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Research Article

An In-Vitro Investigation of the Antibacterial Effects of the Methanol and Aqueous Extracts and the Supernatant of the Algae *Chlorella vulgaris* CCATM 210-1 on Multiantibiotic-Resistant *Staphylococcus aureus* Isolates Causing Urinary Tract Infections

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ABSTRACT:
Algae contain compounds, some of which have antibacterial properties. For example, the antibiotic Chlorellin can be mentioned, which is extracted from *Chlorella* species. The methanol and aqueous extracts, and the supernatant of the algae *Chlorella vulgaris* CCATM- 210-1 were used in this study. After culturing the Algae and preparing the supernatant and extracts, the antibacterial effects of the extracts and supernatant of this algae against multidrug-resistant (MDR) *Staphylococcus aureus* isolates causing urinary tract infections were determined using the microdilution and checkerboard titration methods. In the microdilution method, first, the aqueous extract of this algae at a concentration of 0.72 ± 0.22 mg/ml could inhibit the growth of the bacteria being tested. Then, the methanol extract and the supernatant of this algae at concentrations of 0.98 ± 0.68 and 1 ± 0.26 mg/ml, respectively, inhibited the growth of the bacteria being studied. The sum of the FIC of the methanol and aqueous extracts of the algae *Chlorella vulgaris* CCATM 210-1 in this study, showed the indifference of these two extracts towards each other.

Keywords: *Chlorella vulgaris*, antibacterial effect, microdilution, checkerboard titration

INTRODUCTION

It has been more than 2,000 years that Algae have been used for food and pharmaceutical purposes. Their application in traditional medicine has created an incentive for researchers to begin large-scale studies about their properties. In this regard, different materials have been obtained from Algae, such as: amino acids, terpenoids, florentines, alkanes, halogenated ketones, steroidal compounds, annular polysulfides, fatty acids, phenols, etc., some of which have antibacterial effects. For example, the antibiotic Chlorellin can be mentioned, which is extracted from *Chlorella* species (Taskin et al., 2007). The unicellular algae *Chlorella vulgaris* CCATM-210-1 is one of the most famous micro-Algae with a diameter of 2 to 10 microns, which lives in fresh water. *Chlorella*, similar to plants, has a high density of chlorophyll, and it is very active in

terms of photosynthesis. Part of the healing properties of *Chlorella* in the body, is related to the large amount of chlorophyll and the structure of its cell wall, especially the constituents of this cell wall. This algae enhances the health and defensive power of the body skin (Safari et al., 2011). Urinary tract infections is one of the most common infections diagnosed in outpatients and inpatients, which can lead to significant complications in patients.

These infections cause a large amount of medical expenses each year, which will consequently have undesirable and adverse effects not only on individuals, but also on society. Hence, considering the use of natural medicines has become of particular importance (Pepeljnjak and Kosalec, 2004; Junior et al., 2005; Yakan et al., 2007). The increased number of antibiotic-resistant bacteria causes an increase in the costs of treatment and replacement of undesirable treatments in recurrent urinary tract infections, which causes the host to be inclined towards non-antibiotic treatments to prevent and control urinary tract infections (Wright and Sutherland, 2007; Jiagang, 2009).

Given the importance and role of Algae in controlling pathogenic bacteria, extensive researches have been conducted on various species of them. This study was conducted with the aim of identifying the inhibition properties of the methanol and aqueous extracts and the supernatant of the algae *Chlorella vulgaris* CCATM- 210-1 against multidrug-resistant (MDR) *Staphylococcus aureus* isolates causing urinary tract infections.

MATERIALS AND METHODS

Materials and devices used in this research

Consuming materials:

The Mueller-Hinton agar (MHA) medium, the Trypticase soy broth (TSB) medium, and the Mueller-Hinton Broth (MHB) medium were all prepared from Qlab Company in Canada. Tamiya medium, methanol 99.9%, dimethyl sulfoxide (DMSO) 99.9%, and distilled water (all made by Merck Company in Germany), the physiological serum, Vancomycin Sigma commercial powder, antibiogram disks made by Padtan Teb Company in Iran.

Devices:

Autoclave model D121 manufactured by Iran Tolid Company, Digital Scale model SBA 32 manufactured by SCALTEC Company in Germany, Spectrophotometer model M259 manufactured by Sherwood Company in the U.K., Freeze-Dryer model CHRIST-21165 made in Germany, Elisa Reader model Awareness technology made in the U.S., Tube Shaker LS-100 manufactured by Labtron Company in Iran, Plate Shaker (PARS Teb Novin Iranian International Company), Incubator and Hot Air Oven (both manufactured by Behdad Company in Iran).

The conditions of culturing and preparing the supernatant of the algae *Chlorella vulgaris*:

To conduct this research, the green algae *Chlorella vulgaris* CCATM-210-1 was prepared from the culture collection of Algae of Department of Marine Biology, Tarbiat Modarres University in Noor City.

Sterilization: A UV lamp was used for 20 minutes to sterilize the culture room. All the glass tools were first washed by distilled water, then were dried and sterilized at a temperature of 180 °C in the oven for one hour. The Erlenmeyer flasks containing liquid media, were covered by cotton and aluminum foil, and were placed in the autoclave at a temperature of 121 °c and pressure of 15 Pounds/Inch^2 for 20 minutes, to be sterilized.

Light setting: In this study, fluorescent lamps with an intensity of 60 µM.m^{-2}.s^{-1} and a light/dark cycle of 9:15 were used.

Temperature setting: The cultivation temperature for the algae *Chlorella vulgaris* CCATM 210-1, was set in the range of 27 ± 1 °C.

Cultivation: Cultivation of the Algae *Chlorella vulgaris* CCATM 210-1 was done in a specific cultivation room (Ficolab) with fluorescent lamps on its walls. First, one liter of Tamiya medium

was added to the 2-liter Erlenmeyer flask, and the algael samples were added.

Then during the logarithmic phase and near the flame, the sterile culture media were inoculated to the extent that the optical density was set between 0.03-0.04. The optical density was read by UV-VIS spectrophotometer on a daily basis, and when the optical density reached 1.2 (stationary phase), the suspensions were centrifuged and finally the Algae and supernatant were separated.

To conduct the research, the supernatant was poured into a large glass plate and put at the ambient temperature (25-28 °C) away from sunlight, to be dried (Ghasemi et al., 2007).

Preparing the powder of the algae *Chlorella vulgaris* CCATM 210-1

To prepare the algae powder, first, using a centrifuge machine, the algae paste was obtained from the obtained suspension during a 21-day period. Then, the algae paste was moved to the freeze dryer model CHRIST-21165, and was dried using the freeze drying method (Ghasemi et al., 2007).

The method for extracting the methanol and aqueous extracts of *Chlorella vulgaris* CCATM 210-1

First, the powder of the algae *Chlorella vulgaris* CCATM 210-1 was solved to the ratio of 5 gr per 100 ml of methanol solvent and Merck distilled water separately, and then they were placed on a shaker for 72 hours. After 72 hours, the extract was strained and poured into a large glass plate and then put at the ambient temperature (25-28 °C) away from sunlight, so that the solvent is removed and the extract is dried (Vishnu and Sumathi., 2014).

Preparing a suspension from the extracts and the supernatant

For this purpose, amounts of 1 gr from the powders of the dried extracts (methanol and aqueous) and supernatant were weighed separately and precisely, and inserted into a sterile container, which contained 100 ml of pure dimethyl sulfoxide, and then dilutions of 10 mg/ml were prepared from each extract and the supernatant to conduct the experiment (Annamalai et al., 2012).

The bacteria being tested

This study was conducted on 12 multiantibiotic-resistant *Staphylococcus aureus* isolates causing urinary tract infections, which had been collected from several medical laboratories and hospitals in Isfahan, and which were identified by biochemical tests. In order to investigate the antimicrobial effects, every time, a new 24-hour culture was prepared.

The test of determining the antibacterial effects of the methanol and aqueous extracts of the algae *Chlorella vulgaris* CCATM- 210-1 and examining the antimicrobial interaction between the extracts, using the checkerboard titration method

In the checkerboard titration method, a 96-well microplate was used. To determine the MIC and MBC of the methanol and aqueous extracts of this algae, the amounts of 50 µl from the higher to lower concentrations of the methanol extract were added to the wells of the first column from the top downward, and the methanol extract was not added to the well in the far bottom. The amounts of 50 µl from the higher to lower concentrations of the aqueous extract was poured in the wells of the first row from right to left, and the aqueous extract was not added to the last well. To examine the antimicrobial interaction between the two extracts, the amounts of 25 µl from the higher to lower concentrations of the methanol extract were added to the wells of the second column and the columns after that from the top downward, and the methanol extract was not added to the well in the far bottom. The amounts of 25 µl from the higher to lower concentrations of the aqueous extract was poured in the wells of the second row and the rows after that from right to left, and the aqueous extract was not added to the last well. Then a suspension equivalent to 0.5 McFarland standard (at wavelength of 630 nm and optical density of 0.08-0.13) was prepared from the 24-hour culture of the intended bacterium, and the amount of 10 µl

of it was added to the wells of the microplate, and 50 µl of the Mueller-Hinton Broth medium was added to the wells. The optical density of the microplate wells was read in the Elisa reader at a wavelength of 630 nm. Finally, the microplate was put in the incubator at 37 °C for 24 hours. After 24 hours, again the optical density of the wells was checked using the Elisa reader at a wavelength of 630 nm. By comparing the optical density before and after the incubation of each well as well as visual inspection of the turbidity created in the wells, the lowest dilution of the methanol and aqueous extracts in whose corresponding well no turbidity was observed, was considered as the MIC of the methanol and aqueous extracts. In order to determine the minimum bactericidal concentration (MBC) of the methanol and aqueous extracts, the amounts of 20 µl from the wells corresponding to MIC and three wells corresponding to the higher concentrations of the substance being tested, which had no detectable turbidity, were linearly cultivated on the MHA medium, and put in the incubator at 37 °C for 24 hours. After 24 hours of incubation, we investigated the growth of the bacteria on the plates. The concentration of the extract on whose solid culture medium, we had not seen any growth of the bacteria being tested, was considered as MBC. Finally, the MIC of the methanol and aqueous extracts in the combined state was determined too. In order to verify the obtained results, the experiments were carried out in 3 replications. Then the interaction between these two extracts was determined using the following relation and based on the calculation of the FIC or the fractional inhibitory concentration index (Pillai et al., 2015; Spoorthi et al., 2011).

$$\text{Sum FIC}_{BC} = \frac{\text{MIC B in combination}}{\text{MIC B alone}} + \frac{\text{MIC C in combination}}{\text{MIC C alone}}$$

B: The methanol extract obtained from the algae *Chlorella vulgaris* CCATM 210-1

C: The aqueous extract obtained from the algae *Chlorella vulgaris* CCATM 210-1

Sum FIC_{BC}: The sum of the fractional inhibitory concentrations of the methanol and aqueous extracts of the algae *Chlorella vulgaris* CCATM 210-1

MIC_B: The minimum inhibitory concentration of the methanol extract of the algae *Chlorella vulgaris* CCATM 210-1

MIC_C: The minimum inhibitory concentration of the aqueous extract of the algae *Chlorella vulgaris* CCATM 210-1

After calculating the sum of the FIC of the methanol and aqueous extracts of the algae *Chlorella vulgaris* CCATM 210-1, the results were interpreted based on Spoorthi et al.'s method in 2011, as follows: Values smaller than and equal to 0.5 indicates the synergistic effect, values between 0.5 and 4 and equal to 4 suggests the indifference of these two compounds toward each other, and values greater than 4 were considered to be due to the antagonistic effect between these two compounds.

Preparing a Vancomycin solution from its commercial powder

For this purpose, we weighed 1 gr of the commercial Vancomycin powder and placed it in the sterile container, which contained 100 ml of pure DMSO, and prepared a dilution of 10 mg/ml of the antibiotic Vancomycin for the desired samples.

The test of determining the MIC and MBC of the supernatant of *Chlorella vulgaris* CCATM 210-1 and the antibiotic Vancomycin using the microdilution method

A 96-well microplate was used to determine the MIC and MBC. First, 100 µl of the Mueller-Hinton Broth (MHB) medium were added to the wells number 1 to 10. Then, the amounts of 100 µl of the highest concentration of the supernatant of *Chlorella vulgaris* CCATM 210-1 and the desired antibiotic (10 mg/ml) were separately added to the first well of each row and mixed appropriately. One hundred µl of the solution was taken from the first well and added to the well number 2, which only contained 100 µl of the culture medium. This process was continued from the second well to the

third well and similarly up to the 10th well, so all the desired concentrations are made. One hundred µl of the well number 10 was removed, so the volumes of all the wells become equal. Then, a suspension equivalent to 0.5 McFarland standard was prepared from the 24-hour microbial culture of the bacteria, and 10 µl of it was added to the wells number 1 to 10. One hundred µl of the Mueller-Hinton Broth medium and 10 µl of the suspension of the desired bacterium with a turbidity equivalent to 0.5 McFarland standard was added to the well number 11, and was considered as a positive control, and 110 µl of the Mueller-Hinton Broth medium was added to the well number 12 as a negative control. The optical density of the microplate wells was read in the Elisa reader at a wavelength of 630 nm. Then, the microplate was put in the incubator at 37 °C for 24 hours. After 24 hours, the created optical density was checked again using the Elisa reader at a wavelength of 630 nm. By comparing the optical density before and after the incubation of each well as well as visual inspection of the turbidity created in the wells, the lowest dilution of the substance being tested, in whose corresponding well no turbidity was observed, was considered as the minimum inhibitory concentration (MIC). In order to determine the minimum bactericidal concentration (MBC), the amounts of 20 µl from the wells corresponding to MIC and three wells corresponding to the higher concentrations of the substance being tested, which had no detectable turbidity, were linearly cultivated on the Mueller-Hinton agar (MHA) medium, and put in the incubator at 37 °C for 24 hours. After 24 hours of incubation, we investigated the growth of the bacteria on the plates. The concentration of the desired substance on whose solid culture medium, we had not seen any growth of the bacteria being tested, was considered as MBC. In order to verify the test results of each sample, 3 replications were done (Zare Bidaki et al., 2014).

Statistical analysis:

The mean and standard deviation in this study, were calculated using SPSS software version 23.

Results

In the microdilution method, first, the aqueous extract of this algae at a concentration of 0.72 ± 0.22 mg/ml could inhibit the growth of the bacteria being tested. Then, the methanol extract and the supernatant of this algae at concentrations of 0.98 ± 0.68 and 1 ± 0.26 mg/ml, respectively, inhibited the growth of the bacteria being studied. In the present study, according to the results obtained in Table 3, the sum of the FICs of the methanol and aqueous extracts was obtained in the range of 0.5 to 4, and according to Spoorthi et al.'s justifications, it showed the indifference of these two extracts towards each other.

Table 1. The antibiogram results of the multiantibiotic-resistant *Staphylococcus aureus* isolates causing urinary tract infections collected from several medical laboratories and hospitals in Isfahan

Antibiotics / ***s. aureus***	**Ciprofloxacin** **CP** 5	**Gentamicin** **GM** 10	**Cefazolin** **CZ** 30	**Cefalexin** **CN** 30	**Meticillin** **ME** 5	**Chloramphenicol** **C** 30	**Erythromycin** **E** 15	**Ampicillin** **AM** 10	**Vancomycin** **V** 10	**Penicillin** **P** 10	**Amoxicillin** **AMX** 25	**Azithromycin** **AZM** 15	**Meropenem** **MEN** 10
S_1	**R**	**S**	**S**	**S**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**I**	**S**
S_2	**R**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**R**	**R**	**S**
S_3	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**

S_4	**R**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**R**	**R**	**S**
S_5	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**S**
S_6	**R**	**R**	**R**	**I**	**R**	**S**	**R**	**S**	**S**	**R**	**S**	**R**	**S**
S_7	**R**	**S**	**S**	**S**	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**S**
S_8	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**R**	**R**	**S**
S_9	**R**	**S**	**S**	**S**	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**S**
S_{10}	**R**	**R**	**R**	**I**	**R**	**S**	**I**	**R**	**R**	**R**	**S**	**R**	**R**
S_{11}	**R**	**S**	**I**	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**R**	**R**	**S**
S_{12}	**R**	**S**	**I**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**

Legend: R: Resistance; I: Intermediate ; S: Sensitive

Table 2. The mean MIC and MBC of the methanol and aqueous extracts and the supernatant of *Chlorella vulgaris* and the antibiotic Vancomycin against the MDR *Staphylococcus aureus* isolates causing urinary tract infections (in mg/ml) using the microdilution method

Bacterial isolates	methanol extract of *Chlorella vulgaris*		aqueous extract of *Chlorella vulgaris*		supernatant of *Chlorella vulgaris*		Vancomycin (Positive control)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S_1	0.52	1.04	0.52	1.04	0.83	1.66	0.26	0.52
S_2	1.25	2.5	1.04	2.08	0.83	1.66	0.26	0.52
S_3	0.83	1.66	1.04	2.08	1.04	2.08	0.52	1.04
S_4	1.46	2.91	0.63	1.25	1.25	2.5	0.52	1.04
S_5	0.83	1.66	0.83	1.66	0.83	1.66	0.41	0.83
S_6	0.83	1.66	0.63	1.25	1.04	2.08	0.21	0.41
S_7	2.91	5.83	0.83	1.66	1.04	2.08	0.1	0.21
S_8	0.63	1.25	1.04	2.08	0.63	1.25	0.21	0.41
S_9	0.52	1.04	0.52	1.04	1.25	2.5	0.52	1.04
S_{10}	1.04	2.08	0.63	1.25	1.46	2.91	0.26	0.52
S_{11}	0.52	1.04	0.52	1.04	0.63	1.25	0.31	0.63
S_{12}	0.41	0.83	0.41	0.83	1.25	2.5	0.41	0.83
Mean	0.98	1.96	0.72	1.44	1	2.01	0.33	0.67
Standard Deviation	0.68	1.37	0.22	0.45	0.26	0.52	0.14	0.28

Table 3. The mean MIC of the methanol and aqueous extracts in the combined state, the FIC of these two extracts and the sum of the FIC of the extracts against the MDR *Staphylococcus aureus* isolates causing urinary tract infections (in mg/ml) using the checkerboard titration method

Bacterial isolates	Combined MIC Methanol extract	Combined MIC Aqueous extract	FIC_B^* Methanol extract	FIC_C^{**} Aqueous extract	FIC_{BC}^{***}
S_1	0.41	0.83	0.78	1.59	2.37
S_2	2.08	1.04	1.66	1	2.66
S_3	0.83	1.66	1	1.59	2.59
S_4	0.63	1.25	0.43	1.98	2.41
S_5	2.08	1.04	2.50	1.25	3.75
S_6	0.63	1.25	0.75	0.98	2.73
S_7	0.83	1.66	0.28	2	2.28
S_8	0.63	1.25	1	1.20	2.20
S_9	0.52	1.04	1	2	3
S_{10}	0.63	1.25	0.60	1.98	2.58
S_{11}	0.41	0.83	0.78	1.59	2.37
S_{12}	0.31	0.63	0.75	1.53	2.28

FIC_B *: The fractional inhibitory concentration of the methanol extract of the algae *Chlorella vulgaris* CCATM 210-1

FIC_C **: The fractional inhibitory concentration of the aqueous extract of the algae *Chlorella vulgaris* CCATM 210-1

FIC_{BC} ***: The sum of the fractional inhibitory concentrations of the methanol and aqueous extracts of the algae *Chlorella vulgaris* CCATM 210-1

FIC smaller than and equal to 0.5 indicates the synergistic effect, values between 0.5 and 4 and equal to 4 suggests the indifference of these two compounds toward each other, and values greater than 4 are due to the antagonistic effect between these two extracts.

DISCUSSION

Pratt et al (1944) reported that *Chlorella vulgaris* contains an antibiotic, known as Chlorellin (consisting of a variety of fatty acids), which acts against active gram-positive and gram-negative bacteria. Also in the present study, the methanol and aqueous extracts and the supernatant of this algae could inhibit the growth of the bacteria being tested, which may be due to the presence of the antibiotic Chlorellin in the extracts and supernatant of this algae.

Ghasemi et al (2004) stated that antimicrobial substances produced by microAlgae, such as: *Chlorella* species, *Scenedesmus* species, *Euglena viridis*, *Fischerella ambigua*, *Nostoc* species, *Scytonema hofmanni*, *Hapalosiphon fontinalis*, *Anabaena* species, *Microcystis aeruginosa*, and *Phormidium* species are not only considered as a means of defense for Algae, but they can also be used in the pharmaceutical industry.

DellaGreca et al. (2010) noted that the antibiotic produced by the algae *Chlorella* (Chlorellin) has an inhibitory effect on most bacteria. They also stated that the fatty acids released by *chlorella* in the culture medium, help this algae survive in its surrounding environment. And the fatty acid of this algae damages the bacterial cell membrane. In the present study, the destruction of the tested bacteria may be due to the presence of the antibiotic Chlorellin and the fatty acid produced by it and its impact on the cell wall of the bacteria being studied.

Using the macrodilution method, Annamalai et al. (2012) calculated the MIC values of the acetone extract of *Chlorella vulgaris* against the bacteria *Escherichia coli* and *Pseudomonas aeruginosa* as 6.25 and 3.1 mg/ml, respectively, and the MIC values of the ethanol extract of *Chlorella vulgaris* against the bacteria *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* as 3.12, 25 and 1.5 mg/ml, respectively. They also reported the MIC values of the aqueous extract of *Chlorella vulgaris* against the bacteria *Pseudomonas aeruginosa* and *Bacillus subtilis* as 12.5 and 6.25 mg/ml, respectively. This is while, the aqueous extract of this algae in this study, failed to inhibit the growth of the bacteria *Escherichia coli* and *Staphylococcus aureus*. In the present study, too, according to the results presented in Table 2, the mean MIC of the aqueous extract of *Chlorella vulgaris* CCATM 210-1 against the bacteria *Staphylococcus aureus* was determined as 0.72±0.22 mg/ml. By comparing the present study with the results obtained by Annamalai et al., we can see that the aqueous extract of *Chlorella vulgaris* CCATM 210-1 in the present study, unlike the results obtained by other aforesaid researchers, was able to inhibit the growth of the aforementioned bacteria. This can be justified and interpreted in this way that use of water as a polar solvent can polarize the ambient conditions, so that some phenolic compounds with antibacterial properties and low polarity in the composition of the substance or plant or algae being used, are released.

El-Sheik and Al-Souod (2015) examined the intracellular and extracellular extracts of *Chlorella*

vulgaris prepared with chloroform, ethanol, methanol, and ethyl acetate solvents against the bacterium *Escherichia coli*, and found that the extracellular extract of *Chlorella vulgaris* had a less inhibitory effect on the growth of the above mentioned bacterium than the intracellular extract did. In the present study, too, the extracellular extract of *Chlorella vulgaris* CCATM- 210-1 (supernatant) had a less inhibitory effect on the growth of the clinical isolates of *Staphylococcus aureus* than the intracellular extracts (methanol and aqueous) of this algae did.

CONCLUSION

Therefore, the findings of this study showed that the methanol and aqueous extracts and the supernatant of the algae *Chlorella vulgaris* CCATM- 210-1 had a good inhibitory properties against a number of MDR *Staphylococcus aureus* isolates causing urinary tract infections, and this may be due to the presence of the antibiotic Chlorellin in this algae.It is hoped that after performing some in-vivo tests in the future, these herbal extracts can be used in the pharmaceutical industry.

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