2024, 21(11): 3452-3462
https://doi.org/10.21123/bsj.2024.9824
P-ISSN: 2078-8665 - E-ISSN: 2411-7986
Baghdad Science Journal

Evaluation of Antibiofilm and Cytotoxic Activity of Microalgae Isolated from Different Sites of betwata- Erbil/Iraq

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Received 02/10/2023, Revised 11/12/2023, Accepted 13/12/2023, Published Online First 20/04/2024, Published 01/11/2024

Abstract

This *in vivo* study is aimed to assess the antibiofilm and cytotoxic potential of selected microalgae isolates. Different concentration of microalgal isolates used for each of antibiofilm and cytotoxic studies. A total extract of 10 µL MTT was added to each well with LB broth, plates were read using microtiter plate spectrophotometer at 490 nm and 630 nm, respectively. The cell viability was determined in CCD-18°C cells ($3x10^3$ cells/well) by MTT assay at 590 nm. The microalgae isolates showed antibiofilm affect after 48 hrs on *Pseudomonas aeruginosa* PA01 and this effect was observed to be higher than those of *S. aureus*. *Chlorella* sp. which showed the maximum antibiofilm activity while *Arthrospira platensis* had no biofilm inhibition activity. *Chlorella* sp. inhibited about 82.67% of the biofilm at 5 mg/mL concentration. Moreover, the antibiofilm effects of *Chara* sp. and *Spyrogyra* sp.1 reached 79.01% and 76.31% at 1 mg/mL, respectively. Among all microalgal isolates, a moderate inhibitory effect was observed on *Pseudomonas aeruginosa* biofilm with *Chlorella sorokiniana*. Cell viability was not changed significantly at higher doses extracts. More than 73% of the cells were viable in all concentration of the algae extracts. All of the microalgal samples were found to have biofilm inhibition activity. The findings suggested that future development of microalgal samples as a means of inhibiting *Pseudomonas aeruginosa* biofilms is possible. Colonic epithelial cells may become cytotoxic in response to algae extracts. Further research is necessary to verify that extracts are safe for human consumption, which may have been hypothesized.

Keywords: Antibiofilm, Cell Line, Cytotoxicity, Cell viability, Microalgae.

Introduction

The occurrence of many biofilm-based human infections and their multiple antimicrobial resistance is a major concern in medicine and human health. The treatment of infections caused by biofilms with

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antimicrobial drugs has prompted several studies since biofilms act a significant role in infectious diseases. However, due to the development of multidrug-resistant bacteria, antibiotic therapy in infections brought on by foreign bodies is insufficient [1,2]. Bacterial anti-biofilm actions are thus expected to manage bacterial populations in an ecological niche in a natural way [3]. Compounds with antibacterial action generated by cyanobacteria and microalgae have been reported in several studies. Nevertheless, there are little investigations on the antibiofilm activity of the extracts and/or chemicals these microbes create. Several studies have demonstrated that microalgae play an important role in the pharmaceutical business because of their potential to create secondary metabolites with a wide variety of pharmacological actions, such as anti-inflammatory, antioxidant, and antimicrobial properties, antibacterial, cytotoxic, antiviral, and anticoagulant. Antibacterial activity from microalgae extracts has been examined by several researchers due to the rising number of reported cases of antibiotic resistance[4].

Microalgae are photosynthetic organisms that account for a large amount of freshwater and marine phytoplankton[5]. The rise of antibiotic-resistant bacteria necessitates the investigation of novel microalgal bioactive chemicals that are cost-effective, safe, and potent[6]. Many studies showed that natural products obtained from plant, algae, microorganisms etc. has great diversity of pharmacological activities due to their secondary metabolites contents[7]. Antibody-based antitoxins are a treatment option for neutralizing toxins in people who have been exposed to them or who have been infected with a toxin-producing microbe. The antibiotic activity of microalgae and cyanobacteria has been attributed to compounds belonging to several chemical classes, such as alkaloids, aromatic compounds, acetogenins, cyclophanes and paracyclophanes, dicarboximides, indanes, indoles, pH, lactones, and lipids [8].

Many bioactive molecules obtained from microalgae have cytotoxic effects on cancer cells. However, the fact that a substance has shown cytotoxicity to cancer cells in anticancer drug research is not an adequate result. For a substance to have drug potential, it is important that it exhibits selective cytotoxicity [9].

Concisely, secondary metabolites from microalgae and cyanobacteria are highly regarded and are being extensively explored as replacements in biomedical research because to their potential biological and pharmacological qualities. Numerous scientific reports have been mentioned and are continuously being investigated in this regard. This in vivo study's objective is to assess the antibiofilm and cytotoxic potential of microalgae and cyanobacteria that were isolated from various locations between Erbil and Iraq.

Materials and Methods

Bacteria and Algal Strains:

Staphylococcus aureus ATCC 29213 and *Pseudomonas aeruginosa* PA01 were used in the present study. Both bacterial strains were sub-cultured in Nutrient Broth (NB) and incubated in shaker incubator at 37 ˚C, for 24 hrs. with agitation speed 150 rpm. Each culture was then streaked on a nutrient agar medium and stained with gram stain to confirm purity of strains.

Different algal strains obtained from betwata-Erbil transferred to College of Health Sciences, Microbiology lab; sub cultured on BG11 medium. Furthermore, each of algal colony was identified under microscope based on morphological forms and then transferred to BG11 liquid medium, after 14 days the fresh biomass was dried overnight at 50 ˚C , and was used for further processing (antibiofilm and cytotoxic activities)[10].

Determination of Biofilm Inhibition Effect

The quantity of each living bacteria *S. aureus* ATCC 29213 and *P. aeruginosa* PA01 in the biofilm was monitored by methoxynitrosulfophenyl-tetrazolium carboxanilide (XTT) assay [11-13]. The microalgal isolates at different concentrations (0.125-5.0 mg/mL), and Lysogeny broth (LB) broth were added to a 96 well plate to a final volume of 100 µl and incubated at 37°C for 24-48 hrs. After that, the wells were washed 2 to 3 times with 100 µl Phosphate buffered saline (PBS) and 50 µl XTT reaction solutions. The plate was incubated at 37°C for 5 hrs., later the plates were read in a microtiter plate spectrophotometer at 490, 630 nm, respectively. The

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media sterility control without biofilm was represented as zero indicates, whereas the presence of biofilm creation is revealed by a positive value.

Fluorescence Microscope Analysis

For microscopy analysis, the coverslips prepared determining the method described by[14,15]. The mixture of SYBR Green and Propidium iodide (SYBR Green/PI; z ratio of 1: 3 in 100µL dH_2O) was used and the coverslips were analyzed by Fluorescence Microscope (100x magnification).

Cytotoxic Effect

Cell Culture

The human fibroblast cell line CCD-18Co (CVCL_2379) was obtained from ATCC (American Type Culture Collection). The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, Serox) and 1% penicillin/streptomycin mixture (100x, Capricorn) in 5% CO_2 in humidified air at 37˚C in a 75cm^2 culture flasks (Nest). After the cells reached 90% confluence, the culture medium was replaced every 2–3 days [16].

Viability Assay

The MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was purchased and used by following the procedure of the manufacturer. The cell line CCD-18℃ cells ($3x10^3$ cells/well) were seated in 96-well plates and incubated for 24 hrs. Then, the medium was refreshed with that containing concentration of each extract. The final concentrations of each microalgal strains were 0, 125, 250, 500, 750, and 1000 µg/mL. The experiment was carried out three times, with three replicates included for each treatment. The medium was taken out after 48 hrs, and each well received 10 mL of MTT solution (5 mg/mL) in 100 mL of media [17]. To dissolve the purple-colored formazan products, the solution was withdrawn after 4 hrs and 50 L of dimethyl sulfoxide (DMSO, Carlo Erba) was added. A microplate reader was used to measure absorbance at 590 nm after the plates had been incubated for 30 min at 37 °C. The percentage of cell viability was calculated by using following formula:

Percentage of cell viability = (OD value in the experiment group – OD value in the blank well)/(OD value in the control group – OD value in the blank well) × 100% [18].

Molecular Characterization of Cyanobacteria and Microalgea

For molecular identification, Thermo Scientific GeneJET Genomic DNA Purification Kit was used for DNA isolation of the isolates. The procedure was applied in the user manual of the relevant kit. The amount and purity of DNA obtained after DNA isolation was determined by spectrophotometric measurements in Thermo Scientific Nanodrop 2000 (USA).

CYA781R – CYA359F [19] primers were used in the PCR studies and Amplification was done on the gene areas that were intended for species identification. PCR reactions were performed with Solis Biodyne (Estonia) FIREPol® DNA Polymerase Taq polymerase enzyme. PCR products were sequenced using the same primer pairs on the ABI 3730XL Sanger sequencing instrument (Applied Biosystems, Foster City, CA) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sanger sequencing was performed by a commercial firm (BM LABOSIS, Ankara, Turkey). The obtained DNA sequences were first edited using BioEdit software and then compared with other sequences in the NCBI (National Center for Biotechnology Information) Gene Bank (NCBI database) using the nucleotide BLAST algorithm. For identification at the species level, the criterion of the sequence in question was more than 99% similar to the species in the gene bank.

Statistical Analysis

Vassar Stats, an online statistical software tool, was used to conduct the statistical analyses. The data was presented as means, with standard error of the means included (SEM). The student t-test used to compare the data and ($p \leq 0.05$) was chosen for statistical significance.

Results and Discussion

The observation of samples under microscope showed the following characters Table 1 and Fig. 1 Also, molecular analyze of two species confirmed that one of the cultures belong to *Chlorella* genus and

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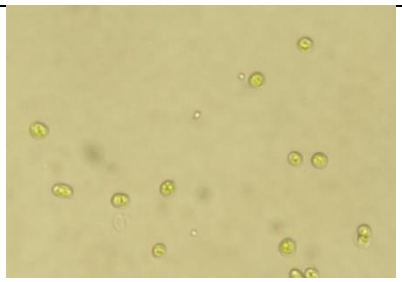
shows that *Chlorella sorokiniana* with similarity of 100% (Accession number: MK177540.1). Another culture was determined to be *Arthrospira platensis* with a 100 % (Accession number: MT426015.1) similarity rate.

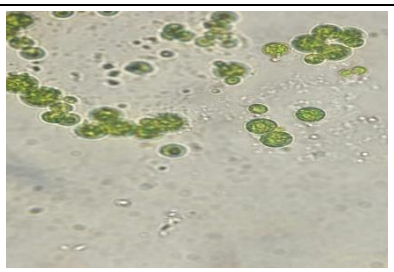
Table 1. Morphological structure of algae

Filament or unicellular	Akinet	Hormogonia	Heterocyst	Colour	Chloroplast	Cell shape	Microalgae strains
Filament (Trichomes cylindrical, sheath absent, coiled into a more or less regular spiral, the apex of Trichome usually not attenuated)	Absent	present	Absent	Colour is variable, blue-green	Chlorophyll- a	Spiral	*Chlorella*
Filament spiral with Short chains of cells (of up to 10 cells), known as hormogonia, are produced as a result of the trichome's fragmentation at the necridia.	Absent	present	Absent	Colour is variable, blue-green	Chlorophyll- a	Spiral	*Arthrospira*
Unicellular single-celled alga	Absent	Absent	Absent	Green algae	Chlorophyll-b	Spherical	*Chlorella*
Filament (Filaments long & unbranched, cells cylindrical short to very long)	Absent	Absent	Absent	Green algae	Chlorophyll-b	cells cylindrical short to very long	*Spirogyra*
Filament (Filaments long & unbranched, cells cylindrical short to very long)	Absent	Absent	Absent	Green algae	Chlorophyll-b	cells cylindrical short to very long	*Spirogyra*
Filamentous algae	Absent	Absent	Absentn	Light green	Discoid shape	The axis branches globule seen above the nuculi	*Chara*



Chlorella sp.

Arthrospira platensis



Chlorella soro kiniana

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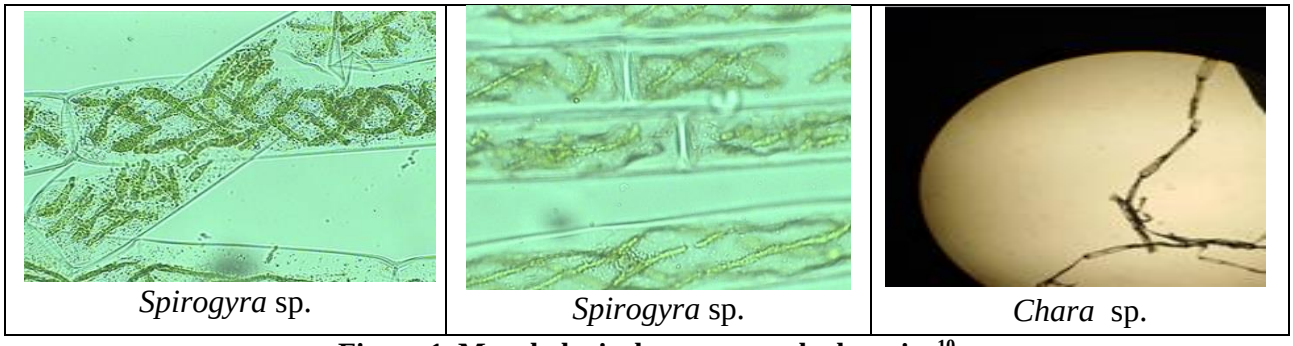

Figure 1. Morphological structures algal strains[10]

The inhibition of metabolic activity was detected by XTT Assay and the percentage of metabolic inhibition was evaluated as biofilm inhibition effect. The antibiofilm activity of algal samples against both pathogenic bacteria appeared to be dosage related. The results of antibiofilm activity of aqueous extracts were given as mean ± standard deviation (SD) for three independent experiments. Statistically there was significant difference compared to the control ($*p \leq 0.05$).

In the first 24 hrs. of incubation, the samples did not show any effect on gram *negative P. aeruginosa*. The samples showed an effect on *P. aeruginosa* 48 hrs. In other words, the microalga and *Cyanobacteria* samples showed antibiofilm affect after 48 hrs on *P. aeruginosa* PA01 and this effect was observed to be higher than those of *S. aureus*. Against *P. aeruginosa* biofilms, *Chlorella* sp. showed the maximum antibiofilm activity while *Arthrospira platensis* had no biofilm inhibition activity. *Chlorella* sp. inhibited about 82.67% of the biofilm at 5 mg/mL concentration. Moreover, the antibiofilm effects of *Chara* sp. and *Spyrogyra* sp.1 reached 79.01% and 76.31% at 1 mg/mL, respectively. Among all microalgal samples, the moderate inhibitory effect was observed on *P. aeruginosa* biofilm with *Chlorella sorokiniana.* In general, it was observed that *S. aureus* were more resistant than *P. aeruginosa* against all samples, Fig. 2. Among the algal samples, only *Chara* sp., *Chlorella* sp. and *Chlorella sorokiniana* samples were found to have a significant biofilm inhibition. This difference in their antibiofilm activities may be related to both differences of microalgal and *Cyanobacteria* samples and the difference in bacterial behavior.

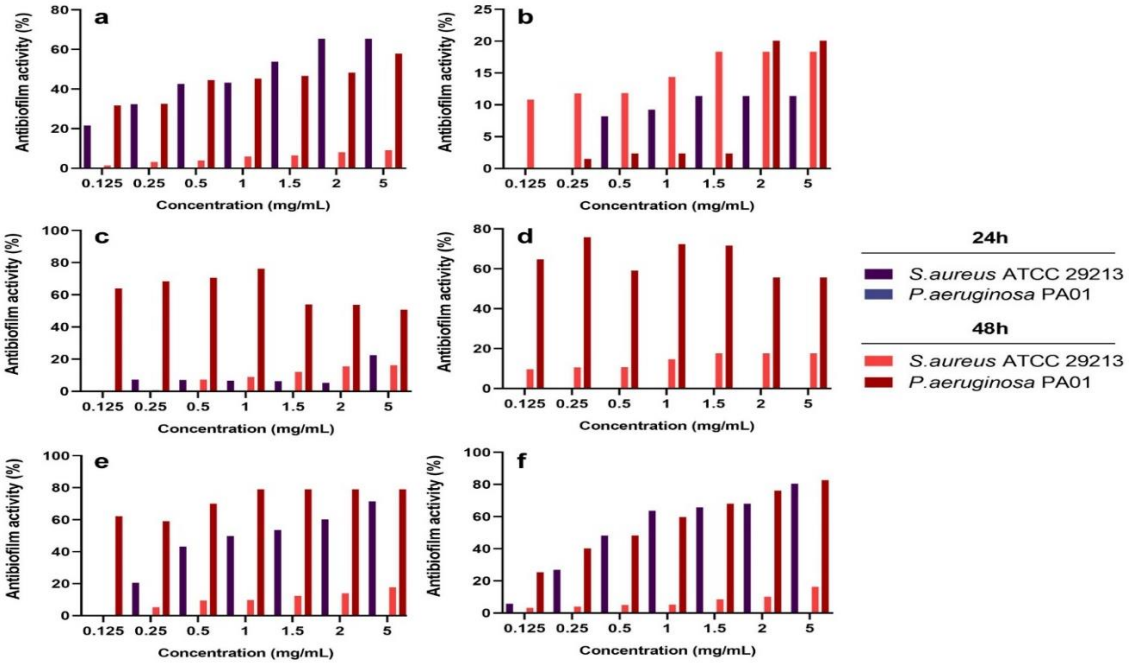

Figure 2. Antibiofilm activity of extracts. a. *Chlorella sorokiniana*, b. *Arthrospira platensis*, c. *Spyrogyra* sp.1, d. *Spyrogyra* sp.2, e. *Chara* sp., f. *Chlorella* sp.

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We now face a huge public health issue since the majority of traditional antimicrobial drugs are no longer effective at this crucial moment when infections have developed diverse resistance routes that have allowed them to surpass our capacity to effectively manage them. In this context, the quest for natural remedies with unique methods to prevent and/or treat life-threatening illnesses might be inspired by the aquatic environment, a place of great biodiversity.

The findings of this investigation revealed that six unprocessed algal extracts exhibit antibacterial properties against *P. aureginosa* and *S. aureus.* According to the findings, all algal extracts had a substantial amount of antibiofilm activity. High antibiofilm activity showed by *Chlorella* against *P. aeruginosa* within 48 hrs while *Arthrospira platensis* showed low antibiofilm activity. The researchers in [20] used different solvents for antibiofilm activity and they concluded that each of solvents showed different antibiofilm activity this discrepancy in the results is likely due to a number of variables that affected the antimicrobial effectiveness of marine algae, including the season and place of algal species, various phases of plant development, extraction ability.

The acetone extract of *Pleurastrum minutum* demonstrated the highest antimicrobial activity among the tested extracts and showed moderate antioxidant activity. Conversely, the methanol extract of the same microalgae exhibited the highest antioxidant activity and moderate antimicrobial activity. These findings underscore the significance of the choice of solvent in the extraction process, as it plays a crucial role in selectively extracting specific bioactive compounds with diverse properties [21].

Anwer and coauthor's isolated phenolic compound and fatty acid properties of some microalgae species and they found that microalgae with a high antioxidant capacity may be investigated for various industrial and pharmaceutical uses [22].

It is known that cell-to-cell intercellular interaction is an important for biofilm formation and structure. For an effective intercellular communication, bacterial cells need to be positioned in close proximity with their neighbors. After the treated with micro-algal and *Cyanobacteria* samples, the bacterial biofilm structure imaged and the close interaction of cells were degraded. The photograph of microscopy images of control group (untreated with samples) and cells treated with micro-algal samples were given in Fig. 3. The general biofilm structure after the treated with algal samples was not a continuous and no dense EPS-matrix. Moreover, the micro-colonies of cell aggregates encapsulated in EPS-matrix and the water channels were degraded.

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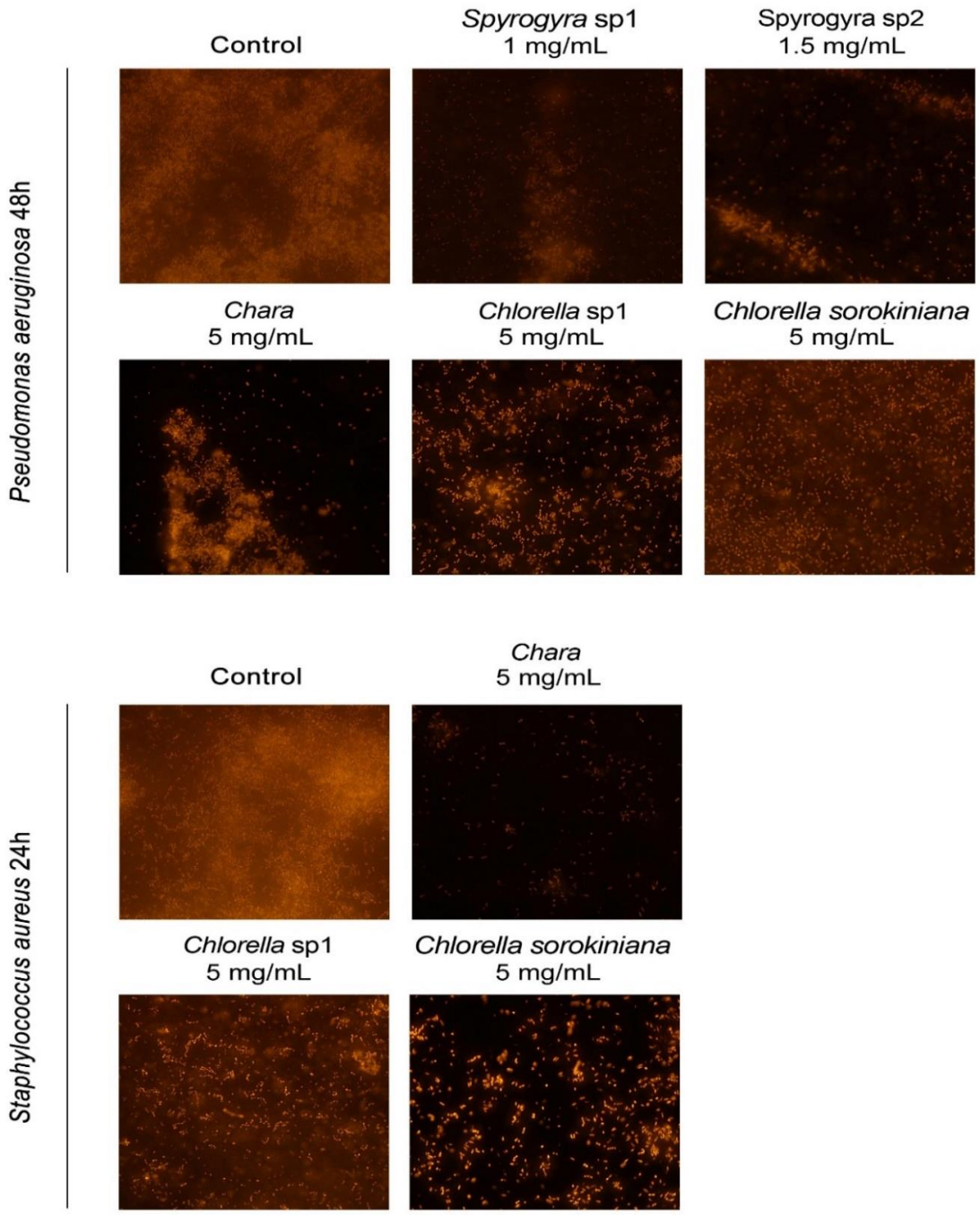

Figure 3. General Biofilm structure before and after the treated with algal samples

To evaluate the cell viability, all extracts were tested by using the MTT assay method. The results for the percentage of cell viability after treatment for 48 hrs., are shown in Fig. 4. As can be seen in Fig. 4, the cell viability was not changed at higher doses and more than 73% of the cells was viable in all concentration of the algae extracts. These results also clearly showed that all algae extracts cannot showed cytotoxic effect on colon cells. It may have speculated that extracts are safe for human usage and further tests will be required to test this hypothesis.

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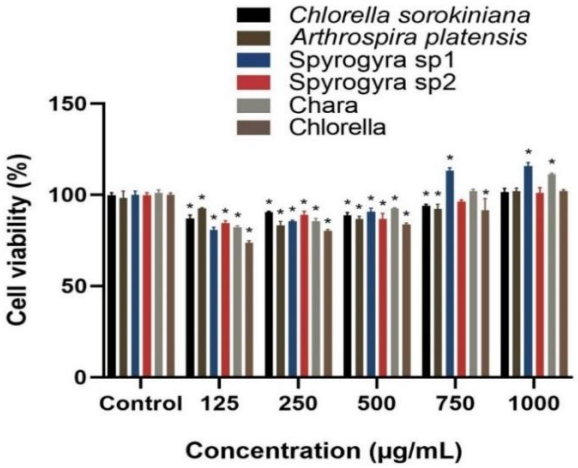

Figure 4. Effects of samples on the CCD-18Co cells after 48 h of treatment. Results were given as mean ± standard deviation (SD) for three independent experiments. Statistically, there was significant difference compared to the control (* $p \leq 0.05$).

CCD-18Co is well-known human fibroblast cell line isolated from normal colon tissue. It is widely used in experimental pharmacology and cancer studies. Moreover, it is important to describe whether secondary metabolites have toxicological effects to gastrointestinal system or not [23-25], Thus, CCD-18°C cell line was used throughout in this study.

The results indicated a minimal decline in the viability even in higher doses of algae extracts after 48 hrs. incubation. These results also clearly showed that all algae extracts cannot showed cytotoxic effect on colon cells. Therefore, it may be speculated that they are safe to use many purposes including antioxidant source. Algae have been shown to contain large amounts of cytotoxic substances with anticancer, antitumor, antiproliferative, and antimetastatic characteristics such fucoidans, laminarians, and terpenoids [26, 27]. In a study done by[28, 29] , it was found that the viability of cells decreased as extract concentrations increase. Bechelli and coauthors[30] implied that algae extracts may be able to suppress primary leukemia blasts and AML cell lines, as well as normal hematopoietic cells. As demonstrated by [31], the methanol extract showed more cytotoxicity oxidative stress than the chloroform extract in the MCF-7 cell line and the tested algae may have potential use for treatment of cancer. These results showed that algae extracts may have the potential to be cytotoxic to cancer cells including gastrointestinal cancers without affecting normal cells. But, further experiments will be required to verify this condition.

Due to its quick growth, ease of large-scale cultivation compared to other microalgae, and valuable nutritional contents, chlorella is one of the microalgae that has been investigated extensively. According to research by[32], oral administration of *Chlorella pyrenoidosa* effectively and dose-dependently prevented increases in blood aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glutamate aminotransferase (GAT) and alkaline phosphatase (ALP) activities in male rats with d-galactosamine-induced hepatic lesions. It also appeared to alleviate hepatic cell necrosis. Additionally, they discovered that *C. pyrenoidosa* reduced lipid peroxide levels and restored normal triglyceride levels in rat serum. When compared to earlier research, the current study revealed limited impacts on cell viability; this could be because the current study utilized a low concentration[33,34].

Conclusion

Antibiotic resistance spreads among bacteria in the world and biofilm formation is effective in spreading this resistance. All algal strains showed antibiofilm activity. *Chlorella* sp. showed maximum antibiofilm activity. It was observed that all microalgal samples had biofilm inhibition activity. And these findings were also confirmed by fluorescent microscopy analysis. Bacterial biofilms will cause a lot of damage in the future, both in the health sector and in the economic field. In order to prevent this, it is important to find new antibiofilm substances. Although the importance of microalgae among new antibiofilm products is increasing, it is necessary to investigate their mechanism of action in detail. Also, the results indicated that microalgal samples have the potential to be developed as an agent to inhibition of *P. aeruginosa* biofilms in the future.

Acknowledgment

The study was carried out at Pamukkale University, Denizli- Turkey and Hawler Medical University, Erbil-Iraq.

Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and Images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at Hawler Medical University

Authors' Contribution Statement

S.S.A. and A. A. contributed in Isolation and identification of Microalgae, R. C. B. was responsible for molecular identification, S. A. and D. M. analyzed cytotoxic activity of samples, N. M. D., N. B. K. and made antibiofilm and V. K. used fluorescence microscopy for analysis. All authors have substantial contributions to the final manuscript and approved this submission. All authors read the manuscript carefully and approve the final version of their MS.

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Baghdad Science Journal

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تقييم التضاد الحيوي والنشاط السمي للخلايا من قبل الطحالب الدقيقة التي تم عزلها من مواقع مختلفة في بيتواتا- اربيل/العراق

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الخلاصة

تمتلك الطحالب الدقيقة, نظراً لسهولة زراعتها ونموها السريع وتجددها, امكانات كمصدر للمركبات النشطة بيولوجياً للاغراض الصناعية والطبية. تهدف هذه الدراسة التي اجريت بشكل (in vivo) الى تقيم القدرة المضادة للاغشية الحيوية والسموم الحيوية لطحالب الدقيقة مختارة وعزلات البكتريا الزرقاء. تم اختيار تراكيز مختلفة من عزلات الطحالب الدقيقة في كل من دراسات المضادات الحيوية والخلايا السامة . تمت اضافة مستخلص اجمالي قدره 10 ميكروليترمن MTT الى كل بئر باستخدام مرق LB , وتمت قراءة اللوحات باستخدام مقياس الطيف الضوئي للوحة ميكروتيتر عند 490 نانوميترو 630 نانوميتر, على التوالي. تم تحديد بقاء قدرة الخلية في خلايا-CCD 18C° ($3x10^3$ cells/well) بواسطة اختبار MTTعند 590 نانوميتر. اظهرت عزلات الطاحالب الدقيقة تاثير مضاد حيوي بعد 48 ساعة على *Pseudomonas aeruginosa* PA01 ولوحظ ان هذا التاثير اعلى من تاثيره على *Chlorella* sp. *S. aureus*. اظهر الحد الاقصى لنشاط المضاد الحيوي بينما لم يكن لدى *Arthrospira platensis* اي نشاط تثبيط للاغشية الحيوية. *Chlorella* sp. تثبط حوالي 82.67% من الغشاء الحيوي في تركيز 5 ملغم \مل . علاوة على ذلك فان التاثيرات المضادة للاغشية الحيوية ل *Chara* sp. و*Spyrogyra* sp.1) وصلت بنسبة الى 79.01% و 76.31% عند 1 ملغم\مل على التوالي. من بين جميع العزلات الطحالب الدقيقة, لوحظ وجود تاثير مثبط معتدل على الاغشية الحيوية ل *Pseudomonas aeruginosa* مع *Chlorella sorokiniana* . لم تتغير قابلية الخلية للبقاء بشكل ملحوظ في مستخلصات جرعات عالية. اكثر من 73% من الخلايا كانت قابلة للحياة في جميع تركيزات مستخلصات الطحالب. وجد بان جميع عينات الطحالب الدقيقة لها نشاط تثبيط الاغشية الحيوية. تشير النتائج الى ان التطوير المستقبلي لعينات الطحالب الدقيقة كوسيلة لتثبيط الاغشية الحيوية ل *Pseudomonas aeruginosa* امر ممكن. قد تصبح الخلايا الطلائية القولونية خلاية سامة استجابة لمستخلصات الطحالب. من الضروري اجراء المزيد من الابحاث للتحقق من ان المستخلصات أمنة للاستهلاك البشري, وهو ما يكون مفترضاً.

الكلمات المفتاحية: المضادات الحيوية، الخط الخلوي، السمية الخلوية، حيوية الخلية، الطحالب الدقيقة.