062	6	7	0.1312	19	7
0.1087	6	7	0.1337	4	6
0.1112	4^1	2^1	0.1362	4	6
0.1137	9	7	0.1387	21^3	6^3
0.1162	9	8	–	–	–

Table 3. Illustrates the results with DNES with *C. vulgaris* in 24.5% heavy and 75.5 % deionized water and *C. vulgaris* in deionized water. The first control sample is heavy water and the second is deionized water.

DNES (eV) ***C. vulgaris*** **24.5 % D_2O; 75.5 % deionized H_2O**	**DNES (eV)** ***C. vulgaris*** **deionized H_2O**	
12.8	10.2	Degree of freedom: f =
12.2	10.5	
12.7	10.8	
12.1	10.1	t – statistics: t = 3.138
12.2	10.5	
11.5	10.6	
12.2	11.4	Significance: p < 0.01
11.6	10.5	
12.9	10.8	
11.7	10.3	
m = 12.11	m = 10.53	Correlation coefficient: r =0.2718
s = 0.5079	s = 0.2706	

molecules is 9 at E=-0.1212 eV and 19 at E= -0.1312 eV. The water cluster is with 10 water molecules. The local maximum for anti-tumor effects is at (E=-0.1387 eV) (λ=8.95 μm)($\tilde{V}$=1117 cm^{-1}). The water cluster is with 21 water molecules.

Table 3 illustrates the results with DNES with *C. vulgaris* in 24.5% heavy and 75.5 % deionized water and *C. vulgaris* in deionized water. The first control sample is heavy water and the second is deionized water.

The data from Table 3 show the DNES of 24.5 % D_2O; 75.5 % deionized H_2O as a sample with 1% suspension of *C. vulgaris* and a control sample of 24.5 % D_2O; 75.5 % deionized H_2O are statistically different with t-test of Student $p < 0.01$ according to DNES of deionized H_2O as a sample with 1% suspension of *C. vulgaris* and a control sample with deionized H_2O.

The beneficial properties of *C. vulgaris* were estimated using other methods for anti-microbial activity (53-55), anti-tumor (55, 56), anti-oxidant (57) and anti-fungal (57).

The present results with spectral analyses of *C. vulgaris* were connected with (E=-0.1312 eV)(λ=9.45 μm) ($\tilde{V}$=1058 cm^{-1}) and were shown anti-inflammatory and anti-oxidant effects of this green algae. Such biological activity of *C. vulgaris* has been reported. The results from spectral tests at (E=-0.1387 eV)(λ=8.95 μm)($\tilde{V}$=1117 cm^{-1}) were connected with anti-oxidant and anti-tumor effects.

These obtained results indicate the potential for the anti-inflammatory and anti-oxidant activity of *C. vulgaris* if administered for prophylactic and therapeutic purposes in animals and humans.

Conclusion

For the first time, spectral analyses of *C. vulgaris* have been performed. The purpose was to determine the health-promoting biological effects of this alga.

The result of the tests of 1% suspension of *C. vulgaris* with NES spectrum was E=-0.1248 eV. Two local maximums were found in the range of the NES (-E) methods. One of these, at (-0.1312 eV)(λ=8.45 μm) ($\tilde{V}$=1058 cm^{-1}), is characteristic for finding out anti-inflammatory and anti-oxidant effects.

The experiments with D_2O revealed new properties of *C. vulgaris* as it can grow on 70 % (v/v) D_2O. The results of 1% suspension (*v/v*) *C. vulgaris* in 24.5% heavy and 75.5% deionized water with NES were: E=- 0.1260 eV. The data from spectral analyses of *C. vulgaris*, connected with (E=-0.1312 eV)(λ=9.45 μm) ($\tilde{V}$=1058 cm^{-1}), showed anti-inflammatory and anti-oxidant effects of this green alga. The data from spectral tests at (E=-0.1387 eV)(λ=8.95 μm) ($\tilde{V}$=1117 cm^{-1}) also revealed the anti-oxidant and anti-tumor effects of *C. vulgaris*.

All the results of the present study show that the green algae *C. vulgaris* can potentially render such beneficial effects if applied for nutritive, prophylactic and therapeutic purposes.

Authors' contributions

I. I., A. I .I. and M. T. I. carried out the spectral analyses, T. P. P., R. B., T. T.-Y., and V.D. performed the biochemical analyses, participated in the sequence alignment, and drafted the manuscript. Ch. D., M. A., T. E. P, and M. D. K. carried out the comparative analyses. S. B. and F.H. participated in the sequence alignment. N. V., Zh. D., and I. G. participated in the design of the study and performed the statistical analysis. I. I. and T. P. P. conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest.

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