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Downregulation of Biofilm formation genes in some pathogenic bacteria by extracts from two algal genera

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Abstract

Most infectious diseases are primarily caused by the growth of microorganisms called biofilms. The formation of bacterial biofilms enables microorganisms to inhabit biotic and abiotic surfaces which increases their resistance to antimicrobials. To control this issue, there is a critical need for novel approaches and compounds can suppress the expression or regulation of virulence genes. A potential method for disarming rather than eliminating bacterial pathogens is antivirulence therapy based on the blockage of biofilm pathways. In current study, the action of water, diethyl ether and acetone extraction on two types of algae namely *Scenedesmus quadricauda* and *Chlorosarcinopsis eremi* in their sub-inhibitory concentration (SIC) was investigated against *Pseudomonas aeroginosa and Escherichia coli* biofilm formation and their gene expression instead of killing them. The SIC values of each extract were determined by minimal inhibitory concentration (MIC) assay then gene expression products were assessed using Real-Time PCR (RT-PCR) when the cells were exposed to the SICs of algal extracts. Results revealed that the expression of *ndvB* (*P. aeroginosa*) & *FimH* (*E. coli*) genes that involved in biofilm formation was reduced by the extracts at their SICs. Diethyl ether was the best solvent with greater inhibitory activity followed by water and acetone against two pathogenic bacteria under this survey. Values of 25mg/ml, 20mg/ml for MIC and 15mg/ml, 10mg/ml for SIC were recorded by diethyl ether solvent against *P. aeroginosa* and *E. coli* respectively. According to biofilm detection, water extract was more efficient in *S. quadricauda* against *P. aeroginosa* and *E. coli.*

Keywords: Algal extract, Biofilm, Downregulation, Sub-inhibitory concentration.

Introduction

Among the most significant risks to public health is resistance to antibiotics, which is primarily caused by the selective pressure of excessive antibiotic use and misuse [1]. Multidrug-resistant (MDR) bacteria, which are linked to both hospital-acquired and community-acquired diseases, are a result of the prolonged and irrational use of antibiotics. The ineffectiveness of presently prescribed antibiotics and the sluggish development of new medications make it difficult to treat MDR bacterial infections [2]. The development of

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other possible strategies and antibiotics that can effectively combat resistant pathogenic microorganisms over the long term is therefore urgently needed [3]. The majority of current treatments work by killing or inhibiting bacterial growth, imposing high selection pressure on bacteria, and increasing the risk of the development of resistance mechanisms [3]. Antivirulence medicines, which are newer avenues of therapy, should target bacterial cellular mechanisms responsible for pathogenesis and virulence rather than growth-related components. Quorum sensing suppression and biofilm formation are two current antivirulence therapies [3].

Microorganisms developed mechanisms for survival via evolutionary changes, enabling them to adapt to harsh situations. Being a biofilm is one such adaptation [4]. Bacterial biofilms are clusters of microorganisms where the cells are immersed in a self-assembled matrix of extracellular polymeric materials. One of the benefits of biofilm production is a safeguard from insults and environmental assaults. The diversity of biofilms differs from its constituent microbes [5]. Bacteria inside biofilm tend to be more resistant to antimicrobials than planktonic forms since bacteria that are non-resistant to antimicrobials can become resistant after biofilm formation [6]; this makes therapies that utilize effective antibiotic dosages on bacteria in planktonic form challenging because it boosts bacterial adaptation or tolerance to environmental stressors and enhances the transfer of antibiotic-resistant genes among various species [7].

Numerous studies have looked at natural resources to understand how to prevent and control biofilms [8]. Humans have traditionally exploited natural compounds and food's secondary metabolites, medicine, and cosmetics [4]. Numerous studies have demonstrated the antibacterial and biofilm-inhibitory properties of a variety of natural compounds, including microalgae [4, 9]. Generally, microalgae are rich in different worthy compounds which supply excellent different biological activities involving, antiviral, anti-inflammatory, antitumor, antioxidant, antibacterial, and finally anti-allergy effects[10, 11].

Some species of microalgae like *Chlorella spp*., *Chlorosarcinopsis spp*., *Dunaliella spp.*, and *Scenedesmus spp.* are recently attracted attention as commercially worth sources for a wide range of compounds as more bioactive. *Scenedesmus* and *Chlorosarcinopsis* are one of the most widespread freshwater algal genera. Due to the facilities of *Scenedesmus* and *Chlorosarcinopsis* planting, harvest, and drying operation, it becomes the most widespread and popular species in microalgal biotechnology surveys. It appears to be a wealthy resource of novel antimicrobial compounds [12]. Previous research has reported that *S. quadricauda and Ch. eremi* extracts can prevent or suppress the growth of many pathogens [13, 14]. To this point, few studies have evaluated the anti-virulence and biofilm of the algal extracts; additionally, their impacts on *ndvB* (*P. aeroginosa*) & *FimH* (*E. coli*) genes at the expression level have not been investigated yet. Hence, this study was performed to observe the role of *S. quadricauda and Ch. eremi* extracts in the weakening of the biofilm formation of *P. aeroginosa* and *E. coli* by decreasing the expression of biofilm formation genes.

Materials and Methods

Isolation and Classification of Algal Species

Algal species were obtained from various locations within Erbil province and identified morphologically by using light microscope their resolution was high with assisting many keys described by [15]. Algae have been isolated by using a streak plating technique [16].

Identification of Algal Species

To obtain pure algal genera, the BG-11 medium was used as a special medium for algal sample enrichment and isolation. Samples of two algal genera inoculated on BG-11 medium containing 15% agar and incubated at 25± 2°C, pH is generally adjusted to around 8.2 and intensity of light 3000-5000 lux for 16 hours light and 8 hours dark for 14 days. This step was repeated several times. To obtain

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algal inoculum, the pure algal colony was transferred to a tube containing 25 ml of BG-11 media and cultured under the same conditions for 14 days. Molecular diagnosis of microalgae using ITS region amplification was employed to confirm the identification.

Biomass Preparation and Harvesting (Algal culturing)

Transferring about 25 ml of separated algae to a flask containing 100 ml of BG-11 and culturing for 14 days under the same conditions as described earlier. Then, this cultured medium was put into a 500ml conical flask containing 100 ml of the BG-11 medium and incubated for 2 weeks under the exact conditions. These steps occurred several times until algal growth arrived at 4 liters existing in the container which was enveloped by pieces of cotton and the air was supplied with rubber [17]. Twenty days later, the culture of algae is harvested by utilizing a centrifuge apparatus at 4000 rpm for 10 minutes [18]. The sample of algae was cleaned and rinsed in sterile water before being dried in an oven set at 38 to 40 degrees Celsius. The dehydrated algal biomass was weighed and preserved in the fridge [16].

Algal extracts preparation

A Soxhlet extractor was used to extract about 30 grams of finely ground powder over 8 hours period using 300 cc of water, diethyl ether, and acetone. About three days of incubation at 37°C needed to evaporate the extracted products. The extracts were then all dissolved in dimethyl sulfoxide (DMSO), 5 ml of DMSO was used to dissolve 1 g of each extract to obtain a stock solution with a concentration 200mg/ml, and all samples were stored at -4 °C until use [19].

Bacterial Isolates

Clinical isolates investigated in this study were; *P.aeruginosa* and *E. coli*. The isolates were identified by the Vitek-2 component obtained from Biology Department-College of Sciences-University of Salahaddin Iraq-Kurdistan Region, Erbil governorate.

The determination of Minimum Inhibitory Concentration (MIC) of Algal Extraction

About 96-well microtiter plates were used to examine different concentrations of algal extracts (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50mg/ml) diluted from stock solution 200mg/ml, by mixing within the nutrient broth. After overnight incubation at 37 °C, 10 µl of the activated *P. aeroginosa* and *E. coli* cultures were added to each well. After that, the MIC was calculated using Elisa reader, and the absorbance at wavelength 490 nm was measured both before and following incubation [20]. Subinhibitory concentrations (sub mic), or levels that were lower than the MICs, were also used to assess the anti-biofilm activity and gene expression in the isolates of *P. aeruginosa* and *E. coli*.

Biofilm Detection

According to O'Toole [21], formation of biofilm in polystyrene microtiter plates was assessed. The wells of the microtiter plates were pipetted with various extracts at various concentrations. Each well received overnight cultures of each bacterial species, which were then introduced and incubated at 37°C for 24 hours. Following incubation, non-adherent cells were eliminated by washing the wells three times with D.W. The microtiter plates were stained with 150 µl of 1% Crystal violet for 5 min, and cells were stained but not the polystyrene. The excess stain was rinsed off with running tap water. At this point purple ring was determined, biofilm formed at the air-liquid interface on the inner surface of the plastic wells and it was dried by oven at 50°C. 150 µl of ethanol was added to each well for 10 min, and the absorbance of each well was monitored with a microtiter plate reader or ELISA reader at 490nm.

RNA Extraction

In order to determine the expression of the *ndvB* and *FimH* genes [22], total RNA Purification Kit, Jena Bioscience, Germany was used to extract RNA from bacterial cells cultured in TSB broth with and without the presence of SICs of the test items [21].

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Table 1. Oligonucleotide sequence used in the study

Primer	Sequence 5---3	Target gene
ndvB FW	GGCCTGAACATCTTCTTCACC	NdvB [23]
ndvB RV	GATCTTGCCGACCTTGAAGAC	
FimH FW	TGCAGAACGGATAAGCCGTGG	FimH [24]
FimH RV	GCAGTCACCTGCCCTCCGGTA	

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR).

To study whether the expression of the genes was modified after treatment by the sub-inhibitory concentration (SIC) of algal extract, real time PCR was used to evaluate the role of algal extracts on the expression levels of the *ndvb* genes of *P. aeruginosa* and *FimH* genes of *E. coli*.

Total RNA was extracted from both untreated bacteria which were used as control and bacteria exposed to the algal extract at SIC following guidance provided by the manufacturer (total RNA kit, Favorgen Biotech, Taiwan). cDNA was synthesized through reverse transcription of the isolated RNA using AddScript cDNA synthesis kit according to the manufacturer protocol (addbio,Koria). RT-PCR reactions were performed utilizing RealQ Plus 2x Master Mix Green (Ampliqon,Denmark) in the PCRmax Eco 48 RT-PCR system. Primer sequences listed in Table 1 were used to analyze candidate genes by qPCR and the results were calculated using ΔCt method [25].

Results and Discussion

We now face a serious public health issue since the majority of conventional 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 alternatives with unique methods to prevent and/or treat life-threatening diseases might be inspired by the marine world, a place of great biodiversity [26]. Algae, a marine organisms, is thought to be one of the possible sources of a wide range of bioactive molecules for preventing the increasing of antimicrobial-resistant and biofilm-forming bacteria [11].

Biofilms play an essential role in our healthcare system. Due to the biofilm's natural resistance to drugs and ability to escape immune responses, it is thought that its development accounts for the majority of persistent microbial infections in humans [27]. According to several studies, biofilm formation can be potentially targeted to fight pathogenic bacteria. In recent years, researchers have used natural products to develop the next generation antimicrobials which can target virulence factors and biofilm formation, without affecting mammalian cells.

In the present study, the antibiofilm characteristics of two species of microalgae *S. quadricauda* and *Ch. eremi* were tested at their sub-inhibitory concentration (SIC) against *P. aeroginosa* and *E. coli*. The MIC and SIC of algal extracts measured at several concentrations against determined bacteria for testing were recorded as shown in Table 2. The diethyl ether extract was the most effective against used pathogenic bacteria. The MIC of *S. quadricauda* was (25mg.ml^{-1}), (20mg.ml^{-1}) and SIC was (15m/ml), (10mg/ml) against *E. coli* and *P. aeroginosa* respectively. *S.quadricauda* exhibited the greatest growth inhibition against pathogenic gram-negative bacteria when using diethyl ether than other solvents. This may be returned to the phytochemical of algal genera which includes used active substances. Toma & Aziz [14], found that *S. quadricauda* contains bioactive chemicals that are crucial for the production of other bioactive compounds as a beneficial precursor. Drugs derived from these algae species are used to precisely limit bacterial development, which improves the effectiveness of controlling vector infections without causing any adverse reactions [14].

Many studies about the antimicrobial activity of *Scenedesmus* species extract concluded that they

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been shown to be very effective against a various of pathogenic bacteria; yet, Most compounds obtained from these species are thought to be unsuitable antibacterial for medical application due of their in vivo toxicity or inactivity [12]. In the same Table, *Ch. eremi* showed more efficiency against *E. coli* than *P. aeroginosa* with MIC of (15mg/ml) and SIC (5mg/ml) when extracted by diethyl ether than water and acetone. *Ch.eremi* has properties of pharmacologically because they were found to contain cardiac glycosides, flavonoids, alkaloids, anthraquinones, saponins, reducing sugars, and terpenoids. It was also present to contain antibacterial activity *versus E. coli*, *Pseudomonas* sp, *Staphylococcus aureus*, *Klebsiella sp*, *Serratia* sp, *Salmonella*, *Proteus* sp, and *Bacillus* sp. [28].

Table 2. MIC and SIC determination of *E. coli* and *P.aeruginosa*

Algal extracts	*MIC(mg/ml)		**SIC(mg/ml)	
	E.coli	***P.aeruginosa***	***E.coli***	***P.aeruginosa***
***S-water**	30	50	10	30
S- diethyl ether	25	20	15	10
S- acetone	45	50	15	35
****Ch- water**	30	30	20	20
Ch- diethyl ether	15	40	5	15
Ch- acetone	40	50	25	25

***MIC: minimum inhibitory concentration, **SIC: sub-inhibitory concentration**
S: *Scenedesmusquadricauda*, ****Ch: *Chlorosarcinopsiseremi

Concerning the biofilm production which was measured by the microtiter plate method as shown in Table 3, it's clear that most algal extracts reduced biofilm formation by *E. coli* and *P. aeruginosa*. The water extract of *Ch. eremi* has the most effective in reducing biofilm formation in *E. coli* while the diethyl ether extract of *S. quadricauda* reduced biofilm formation in *P. aeruginosa* more than other algal extracts. This is consistent with other research' findings, which showed that algal extracts inhibit biofilm formation among pathogenic bacteria[29-32].

Table 3. Effect of algal extracts on biofilm production by *E. coli* and *P. aeroginosa*

Bacteria	Algal extract						
	Control	**S-W**	**S-D**	**S-A**	**S-A**	**C-W**	**C-D**
E. coli	0.184	0.201	0.131	0.189	0.114	0.165	0.140
P. aerogenosa	0.196	0.216	0.145	0.219	0.209	0.180	0.182

S=*Scenedesmus quadricauda*, C=*Chlorocinopsis eremi*, W=Water, D=Diethyl ether, A=Acetone.

The results of RT-PCR as shown in Figs. 1 and 2 which indicated that all algal extracts downregulated *FimH* gene in *E. coli* in different ratios and the most potent extract is a water extract of *Ch.eremi*, while most algal extracts reduced expression of *ndvB* gene in *P. aeruginosa* likewise water extract of *Ch.eremi* reduced expression of *ndvB* gene more than the other extracts. Regarding the diethyl ether and acetone extract of *S.quadricauda* have no effect in a decline of *ndvB* gene expression.

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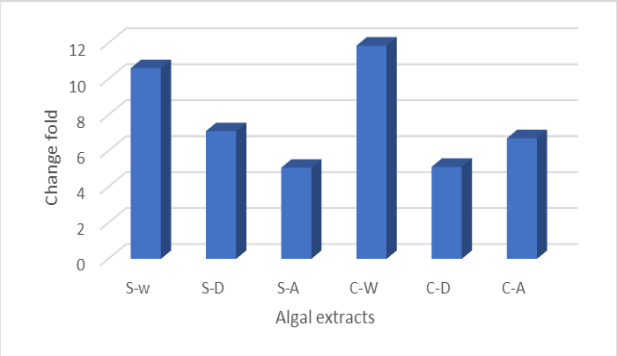
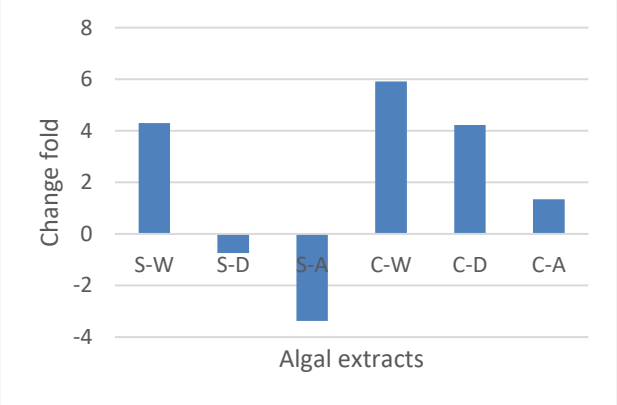


S=*Scenedesmusquadricauda*, C=*Chlorocinopsiseremi*, W=Water, D=Diethyl ether, A=Acetone.

Figure 1. Effect of algal extracts by different solvents on the expression of biofilm gene (*FimH*) in *E. coli*



S=Scenedesmus*quadricauda*, C=*Chlorocinopsiseremi*, W=water, D=Diethyl ether, A=Acetone.

Figure 2. Effect of algal extracts by different solvents on the expression of biofilm gene (*ndvB*) in *P. aeruginosa*

Nowadays, an alternate strategy for managing bacterial infections is the use of organic and conventional plant substances to reduce, disperse, and/or eliminate bacteria's quorum-sensing (QS) systems [33, 34]. Quorum sensing, a method of cell communication that is frequently used by bacterial pathogens to coordinate the expression of many collective properties, such as the production of several virulence factors and biofilm formation, is one of the main targets of anti-virulence agents [34, 35].

By comparing the relative levels of expression of genes involved in biofilm formation and QS, which are regulated by several signaling molecules in bacteria, the impact of algal extracts on gene expression was determined.

This downregulation of genes means that the algal extracts disrupt the signaling pathways that control the formation of biofilms and QS in bacteria, decreasing their ability to spread infections and resist antibiotics. According to previous studies, *Asparagopsis taxiformis* marine algae's methanol extracts exhibits antibacterial and quorum-quenching properties against Serratia liquefaciens [36].

Algal extracts possess many mechanisms that explain their antibacterial, anti-biofilm, and gene regulatory properties. Bioactive substances that directly damage bacterial cell membranes, disrupt QS signals, or alter the expression of virulence genes may be present in algae extracts. Secondary metabolites, polyphenols, terpenoids, and fatty acids are among the specific substances that may be causing these effects. Furthermore, it is unclear how algal extracts affect gene expression, but they may do so by attaching to or blocking the receptors or enzymes that are a part of these processes.

Conclusion

Overall, the findings from the present investigation highlight that algae may be a valuable source of antibiofilm compounds, which improve the effectiveness of antibiotics and decrease the pathogenicity of bacterial pathogens. Furthermore, with exposure to the sub-inhibitory concentrations of algal extracts, the major virulence factors including biofilm formation of the tested organism were downregulated, which may alter the pathogenesis mechanisms of pathogens in the hosts. Future treatments for drug-resistant *P. aeruginosa* and *E. coli* infections could benefit from the potential for algae extracts to reduce the expression of these genes. As a result, further research is needed to determine whether their usage in therapeutic therapies, either alone or in combination with well-known antibiotics, is a viable alternative to the present pharmaceuticals.

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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 University of Salahaddin.

Authors' Contribution Statement

N. S. H. and J. J. T., A. S. I., S. A. Sh. and M. J. a. Contributed to the design and implementation of the research, to the analysis of the results and to the writing and translation of the manuscript.

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تقليل تنظيم الجينات المكونة للاغشية الحيوية في بعض انواع البكتريا المسببة للامراض عن طريق مستخلصات من جنسين من الطحالب

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الخلاصة

يُمّكن تكوين الأغشية الحيوية البكتيرية الكائنات الدقيقة من العيش باستمرار على الاسطح الحيوية وغير الحيوية مما يزيد من مقاومة مضادات الميكروبات. للسيطرة على هذه المشكلة، هناك حاجة ماسة لاستراتيجيات ومركبات جديدة يمكنها قمع وتثبيط تعبير أو تنظيم جينات الضراوة. تُعد العلاجات المضادة لعوامل الضراوة والقائمة على مقاطعة مسارات الأغشية الحيوية واحدة من العديد من هذه الاستراتيجيات الواعدة التي تهدف إلى إضعاف مسببات الأمراض البكتيرية بدلاً من القضاء عليها أثناء العدوى. لذلك، في الدراسة الحالية، تم اختبار تأثير فاعلية المستخلص المائي، مستخلص ثنائي إيثيل الأثير ومستخلص الأسيتون لنوعين من الطحالب هما *S. quadricauda* (*Scenedesmus quadricauda*) و *Ch. eremi* (*Chlorosarcinopsis eremi*) في تركيزها دون المثبط (SIC) ضد تكوين الاغشية الحيوية والتعبير الجيني لكل من (*P. aeroginosa* (*Pseudomona aeroginosa* و *E. coli*. (*Eschershia coli*) بدلاً من قتلها. تم تحديد قيم SIC لكل مستخلص بمقايسة التركيز المثبط الأدنى (MIC) ثم تم قياس منتجات التعبير الجيني بواسطة Real Time PCR (RT-PCR) عندما تعرضت الخلايا لـ SICs لمستخلصات الطحالب المذكورة. أوضحت النتائج أن التعبير الجيني ل (*P. aeroginosa*) *ndvB* و (*E. coli*) *FimH* التي تشارك في تكوين الأغشية الحيوية قد تم تقليله بواسطة المستخلصات الموجودة في SIC. كان مستخلص ثنائي إيثيل الأثير هو أفضل مذيب مع نشاط مثبط أكبر يليه الماء والأسيتون ضد نوعين من البكتيريا الممرضة في هذه المسحة. تم تسجيل قيم 25 مجم / مل، 20 مجم / مل لـ MIC و15 مجم / مل، 10 مجم / مل لـ SIC بواسطة مذيب إيثيل إيثر ضد *P. aeroginosa* و *E. coli* على التوالي. وفقًا لكشف الأغشية الحيوية، كان مستخلص الماء أكثر كفاءة في *S. quadricauda* ضد *P. aeroginosa* و *E. coli*.

الكلمات المفتاحية: المضاد الميكروبي، مستخلص الطحالب، الاغشية الحيوية، التنظيم الانحداري، تركيز دون المثبط.