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Research Article

AN *IN-VITRO* ANTIBIOFILM ACTIVITY OF *CHLORELLA VULGARIS*

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ABSTRACT

Objective: Most of the microbial infection in the body is through biofilm formation. Quorum sensing (QS) is the key regulator in the biofilm formation in both Gram-negative and Gram-positive bacteria. Therefore, interfering with QS is the current strategy to prevent bacterial infection.

Methods: In this study, the effect of various extracts of freshwater microalgae – *Chlorella vulgaris* on the growth of clinical pathogens – *Pseudomonas aeruginosa* and *Staphylococcus aureus* which were studied using minimum inhibitory concentration (MIC), antibiofilm activity, and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay using 96-well flat bottom microtiter plates. The phytochemical analysis of *C. vulgaris* was also carried using standard procedure.

Results: The petroleum ether, dichloromethane, chloroform, ethyl acetate, and methanolic and acetone extract of *C. vulgaris* showed the presence of carbohydrates, amino acids, proteins, steroids, flavonoids, alkaloids, saponins, and phenolic compounds. The MIC value of methanolic extract of *C. vulgaris* was found to be 1 mg/ml. The highest inhibition of 82.5% and 88.0% was shown by methanolic extract at a concentration of 1 mg/ml for *P. aeruginosa* and *S. aureus*, respectively. The antibiofilm activity by crystal violet and MTT assay confirmed the reduction of biofilm formation in both pathogenic organisms.

Conclusion: The present results suggested the possible use of *C. vulgaris* in attenuation of QS and biofilm formation to control pathogenic bacteria – *P. aeruginosa* and *S. aureus*.

Keywords: Antibiofilm, Quorum sensing, Freshwater algae, *Chlorella vulgaris*, Phytochemicals.

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INTRODUCTION

Bacterial biofilms are sessile communities of bacteria encased in a self-synthesized exopolysaccharide matrix [1]. Bacteria in biofilms show distinct features from their free-living planktonic cells, such as different physiology and high resistance to the immune system and antibiotics that make the biofilm a source of chronic and persistent infections [2]. Hence, it is necessary for the development of drugs to prevent bacterial infections because of increasing emergence in the multidrug resistance among pathogens for antibiotics.

Pseudomonas aeruginosa and *Staphylococcus aureus* are the important opportunistic human pathogens which can cause a variety of infections [3-8]. Majority of microbial infections in the body are due to quorum sensing (QS)-mediated biofilm formation as well as toxins for the further establishment of host infections [9-12]. QS controlled virulence factors, and biofilm formation is vital for the development of acute and chronic diseases of *P. aeruginosa* and *S. aureus.* Bacteria in biofilms show distinct features from their free-living planktonic cells, such as different physiology and high resistance to the immune system and antibiotics that make the biofilm a source of chronic and persistent infections [10]. Hence, there is a need for the search of potential anti-QS and antibiofilm compounds from natural sources to prevent bacterial infections because of increasing emergence in the multidrug resistance among pathogens for antibiotics.

A promising approach is to target cell–cell communication QS which utilizes autoinducer to sense information from other cells which accumulates in the environment in proportion to cell density. Gram-negative and Gram-positive bacteria use acylated homoserine lactones and oligopeptides as autoinducer which induces the expression of QS genes [13-15].

Freshwater algae are rich sources of novel and biologically active secondary metabolites which find various applications in pharmaceutical industries such as antibiotic, antiviral, antioxidant, antifouling, anti-inflammatory, cytotoxic, and antimitotic activities [16,17]. At present, freshwater algae are gaining importance for the studies on biofilm and QS.

The unicellular freshwater algae – *Chlorella vulgaris* – comprise a lot of bioactive compounds with therapeutic properties. Studies carried out using *Chlorella* are mainly known for its antitumor effect, cancer chemoprevention properties, anti-inflammatory activity, antioxidant activity, and antimicrobial activity [18]. With this background knowledge, the study was aimed to investigate the antibiofilm activity of *C. vulgaris* extract against the clinical pathogens – *P. aeruginosa* and *S. aureus*.

METHODS

Collection of sample

C. vulgaris powder was collected from the parry nutraceutical division of EID Parry (India) limited, Tamil Nadu, India.

Preparation of extracts

The samples were extracted using a sequential extraction method of the increasing polarity of the selected solvents. 10 g of the *Chlorella* powder was dissolved in 100 ml of petroleum ether and kept in the shaker for 24 h at 37°C. The extract was filtered using Whatman No.1 filter paper, and the filtrate was kept for evaporation. The collected residue was again extracted using different solvents, namely dichloromethane, chloroform, ethyl acetate, methanol, and acetone by the following procedure as mentioned above. The crude substance obtained after the evaporation was dissolved in dimethyl sulfoxide and used for further experiments.

Collection of microbial strains

The microbial strains selected are *P. aeruginosa* and *S. aureus* and were collected from the Micro Lab, Coimbatore. The stock cultures were maintained in nutrient agar slants at 4°C.

Phytochemical analysis

The various phytochemical screening such as carbohydrates, proteins, phenols, flavonoids, alkaloids, saponins, phenolic content, and phytosterol present in the petroleum ether, dichloromethane, chloroform, ethyl acetate, methanol, and acetone extracts of *C. vulgaris* was carried as per the standard procedure is given by Harborne [19].

Determination of antibiofilm activity

Minimum inhibitory concentration (MIC)

MIC of different extracts of *C. vulgaris* and azithromycin was performed against *P. aeruginosa* and *S. aureus,* respectively, using standard broth microdilution assay [20]. Test inoculums (100 µL of 18 h cultures) were added in 96-well microtiter plate containing algal extract and standard antibiotics in 250 µL of Luria-Bertani (LB) broth media. The plates were incubated at 37°C for 24 h, and the optical density (OD) was recorded at 595 nm. The MIC was the lowest concentration in which there would be no visible bacterial growth after 24 h of incubation at 37°C.

Biofilm formation assay

An overnight culture of *P. aeruginosa* and *S. aureus* was diluted to 1:100 in fresh medium for biofilm assays. 100 µL of the dilution was added in a 96-well plate microtiter plates which were used and incubated for 24 h at 37°C.

After incubation, the cells were removed by turning the plate over and shaking out the liquid. The plate was gently submerged in a small tub of water, and then, water was taken out. This process was repeated twice. 125 µL of a 0.1% solution of crystal violet was added to each well and incubated at room temperature for 10–15 min. The plate was rinsed 3–4 times with water by submerging in a tub of water. The excess cells and dye were removed by shakes out and blots vigorously on a stack of paper towels and kept for drying.

To quantify the biofilm, 125 µL of 30% acetic acid was added to each well of the microtiter plate to solubilize the crystal violet. Then, the microtiter plate was incubated at room temperature for 10-15 min. OD was measured using the ELISA reader at 595 nm [21].

Biofilm inhibition assay

The antibiofilm activity of methanolic extract of *C. vulgaris* was determined by inoculating clinical pathogens *P. aeruginosa* and *S. aureus* in a 96-well microtiter plate containing different concentrations (0.25, 0.5 and 1.0 mg/ml) of extract. The algal extract and standard antibiotics (azithromycin) were separately added to both organisms and incubated at 37°C for 24 h. The planktonic cells were removed out, and the tubes were washed with sterile water. 125 µL crystal violet (0.1%) was added to all the wells and incubated for 30 min at room temperature. After 30 min, the stain was removed and washed with sterile distilled water to remove unbound crystal violet stain. Finally, the adhered biofilm bound crystal violet was eluted in absolute ethanol (200 µL), and the absorbance was measured at 595 nm [22].

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The effect of *Chlorella* extract on biofilm formation of *P. aeruginosa* and *S. aureus* was studied using MTT assay. An overnight culture of *P. aeruginosa* and *S. aureus* was prepared using LB broth. 100 µL of the diluents were loaded in a 96-well microtiter plate. Different concentrations of 5 µL, 10 µL, and 20 µL methanolic extract of *C. vulgaris* were added to microtiter plate. The microtiter plate was then incubated at 37˚C for overnight. The biofilm was then incubated with 0.5 mg/ml of MTT, a yellow tetrazole and kept at 37°C for 10 min. After incubation, the supernatant was discarded and washed with phosphate-buffered saline. The number of surviving bacteria was determined by measuring the ability to reduce the yellow tetrazolium salt to purple formazan product at 570 nm [23]. Higher the OD indicates an increased number of surviving microorganisms in the biofilm. Percentage of inhibition was calculated using the equation.

$$\%\text{inhibition} = 100-([At-Ab]/[Ac-Ab])\times 100$$

Where,
At=Absorbance value of test compound
Ab=Absorbance value of blank
Ac=Absorbance value of control.

Statistical analysis

All the experiments in this study were conducted in triplicates, and the data are presented as mean±standard deviation.

RESULTS AND DISCUSSION

Phytochemical analysis

The results of qualitative phytochemical analysis of the various extracts of *C. vulgaris* are shown in Table 1. From Table 1, we see the presence of carbohydrates, amino acids, proteins, phenolics, flavonoids, alkaloids, saponins, and steroids in the extracts of petroleum ether, dichloromethane, chloroform, ethyl acetate, methanolic, and acetone extract of *C. vulgaris*. The presence of steroids was observed only in dichloromethane, chloroform, and methanolic extract of the algae. Alkaloids and saponins were found to be absent in the petroleum ether and the ethyl acetate extract of *C. vulgaris*. The glycosides and terpenoids were absent in all the extracts of *C. vulgaris*. A similar result was reported by Annamalai and Nallamuthu [24].

The most commonly known phytochemical constituents with medicinal value are flavonoids and phenolic compounds. The obtained results indicated that *C. vulgaris* is proving to be a valuable resource of bioactive compounds with the medicinal property.

Antibiofilm activity

MIC

The MIC values of different solvent extracts of *C. vulgaris* were evaluated against *P. aeruginosa* and *S. aureus* and are shown in Table 2. Except for ethyl acetate, all other extracts showed inhibitory activity against *P. aeruginosa.* Dichloromethane and petroleum ether extract showed no effect on the growth of *S. aureus*. The MIC of 1 mg/ml was observed for the methanolic extract of *C. vulgaris* against both pathogenic organisms – *P. aeruginosa* and *S. aureus.* The least concentration 1 mg/ml of methanolic extract was taken for further studies.

Biofilm formation

The OD value of the clinical strains of *P. aeruginosa* and *S. aureus* was found to be 0.914 and 0.902, respectively (Fig. 1). According to Naves

Table 1: Phytochemical screening of different extracts of *C. vulgaris*

S. No.	Phytochemical constituents	Extracts of *C. vulgaris*					
		PE	DI	CF	EA	MN	AE
1.	Carbohydrates	+	+	+	+	+	+
2.	Amino acids	+	+	+	+	+	+
3.	Proteins	+	+	+	+	+	+
4.	Phenols	+	+	+	+	+	+
5.	Flavonoids	+	+	+	+	+	+
6.	Alkaloids	–	+	+	–	+	+
7.	Glycosides	–	–	–	–	–	–
8.	Saponins	–	+	+	–	+	+
9.	Terpenoids	–	–	–	–	–	–
10.	Steroids	–	+	+	–	+	–

"+" represents the presence of phytochemicals, "–" represents the absence of phytochemicals. PE: Petroleum ether, DI: Dichloromethane, CF: Chloroform, EA: Ethyl acetate, MN: Methanol, AE: acetone

Table 2: Minimal inhibitory concentration of different solvent extracts of *C. vulgaris against P. aeruginosa* and *S. aureus*

C. vulgaris extract	Minimal inhibitory concentration (mg/ml)															
	P. aeruginosa								*S. aureus*							
	7.0	6.0	5.0	4.0	3.0	2.0	1.0	0.5	7.0	6.0	5.0	4.0	3.0	2.0	1.0	0.5
Methanol	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+
Chloroform	–	–	–	–	–	–	–	+	–	–	+	+	+	+	+	+
Ethanol	–	–	–	+	+	+	+	+	–	–	–	–	–	+	+	+
Ethyl acetate	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+
Dichloromethane	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Petroleum ether	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+

"-" represents the inhibitory activity of the extract and "+" represents ineffectiveness of the extract. *C. vulgaris*: *Chlorella vulgaris*. *P. aeruginosa*: *Pseudomonas aeruginosa*. *S. aureus*: *Staphylococcus aureus*

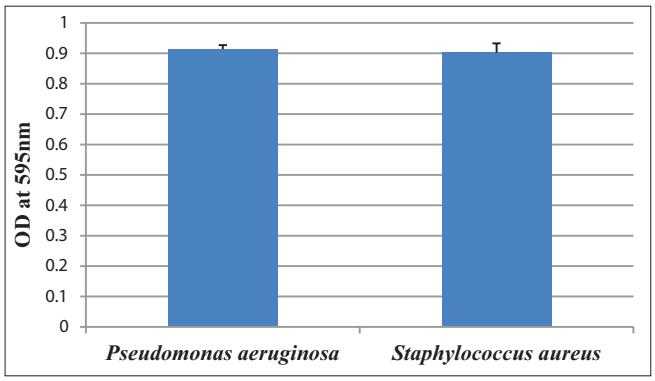

Fig. 1: Biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Values are expressed as mean±SD)

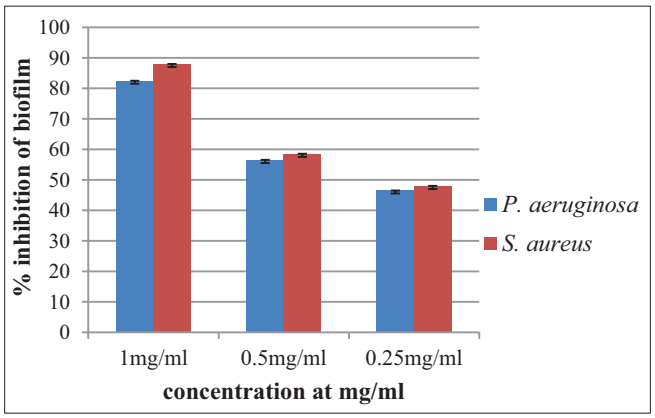

Fig. 2: Inhibition of biofilm formation in *Pseudomonas aeruginosa* and *Staphylococcus aureus* treated with methanolic extract of *Chlorella vulgaris* (Values are expressed as mean±SD)

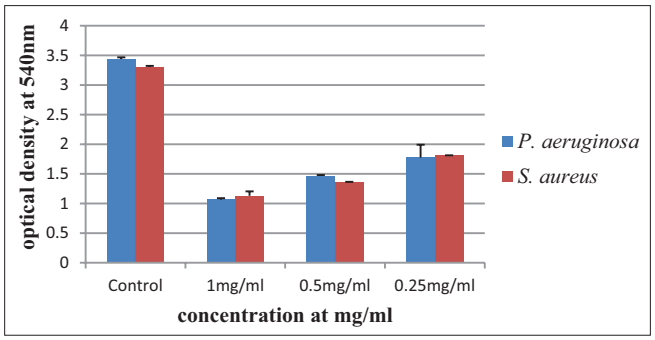

Fig. 3: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on the viability of *Pseudomonas aeruginosa* and *Staphylococcus aureus* treated with methanolic extract of *Chlorella vulgaris* (Values are expressed as mean±SD)

et al., the OD ≥0.3 was considered as active biofilm-forming bacteria. Both the clinical strains were considered as strong biofilm formers [25].

Biofilm inhibition assay

The present work was carried out to find the antibiofilm activity of methanolic extract of *C. vulgaris* against the pathogenic bacteria – *P. aeruginosa* and *S. aureus* and is shown in Fig. 2. The treatment with 1.0 mg/ml, 0.5 mg/ml, and 0.25 mg/ml of the methanolic extract of *C. vulgaris* has shown significant reduction in biofilm formation (82.5%, 56.5%, and 46.5%, respectively) for *P. aeruginosa* and (88.0%, 58.5%, and 48.0%, respectively) for *S. aureus*.

Mutungwa *et al*. reported only 49.36% inhibition of biofilm formation in *P. aeruginosa* when treated with *Syzygium aromaticum* at a concentration of 200 mg/ml [26]. Whereas, methanol extract of *C. vulgaris* showed 82% inhibition even at a concentration of 1 mg/ml. Pratiwi *et al.* reported that the ethanol extract of *Caesalpinia sappan*, *Kaempferia rotunda,* and *Nymphaea nouchali* showed the antibiofilm activity against *P. aeruginosa* and *S. aureus* at a concentration of 0.25 mg/ml [27].

MTT assay

The methanolic extract of *C. vulgaris* at different concentration of 1.0, 0.5, and 0.25 mg/ml reduced the viability of *P. aeruginosa* and *S. aureus* biofilm (Fig. 3). The maximum reduction in the viability of *P. aeruginosa* and *S. aureus* biofilm was found to be given in terms of OD value of 1.065 and 1.129 at 1 mg when compared to the untreated sample. The OD value for 0.5 mg/ml and 0.25 mg/ml extract of *C. vulgaris* was found to be 1.466, 1.775, and 1.361, 2.0 for *P. aeruginosa* and *S. aureus,* respectively. Gayatri *et al*. reported that the ethanolic extract of *C. vulgaris* reduced the viability of *P. aeruginosa* was found to be 0.171, 0.198, and 0.247 OD at different concentration of 1 mg, 0.5 mg, and 0.25 mg/ml when compared to untreated sample [28].

In our experiments, the methanolic extract of *C. vulgaris* at a concentration of 1 mg/ml showed the antibiofilm activity against the clinical pathogens studied. The biofilm inhibition property of the methanolic extract was confirmed by the results obtained in MTT assay. Thus, potential microalgal-derived compounds will have a major footprint in the treatment of antibiotic-resistant pathogenic bacteria.

CONCLUSION

P. aeruginosa and *S. aureus* are responsible for nosocomial infections to severe tissue infections. In this study, the methanolic extract of *C. vulgaris* significantly inhibits the biofilm formation and QS controlled virulence factors in *P. aeruginosa* and *S. aureus.*

AUTHORS' CONTRIBUTION

The design of the work was carried out by Dr. N. Santhi (corresponding author) and Dr. M. Rajeswari; the laboratory work was carried out by Dhanusha V and Sridevi N.S. The manuscript was designed by the authors and reviewed by the corresponding author.

CONFLICTS OF INTEREST

None declared.

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