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Antibiofilm and Antiquorum Sensing Potential of Microalgae: A Study on *Spirulina platensis*

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ABSTRACT

The rise of multi-drug resistant microorganisms has become a major global challenge. This is mainly due to the over-use of antibiotics and the formation of biofilms. Quorum-sensing processes control biofilms, which are responsible for increased bacterial resistance. The present study was aimed to evaluate the antibiofilm and antiquorum sensing properties of Spirulina platensis. Antibacterial activity of several solvent extracts of against Pseudomonas aeruginosa and Staphylococcus aureus. The methanolic extracts were found to be more effective in considerably reducing biofilm formation, showing bacterial viability and affecting quorum sensing processes. The results suggest that microalgae are promising sources of alternative antibacterial chemicals.

Keywords: multi-drug resistant, antibiofilm, antiquorum sensing, *Spirulina platensis*, methanolic extracts.

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1. INTRODUCTION

Over-prescription and inadequate use of antimicrobial drugs lead to increased antibiotic resistance. Biofilms are organised microbial communities in a self-produced extracellular matrix that protects them. This matrix protects the bacterium from drugs and host immunological reaction. Quorum sensing is a relevant target for antimicrobial therapy due to its regulation of biofilm formation and activation of virulence traits.

Antimicrobial resistance (AMR) is a significant global health issue, primarily due to the overuse and misuse of antibiotics. As a result, resistant strains of microorganisms are appearing which have decreased the efficacy of common treatment and increased the impact of infectious diseases (Ventola C., 2015). In addition to genetic mutation, bacteria have several ways to enhance their resistance to antimicrobial treatments, such as biofilm formation.

Biofilms are complex populations of bacteria adhered to surfaces and embedded within a self-produced matrix of extracellular polymeric substance (EPS). The matrix inhibits medications from entering and shields the bacteria from environmental stimuli and the human immune response (Flemming, 2008). This indicates that bacteria in biofilms are substantially more resistant than their planktonic counterparts (Costerton, 1999). Pseudomonas aeruginosa and the Staphylococcus aureus are able to create biofilm firmly and are generally employed and associated with resistant and health related illnesses.

Quorum sensing (QS) is a major mechanism regulating biofilm formation and pathogenicity of bacteria in a cell-density dependent way. This system involves bacteria that produce and sense signalling molecules that co-ordinate group actions such as production of virulence factors, motility, and biofilm formation (Bassler, 2002). In Gram-negative bacteria, QS is mostly controlled by systems like Las and Rhl, like Pseudomonas aeruginosa however in Gram-positive bacteria like Staphylococcus aureus Agr system is particularly significant (Rutherford, 2012) & (Bassler, 2002). An interesting technique is to target the pathways involved in quorum sensing, which decreases the virulence of the bacteria without strongly promoting the development of resistance.

Nowadays, there has been a significant interest in naturally synthesized substances as another antimicrobial tectis due to their different chemical composition and lesser harmful reactions. Microalgae, especially, have become prominent for their abundance of biologically active substances. Microalgae like Chlorella vulgaris and Spirulina platensis producing a kind of compounds such as phenolics, flavonoids, alkaloids, and fatty acids, which shows antimicrobial, antioxidant, and anti-inflammatory characteristics.

The healing properties of microalgae have been known for many years, and several studies have shown their antibacterial properties . For example, Spirulina platensis has been shown to inhibit the growth of Gram-positive and Gram-negative bacteria, and Chlorella vulgaris has been described as having strong antimicrobial and antioxidant properties (Kaushik, 2008) & (Safi, 2014). However, the effect of this kind of research on biofilm inhibition and quorum sensing has not been extensively studied.

Recent research indicate that chemicals found in algae can interfere with the communication mechanism of bacteria, lowering their pathogenicity and inhibiting the production of biofilms (Natrah, 2011). These chemicals may interfere with the routes employed for group coordination in bacterial population or inhibit signalling reception. Also, chemical components in algal product such as hydroxyl and amide groups improve the bond with microbial cells and so increase their efficiency.

The present study termed as “Evaluation of Spirulina platensis for its Antibiofilm and Antiquorum Sensing Activities using in Vitro and in silico Studies,” is aimed to explore the potential of these microalgae to act as antibiofilm and antiquorum sensing agents.

The main aim of the study was to analyse the effect of these extracts on the major pathogens like Pseudomonas aeruginosa and Staphylococcus aureus . The investigation identified the active chemicals responsible for these effects.

The emergence of multidrug-resistant bacteria and the limited efficacy of the currently available antibiotics make the search for bioactive chemicals from microalgae a promising and sustainable route for the development of novel antimicrobial strategies.

2. MATERIALS AND METHODOLOGY

Culture Media and Reagents

Different microbiological substances were employed for growing bacteria and conducting experiments, which included Mueller–Hinton agar/broth and Luria–Bertani (LB) medium. *Spirulina platensis* was cultivated using Zarrouk medium. The composition of LB medium was tryptone (10 g/L), yeast extract (5 g/L), sodium chloride (10 g/L), and agar (18 g/L), with pH adjusted to 7.0.

Zarrouk medium included necessary nutrients like sodium bicarbonate, sodium nitrate, potassium phosphate, magnesium sulfate, calcium chloride, iron sulfate, and trace element solutions (A and B) that promote the growth of algae. (Zarrouk, 1966)

Extra materials utilized in this research contain crystal violet (0.4%) for staining biofilms, phosphate-buffered saline (PBS, pH 7.4), and elastin-Congo red substrate. A stock solution of gentamicin antibiotic (100 mg/mL) was made by dissolving it in clean Milli-Q water and then filtering it before application.

Microbial Strains and Culture Conditions

Several bacterial strains were used: diverse strains of Pseudomonas aeruginosa (clinical and reference strains), Staphylococcus aureus, and Chromobacterium violaceum. Most of the bacterial strains were cultured in LB medium at 35°C, except for Chromobacterium violaceum, which was maintained at 30°C. These temperatures were maintained to produce optimum conditions for growth and biofilm production.

Spirulina platensis: Cultivation and Maintenance

Micro-algae Spirulina platensis was cultured in Zarrouk's medium. Cultivation was conducted in controlled laboratory environment with temperature of 25 ± 2°C, light-dark cycle of 16:8 h and light exposure of about 1500 lx. (Zarrouk, 1966)

After the algae have grown, the biomass is harvested and freeze-dried using a lyophilizer to produce a dry powder for further extraction.

Extraction of Algal Biomass

5 grams of dried *Spirulina platensis* biomass was extracted by using a Soxhlet device with solvents of ascending polarity, such as hexane, dichloromethane, chloroform, ethyl acetate, methanol, and acetone. The resulting extracts underwent filtration using a 0.2 µm membrane and were then dissolved in a mixture of methanol and Milli-Q water (1:9, v/v). These extracts were utilized in all subsequent bioassays.

Antibiofilm Assay

The ability of algal extracts to control biofilm formation by Pseudomonas aeruginosa was tested by microtiter plate method. Bacterial cultures (approx. 10^7 CFU/mL) were inoculated into LB broth and subjected to different doses of the extracts (0.1–250 µg/mL). The free cells were washed away, and after 24 h incubation at 37 °C, the biofilm was stained with 0.4 % crystal violet. Ethanol was added to dissolve the stained biofilm, and the optical density was measured at 570 nm. The percentage of inhibition in biofilm formation was calculated by comparing with the untreated sample. (Nithya, 2010)

Antimicrobial activity

Antibacterial properties of the extract were evaluated by agar well diffusion technique. Overnight cultures of P. aeruginosa were plated on Mueller–Hinton agar and placed in wells with the algal extracts. After incubation at 37°C, the zones of inhibition were measured to assess antibacterial activity.

Minimal Inhibitory Concentration determination

The minimal Inhibitory concentration (MIC) Spirulina extract was determined using 0.1 to 5.0 mg/ml concentration in tubes. The tube with solvent alone was act as a control. 0.1 ml of young culture of Psudomonas spp. And Staphylococcus Spp. was added in each tubes containing different concentration of SME and incubate all the tubes at 37ºC for 24 hrs. After incubation optical density was measured at 595 nm and determined the MIC upon the tubes having no growth.

Microscopic Analysis of Biofilms

The biofilm architecture was examined using light microscopy. Biofilms were grown on glass slides and stained with crystal violet dye. Structural characteristics, such as biofilm thickness and surface coverage, were analyzed by microscope.

Cell Surface Hydrophobicity (CSH) Assay

The bacterial adhesion to hydrocarbons (BATH) assay was performed as described by Thenmozhi et al. to evaluate cell surface hydrophobicity. Treated and untreated bacterial cultures were mixed with toluene, vortexed, and the absorbance of the aqueous phase was measured at 600 nm. The hydrophobicity percentage was calculated based on the reduction in optical density.

EPS Quantification

Extracellular polymeric substances (EPS) were extracted from treated and untreated cultures by centrifugation, followed by precipitation using trichloroacetic acid and acetone. The resulting pellet was collected and weighed to determine the EPS production.

Microbial cell viability assay (Nemati, 2013)

100µl of the diluted cultures were added to each wells of the microtiter plates, and then 30 µl and 15 µl of the methanolic extract of Spirulina platensis were added. The microtitre plates were incubated at 37°C for 24 hours. After that, biofilms were incubated with MTT (3-(4, 5- dimethyl thiazol-2yl) - 2, 5-diphenyl tetrazolium bromide) (0.5mg/ml in PBS) at 37°C for3 hours. MTT was removed from all the wells. After incubation, the purple formazan that formed inside the bacterial cells was dissolved by DMSO, and then the absorbance was measured at 570 nm.

3. RESULTS AND DISCUSSION

Antibacterial and Antibiofilm Efficacy

The research displayed that Spirulina platensis Methanolic extracts show notable antibacterial and antibiofilm properties against both Gram-negative (*P. aeruginosa*) and Gram-positive (*S. aureus)* bacteria. **(Image 1)** Initial assessments using the agar well diffusion method confirmed the existence of areas where bacterial growth was inhibited, indicating the presence of active compounds like phenolic, flavonoids, and alkaloids.

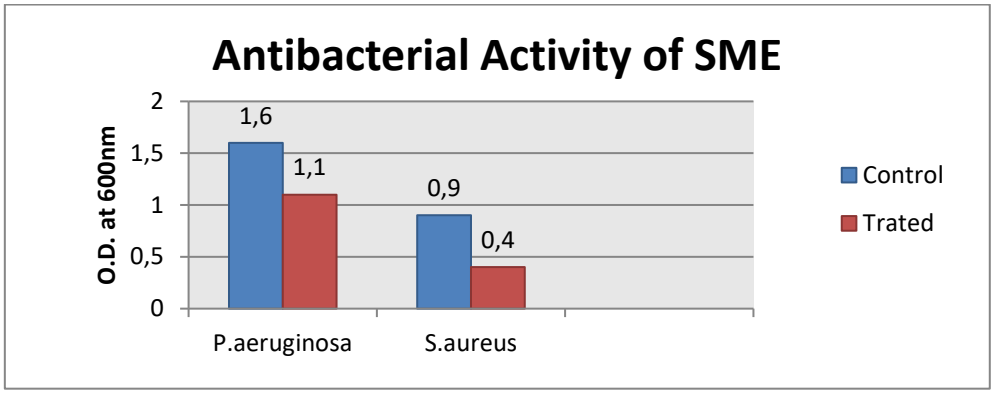

Image 1. Graphical representation of antibacterial activity of Spirulina methanolic extract against hospital isolates.

The antibiofilm test demonstrated a dose-dependent decrease in biofilm formation due to the extracts. At the highest concentrations used, the extracts markedly decreased bacterial attachment to surfaces. This is crucial as biofilms create protective shields, often enhancing bacterial resistance compared to their free-floating counterparts.

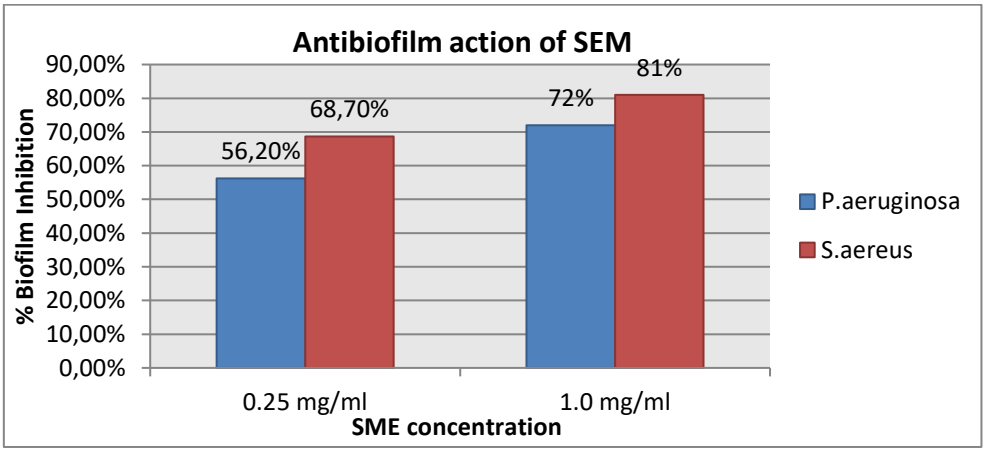

Image 2. Graphical representation of antibiofilm action of Spirulina methanolic extract against hospital isolates

Minimal Inhibitory Concentration (MIC)

Methanolic extract of Spirulina platensis revealed to possess growth-inhibitory activity at 3 mg/ml dose. However, the extract of Spirulina platensis in ethyl acetate, dichloromethane, or petroleum ether did not show any inhibition of the growth of these pathogens. The minimum inhibitory concentration (MIC) of Spirulina platensis ethanol extract was 3.0mg/ml for Pseudomonas aeruginosa and 4.0mg/ml for Staphylococcus aureus. The chloroform extract showed the MIC values of 4.0 and 3.0 mg/ml against Pseudomonas aeruginosa and Staphylococcus aureus, respectively. The methanol extract of Spirulina platensis showed the minimum inhibitory concentration (MIC) 3.0 mg/ml for both the clinical isolates. Therefore, the methanol extract of Spirulina platensis was selected for further study.

Biofilm generating bacteria have 1000 fold tolerance to medicines and escape from the immune system of the host (Hofman et al., 2005). Chauhan and Kaushik (2008) showed that the minimal inhibitory doses of the Spirulina platensis methanolic extract were 256 micrograms/ml for *Escherichia coli*, 128 micrograms/ml for *Staphylococcus aureus,* and more than 512 micrograms/ml against *Pseudomonas aeruginosa.* On the other hand, the lowest inhibitory concentration (MIC) of the Spirulina platensis methanol extract in this study was 3.0 mg/ml for gram-positive and gram-negative bacteria .

The obtained MIC values against *Pseudomonas aeruginosa* and *Staphylococcus aureus* were higher than prior investigations. The increase of the MIC value could be due to the test organisms that are clinical isolates employed in this experiment.

Table 1. MIC determination of Spirulina Extract in different Solvent against isolated *Pseudomonas Spp.*

Extract	Concentration (mg/ml)						
	4.0	3.0	2.0	1.0	0.5	0.2	0.1
Chloroform	-	+	+	+	+	+	+
Ethyl acetate	+	+	+	+	+	+	+
Methanol	-	-	+	+	+	+	+
Ethanol	-	-	+	+	+	+	+

Table 2. MIC determination of Spirulina Extarct in different solvent against *Staphylococcus spp.*

Extract	Concentration (mg/ml)						
	4.0	3.0	2.0	1.0	0.5	0.2	0.1
Chloroform	-	-	+	+	+	+	+
Ethyl acetate	+	+	+	+	+	+	+
Methanol	-	-	+	+	+	+	+
Ethanol	-	+	+	+	+	+	+

Impact on Extracellular Polymeric Substances (EPS)

The primary mechanism underlying the observed antibiofilm activity was the reduction in EPS production. EPS is vital for biofilm stability as it shields bacteria from antibiotics and host immune responses.

Quantitative analysis of EPS **(Table 3)** showed a substantial decrease in content when treated with *S. platensis* extracts.

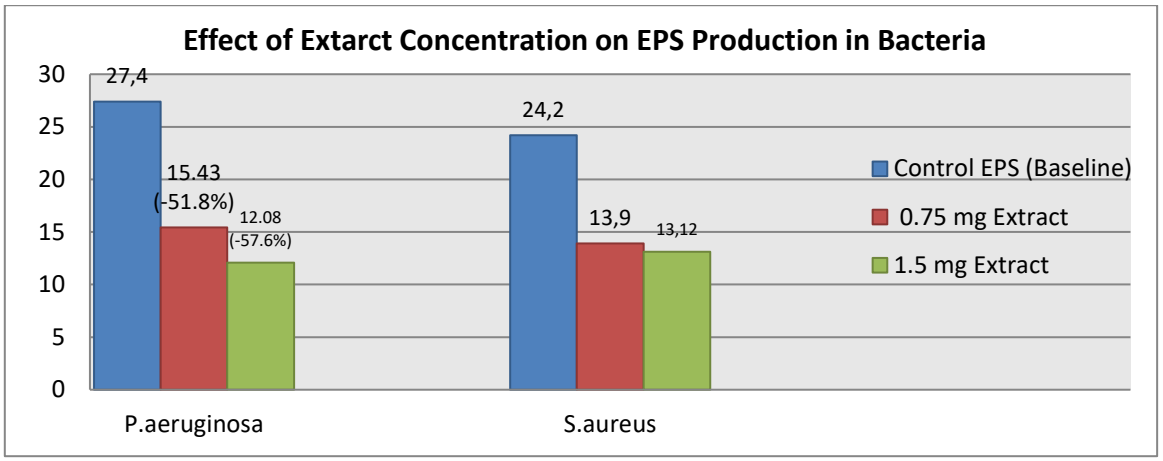

Table 3. Quantitative analysis of EPS against hospital isolates.

The extract showed a peak reduction of 57.6% in EPS of *P. aeruginosa* at 1.5 mg and 50.9% in S. aureus. The reduction in the biofilm's "molecular glue" is directly related to the reduced structural integrity seen under the microscope.

Cell Surface Hydrophobicity

Additionally, based on the Cell Surface Hydrophobicity (CSH) assay, there was a decrease in bacterial attachment to hydrocarbons. Since greater hydrophobicity is linked to improve initial bonding to surfaces, the decline in CSH indicates that the substances alter the outer layer of bacterial cells, making it tougher for pathogens to start forming biofilms.

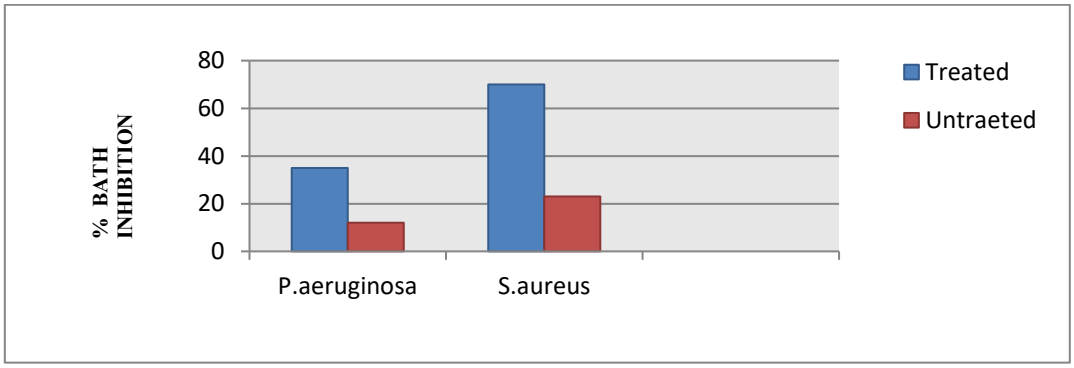

Table 4. CSH inhibition by SME against isolates.

Microbial Cell viability Assay

In contrast, (Gayatri, 2019) showed that the ethanolic extract of Chlorella at a concentration of 2.0 mg/ml reduced Pseudomonas aeruginosa's metabolic activity by up to 90% and biofilm formation by 85% at a concentration of 1.0 mg/ml.

According to (Jadhav, 2013) arrow essential oil reduced Listeria's metabolic activity. In this investigation, both CVM and SPM significantly decreased the viability of bacterial cells against Pseudomonas aeruginosa and Staphylococcus aureus.

In this investigation, SPM significantly decreased the viability of bacterial cells against Pseudomonas aeruginosa and Staphylococcus aureus **(Table 5).**

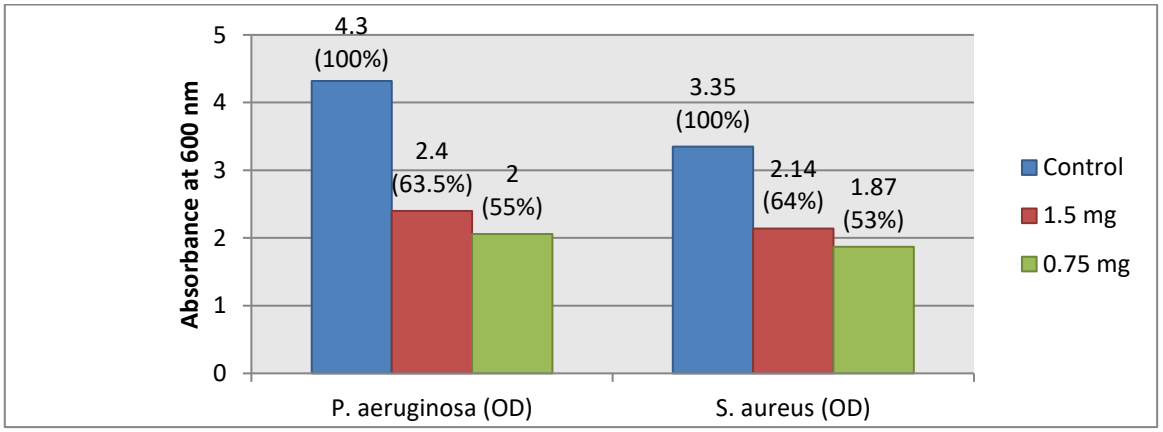

Table 5. MTT Assay for Clinical Isolates by Using SME.

4. CONCLUSION

Cyanobacteria Spirulina platensis has many useful properties that have been studied for their ability to break down biofilms made by certain Gram Positive and Gram Negative bacteria. The present study offers experimental evidence of biofilm inhibition, in addition to a reduction in the EPS and CSH of the tested bacterial strains. In addition to being good for you, *S. platensis* formulations could also be used to treat long-lasting illnesses caused by bacteria that form biofilm. The active principle that causes this activity could be better understood and cleaned up to help make new or better agents, but this has only been partially successful so far. The extracts are made from Spirulina powders that are safe for humans to eat, so using them to fight infectious diseases could be very useful.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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