

The effect of methanolic extract of *Spirogyra* varians on biofilm genes expression of *Staphylococcus aureus*

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Abstract

Background and goals: Methicillin resistance *Staphylococcus aureus*

(MRSA) is one of the most significant antibiotic resistant bacteria, that can produce biofilm. Which has seriously hampered patient care, Twenty to thirty percent of people carry this bacteria with no clinical symptoms appear. New strategies were required to eradicate the effect of this bacteria on health care, so this study aimed to evaluate biofilms gene expression levels in MRSA after treated with alga *Spirogyra* extract.

Materials and Methods

Soxhlet extract of the *Spirogyra varians* species was made in methanol, the minimum inhibitory concentration (MIC) of methanol extract of *Spirogyra* toward *S. aureus* was detect. PCR technique was used to Real Time PCR was used to measure the desired genes' expression levels at doses below the minimum inhibitory concentration (MIC).

Results

the *Spirogyra* extract MIC toward *S. aureus* was 200 mg/ml, the effect of extract on genes the significantly changed the expression of genes involved in MRSA biofilm formation. After being treated with algal extracts from *Spirogyra*, the expression level of *icaA* and *icaD* were 0.480, 0.688, respectively, *icaD* expression level was correspondingly high.

Conclusion

This study showed that *Spirogyra* methanol extract can prevent *S. aureus* from forming biofilms.

1. Introduction

Bacterial infection is the primary factor in many serious issues that result in physical impairments, health issues, and fatalities every day in the world. One of the important human pathogens that can adapt to a variety of hosts and environmental circumstances and produce a wide range of diseases is *Staphylococcus aureus*. It is also a key contributor to infections acquired in hospitals and the community [1]. It can result in infections of the lower respiratory tract, skin and soft tissues, bloodstream, and medical equipment, including central-line-associated bloodstream infection (CLABSI) and some very dangerous deep-seated infections such endocarditis and osteomyelitis [2]. A variety of virulence factors

and toxins are available to *S. aureus*, the latter can cause a wide range of toxin-mediated illnesses, such as foodborne illnesses, scalded skin syndrome, and staphylococcal toxic shock syndrome [3].

One of the main problems with *S. aureus* is that it can become resistant to most medications so the use of alternative therapeutic agents is receiving more attention as a result of the development of bacterial resistance to antibiotics, which is a significant barrier, as well as the sometimes-severe side effects of many antibiotics, which are enough to cause aversion to many synthetic drugs [4]. There is a high demand for the development of novel medicines using essential resources like algae. Following the rise in demand for biodiversity, algae are receiving more attention since they have a more specific impact on traditional treatments. Long-standing use of algae in traditional medicine and evidence of some algae's bactericidal, bacteriostatic, antiviral, antifungal, and anticancer properties [5]. *Spirogyra* is filamentous green algae belonging to the Zygnematales order, *Spirogyra*, has new physiologically active substances that include antiviral, antioxidant, anti-inflammatory, and antibacterial properties [6]. In contrast, *Staphylococcus aureus*, a human pathogen and Gram-positive bacteria, can cause a variety of illnesses include endocarditis, bacteremia, furthermore, infections of the skin and soft tissues [7]. MRSA is a resistant bacterial strain that can develop when antibiotics like penicillin are used incorrectly or excessively against *S. aureus* [8]. Microbial biofilms are bacterial colonies that grow on both living and nonliving solid surfaces. They are immersed in a self-producing matrix. Biofilm-associated cells can cling permanently to a range of surfaces, such as live tissues and implanted medical devices including valves, prostheses, catheters, and so on [9]. Since biofilms are extremely resistant to both human immune systems and antibiotics, they are thought to be a significant virulence component that results in chronic, recurring infections [10].

In comparison to free-floating planktonic cells, exopolysaccharide-protected bacteria in biofilms can be up to 1,000 times more resistant to a medication [9], which has significant consequences for therapy and makes finding alternative treatments exceedingly challenging. Antibiotics that cause biofilm resistance can result from a number of factors, such as the emergence of multidrug efflux pumps, type IV secretion systems, decreased permeability, and enzyme activity. The need for new control measures to be developed is heightened by the growing biofilm resistance to existing therapies [11]. Inhibition of biofilm formation is seen as an important therapeutic target for the treatment of many bacterial and fungal infections, and much research is now being done on the pharmacological development of these medications. Many environmentally friendly, non-lethal options for controlling biofilms have recently been developed since the mechanism of action of these cutting-edge antibiofilm medications is far less vulnerable to the development of resistance [12].

Though promising, each of these strategies has its shortcomings as none have been fully effective [13]. An approach is to search for naturally occurring compounds of algal origin that can inhibit the production of biofilms [14]. The physiologically active compounds found in plant extracts have long been a valuable source of natural goods that have greatly aided in diagnosis, maintenance and care of human health [15]. They have a long history of usage in folk medicine to heal illnesses and afflictions since ancient times, which contributes to their popularity. People also think they are safe [16]. Thus, this study aimed to

assess the efficacy of algal organic extracts against MRSA and emphasize the importance of new materials as potential antibiotics.

2. MATERIALS AND METHODS

2.1. Collection and Preparation of algae Samples

Six samples were collected from various regions of Baghdad and its surrounding area During the summer of 2022, only one species, *Spirogyra*, was isolated from the University of Baghdad-aljadria. Manual collection of *Spirogyra* variant samples from the water was done. The collected macro algae were brought to the lab in polypropylene bags for storage. According to Bellis *et al.* (1986) voucher specimens of several species were compressed and kept in 5% formalin for identification [17]. To get rid of extraneous substances like sand, shells, etc., fresh water was used to rinse the biomass. The macro algae were kept in the labs, allowed to dry at room temperature, and then pulverized into a powder using a blender.

2.2. Extract of *Spirogyra*

extract the active compounds was done by soxhlet apparatus, 20 gm of algal powder and 600 ml of methanol were taken and extracted within 6 hours [18]. Then it was dried by the incubator and the dry extract was weighed and dissolved with DMSO and then kept in the refrigerator until use.

2.3. Bacterial strain and culture conditions

An assessment was conducted using a standard strain of *S. aureus* obtained from Mustansiriyah University/ College of Science/ bacteriology lab, to assess the antibacterial effects of *Spirogyra* extract on bacterial biofilm genes expression. The strain was first cultivated on BHA (Brain Heart Agar/India) and in MHB (Muller Hinton Broth). The isolate was then utilized for further analyses after being incubated for 24 hours at 37°C. a modified version of the Micro titer Plate method was employed to evaluate the development of biofilms [19]. As list below:

- Agar for brain and heart infusion was made using 2% sucrose.
- After being sanitized with alcohol and UV light, the plastic plate. For twenty-four hours, the plate was incubated at 37°C after medium was introduced to the wells and 75 micrometers of bacterial broth were added.
- The liquid mixture was emptied after incubation and rinsed three times with sterile phosphate buffer (PBS) to remove any surplus.
- 200 liters of 95% ethanol were used to wash the plate after it had been dipped in crystal violet dye and left for 1 minute.
- At 620 nanometers in wavelength, the plate was read. [20, 21].
- Weak or non-biofilm formation at 0.1 nm.
- Intermediate or good biofilm formation at 0.5 nm.

- Strong or strong biofilm formation at 0.500 nm

2.4 Antibigram and Minimum inhibitory concentration (MIC)

A test for antibiotic susceptibility against six different antimicrobial agents was done by disc diffusion method, including ceftazidime (CAZ 30 µg), levofloxacin (LEV 5 µg), Tetracycline (TE 10 µg), imipenem (IMP 10 µg), Erythromycin (E 15 µg), and gentamicin (CN 10 µg), to determine the antibiogram activity of the bacterial isolates according CLSI 2022 guidelines.

The microdilution technique was used to ascertain the antibacterial activity of metabolic extract of *Spirogyra*. One colony from a 24-hour-grown culture of MRSA on BHA was selected and dissolved in MHB to create bacterial suspensions. 0.5 McFarland standard concentrations of comparable turbidity were present. Serial dilutions of the algal extracts starting at 200 mg/ml for extract were first made. At first, 100 µl of culture media and 100 µl of algal extract were placed in each well of the microtiter plate. Then, 100 µl from this well was put to the second well, 100 µl from the second was taken out, and the process was repeated for the last well of the micro titer plate. After that, Except for the well that had no bacterial culture in it and was designated as the negative control while the last well was the positive control, 50 µl of microbial suspension was put to each well and incubation 24 hour at 37 degrees. When evaluated in relation to the positive control well, turbidity revealed bacterial growth. A concentration was said to be at which no growth was seen, or MIC. For each experiment, three repetitions were conducted [22].

2.5 Evaluated biofilm gene expression

Real time PCR technique was used to evaluate the level of biofilm genes (*icaA* and *icaD*) expression. In order to compare the expression of the biofilm genes *icaA* and *icaD* with and without the application of *spirogyra* extract on MRSA isolates. The procedure was carried out in many steps, including:

- **RNA extraction**

Total RNA was extracted from two groups (first with presence algae extract, second with absent) by using TransZol Up Kit (Trans/China) by centrifuge (2 m/8000 rpm) the suspension of bacteria, discarded the supernatant and pellet suspended in 1000 µl TransZol Up Kit, the eluted RNA was kept at -80°C and extracted according manufacture, Nanodrop examined the RNA's purity and concentration.

- **Genomic DNA removing**

The Easy Script® One-Step gDNA Removal and cDNA Synthesis Super Mix (Trans/China) Kit method was used to carry out the cDNA synthesis.

- **2.6.3. Oligonucleotides sequences of primers and Real-time PCR conditions**

This procedure was carried out by using primers as list in table (1). RT-PCR mixture contained 10 µl of Master mix syper green, 3 µl cDNA, 1 µl of each primers and completed the volume with Nuclease free water until 20 µl. RT-PCR amplification was done by CFX96 (Bio-Rad/USA). Denaturation at 94 C°/ 1 min and then 40 time repeat denaturation at 94 C°/ 10 Sec., annealing at 58 C°/ 15 Sec. and extension at 72 C°/ 20 Sec. the plot of RT-PCR appeared as fluorescence from the amplification reaction and cycle numbers on X-axis, while the proportional amount of amplified product appeared on Y-axis.

Table (1): Primer's oligonucleotides sequence

Primer	Sequence (5′→3′ direction)
<i>icaA</i>	
Forward	GAGGTAAAGCCAACGCACTC
Reverse	CCTGTAACCGCACCAAGTTT
<i>icaD</i>	
Forward	ACCCAACGCTAAAATCATCG
Reverse	GCGAAAATGCCCATAGTTTC
<i>gyrB</i>	
Forward	GTCGAAGGGGACTCTG
Reverse	GCTCCATCCACATCGG

Statistics analysis

The expression of genes was evaluated according to Duncan’s multiple range comparisons (DMRTs) test considering 95% confidence interval (CI) and by consideration of *p* value < 0.001 as a significant result.

3. Results

3.1. Morphological Structure of *Spirogyra varians*

Unbranched filamentous green algae belonging to the genus *Spirogyra spp.* produce mats in shallow seas. It frequently grows in shaded littoral zones of lakes, meandering streams, and stagnant waters like ponds and canals They has a vegetative structure easily distinguishable due to its distinctive structure spirally coiled chloroplast present in their cells (Fig. 1). Since the genus *Spirogyra* is photosynthetic, it plays a part in the overall process of fixing carbon dioxide in their environment, they raise the oxygen content and they are eaten by a variety of aquatic life [23].

Figure (1): Spirogyra spp. Under microscopy

3.2. Antibacterial activities of crude extracts of algae

According to the results of the antibiogram test, all six isolates exhibited multidrug resistance (Fig. 2). The high sensitive rate became apparent towards Imipenem (32mm), Levofloxacin (28mm) and Gentamicin (20mm), although resistance was seen both sensitively and moderately toward Erythromycin (18mm), and resistant towards Ceftazidime (1mm) and tetracycline (12mm). Due to the widespread resistance of bacteria to antibiotics, a search was made for natural antibacterial alternatives, by using alga. Algae proved that methanol extracts of *Spirogyra* was good against the human pathogenic bacteria with an inhibition zone (28mm) at a concentration 250mg/ml. Over the last decades several studies on algal extracts antimicrobial activity have been reported in figure (3).

Figure (2): Antibiotic resistance profile of *S. aureus* isolates. CAZ: Ceftazidime; LEV: Levofloxacin; TET: Tetracycline; E: Erythromycin; GEN: Gentamicin; IMP: imipenem and SPI: *Spirogyra* extract

Figure (3): show the effect of *Spirogyra* effect on *S. aureus* biofilm in microtiter plate

3.3. Results of Gene Expression

The amount of MRSA susceptibility to the effects of *Spirogyra* methane extracts was assessed using the MIC technique. The *Spirogyra* methanol extract had a minimum inhibitory concentration of 200 mg/ml. At 18 hours after being treated with herbal extracts of *Spirogyra*, the expression level of the genes involved in biofilm formation (*icaA* and *icaD*) was statistically significant ($p < 0.001$) in comparison to the housekeeping gene (*gyrB*). In actuality, as concentration was increased, the expression levels of the genes under study declined. The relative reaction after receiving treated with *Spirogyra* shown in Table (2 and 3).

Expression of the target genes (*icaA*, *icaD* and *gyrB*), which are considered as important resistance genes in *S. aureus* were done in three replicates.

Figure (5): Comparison of average decrease in gene expression in three groups *icaA*, *icaD* and control

Traditionally, reference or housekeeping genes have been utilized in RNA expression analysis utilizing real-time PCR methods to account for sample variation [27]. As it becomes more obvious that some housekeeping genes may differ significantly in different biological samples, this technique is coming under scrutiny. Using the RT-PCR method, genotypic expression was found for the genes *icaA*, *icaB*, and *gyrB* in *S. aureus* isolates by RNA extraction. The comparison of average decrease in gene expression in two group *icaA* group and

icaD group is shown in (Fig. 5) while the mean *icaA* gene's Ct values range from 0.8013 to 0.4900 is shown in (Fig. 4-A) and the mean *icaD* gene's Ct values varied from 6.3880 to 0.6490 is shown in (Fig. 4-B)

Table (2) Fold of *icaA* Gene expression Depending on $2^{-\Delta C_t}$ Method

Groups	Mean	Std. Deviation	Std. Error of Mean	p-value
200 μ l	0.8013 b	0.14142	0.10000	0.001**
400 μ l	0.6364 c	0.07071	0.05000	
600 μ l	0.5746 c	0.02828	0.02000	
1000 μ	0.4760 c	0.01414	0.01000	
1200 μ	0.4900 c	0.01414	0.01000	
Control	0.9950 a	0.00707	0.00500	

Table (3) Fold of *icaD* Gene expression Depending on $2^{-\Delta C_t}$ Methods

Groups	Mean	Std. Deviation	Std. Error of Mean	p-value
200 μ l	6.3880 a	0.15839	0.11200	0.001**
400 μ l	4.4560 b	0.48649	0.34400	
600 μ l	5.9485 a	0.07283	0.05150	
1000 μ	2.2220 c	0.11031	0.07800	
1200 μ	0.6490 d	0.05515	0.03900	
Control	0.9900 d	0.01414	0.01000	

4. Discussion

In recent years the increasing in multi drug resistance bacteria was emerging [28, 29], causing increase in public health problems that is not affect of health sector only but also in economics [30]. the employing natural substances like medicinal herbs appears to be the most viable way to treat these diseases. Numerous research on this subject and the impact of therapeutic herbs on different microorganisms,

such as *H. pylori*, *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, and many others, have been done by now[31]. This study has demonstrated that medicinal herbs, like *Spirogyra*, alter the bacterial cell wall and prevent the growth of these bacteria. Since 20–50% of hospital medication expenditures are related to the overuse of antibiotics, financial loss and resource waste result from this [32]. Numerous recent studies in various sectors of these compounds' ability to regulate biofilm have been cited, in accordance with the significance of alga chemicals in the treatment of bacterial illnesses and also the increasing attention that researchers have paid to them. Over the last decades several studies on algal extracts antimicrobial activity have been reported. Even though the majority of these studies showed inconsistent activities against the tested microorganisms, it is challenging to compare the findings from these studies because a variety of factors can affect the antimicrobial activity of algal extracts, such as the type of algae involved [24], the extraction process, the testing procedure, the solvent used in the extraction [25], the season or time of year the samples were collected, and the location of the sample collection [26].

The results of this investigation, which examined an MRSA strain with the *icaA*, *icaD*, and *gyrB* genes, demonstrated that applying *Spirogyra* extracts had the ability to inhibit the methicillin-resistant *S. aureus* bacteria's biofilm-forming genes. The effects of *Artemisia princeps* ethanolic extract on MRSA were assessed in a 2015 research by Yang Chio. The study's findings were reported as the suppression of genes related to pathogenicity, biofilm formation, and acid generation [11]. Chio's work examined the esteemed genes *sea*, *agrA*, and *sarA*, which vary from our target genes. Furthermore, the concentration used in our study differs from the concentration used by Choi, as Choi used a concentration of 2 mg/mL from the *Artemisia princeps* extract, while we used a concentration 200 mg/ml of *spirogyra* extract was the effective dosage we used.

Aloevera leaf aqueous extract was found to have antibacterial properties against six of methicillin-resistant *S. aureus* strains in a 2018 study conducted by Saddiq *et al*. The findings showed that, following 24 and/or 48 hours of exposure, the concentrations of plant extract was 15–20 mg/ml significantly decreased the dry weights of the majority of *S. aureus* strains [32]. The results of our investigation demonstrated that these extracts had a more powerful effect on MRSA biofilm formation due to a greater down regulation of target gene expression.

Overall, based on the data from this investigation, it can be said that therapeutic algae extract from *Spirogyra* has potent compounds that prevent the gene expression necessary for MRSA biofilm development.

In this piece of art, we discovered that algal extract had a positive impact on the gene expression of biofilm development and may warrant further investigation.

Conclusion

Several genes are involved in the development of biofilms, which is one of the most significant microbial pathogenic tactics. However, these genes are good candidates for novel components that could have an impact on their expression. Therefore, evaluating the impact of different natural and synthetic

components on biofilm gene expression is of interest to scientists. According to our survey, alga and respected alga extracts have a strong influence on the genes implicated in MRSA biofilm formation strain and may indicate the need for further assessment.

Declarations

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Consent to publish (Ethics): All authors agree to publish this work.

Research involving human & animals' participants: Not applicable.

Conflicts of interest: there are no conflicts of interest.

Data Availability Statement: No Data associated in the manuscript.

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Figures

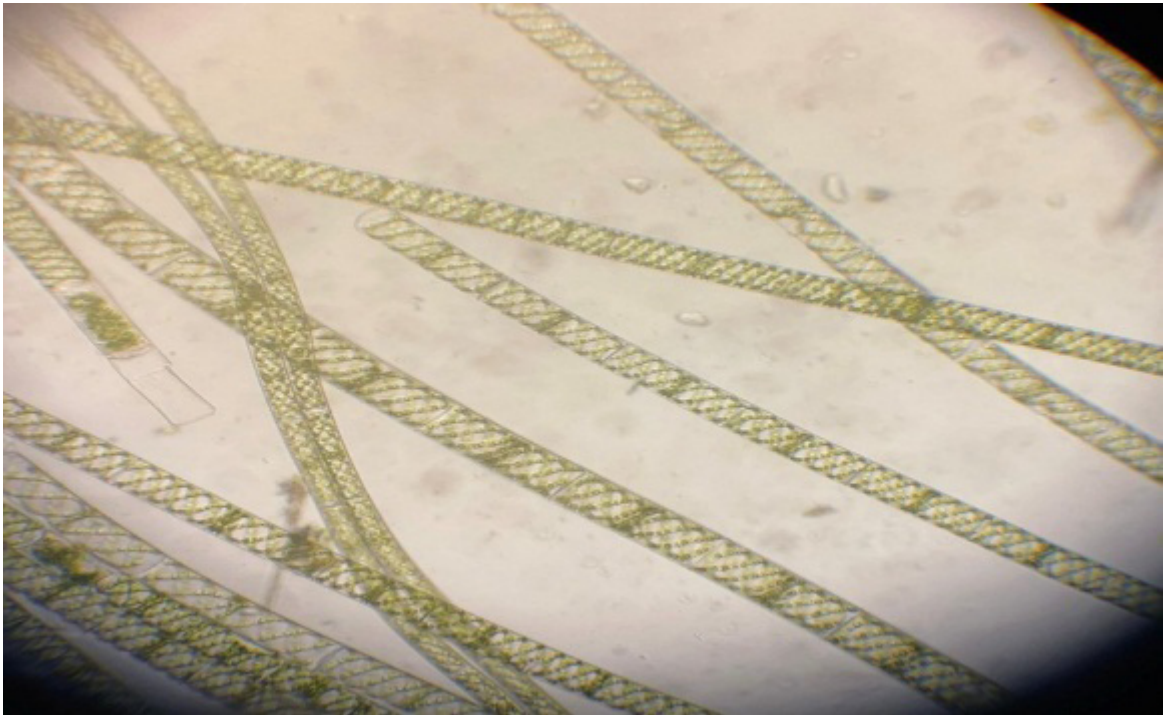


Figure 1

Spirogyra spp. Under microscopy

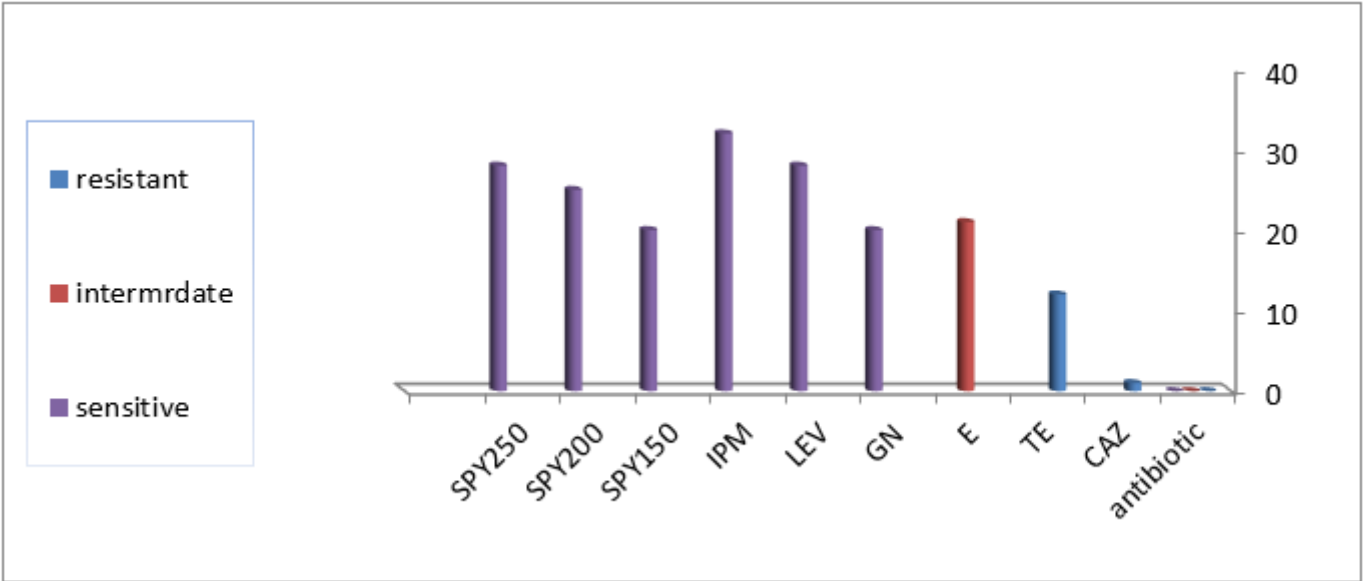


Figure 2

Antibiotic resistance profile of *S. aureus* isolates. CAZ: Ceftazidime; LEV: Levofloxacin; TET: Tetracycline; E: Erythromycin; GEN: Gentamicin; IMP: imipenem and SPI: *Spirogyra* extract

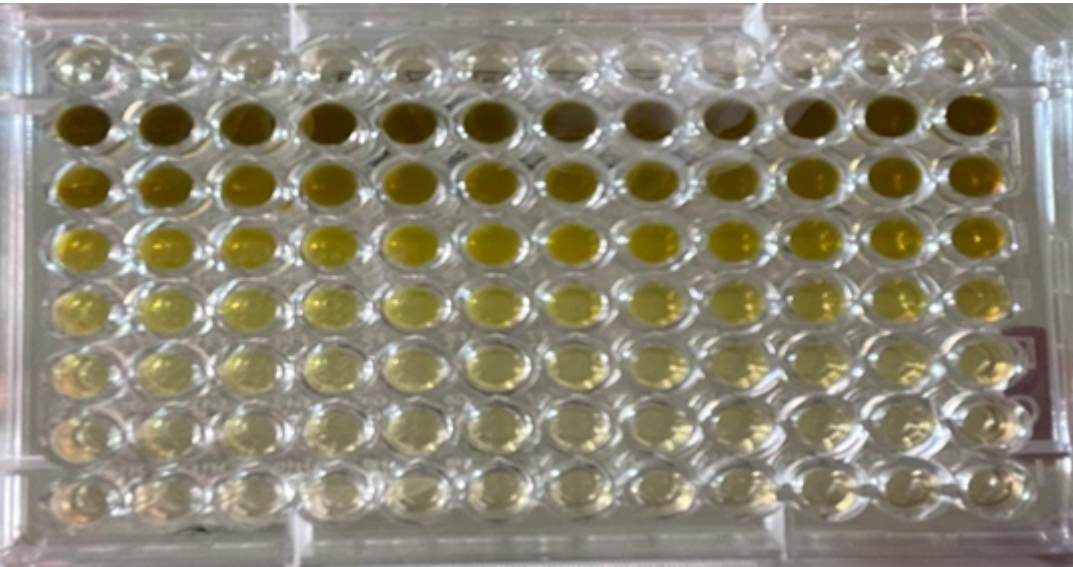


Figure 3

show the effect of *Spirogyra* effect on *S. aureus* biofilm in microtiter plate

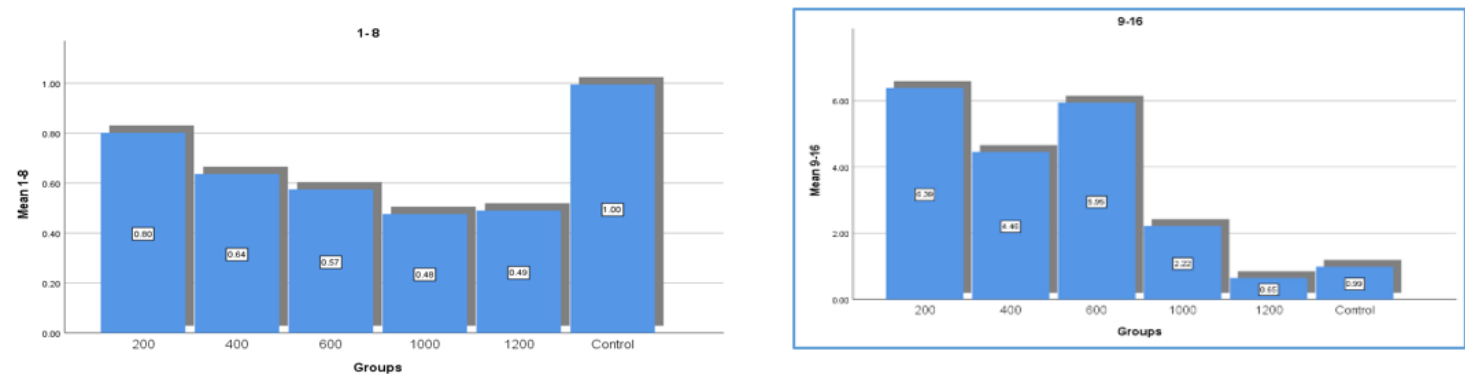


Figure 4: show the mean of Ct values ; A is the mean *jcaA* gene's Ct values while B is the mean *jcaD* gene's Ct values

Figure 4

See image above for figure legend

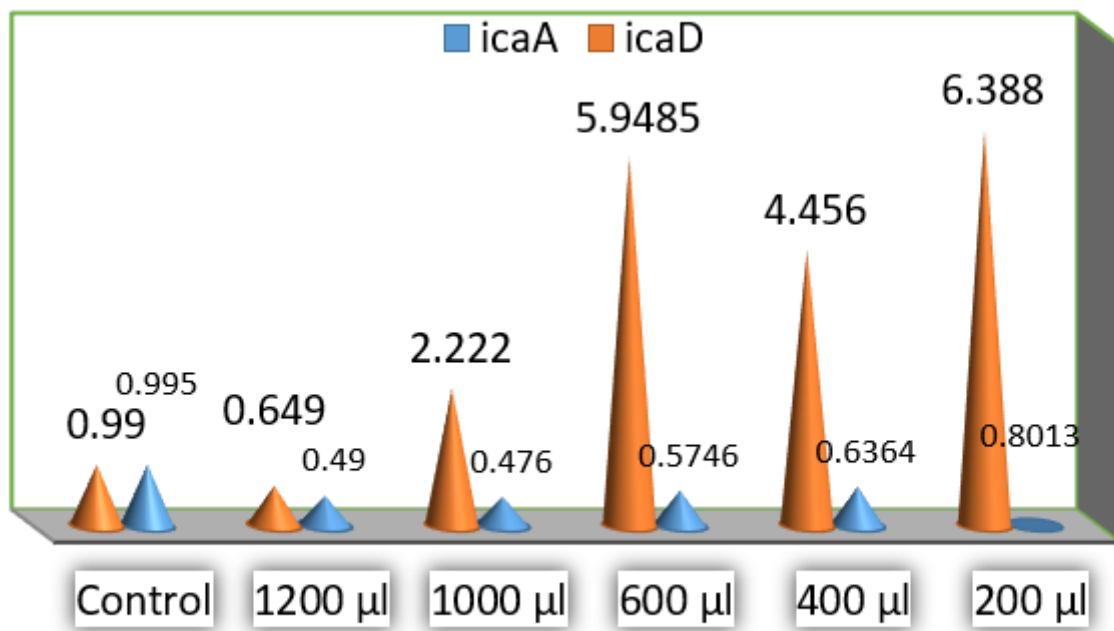


Figure 5

Comparison of average decrease in gene expression in three groups *icaA*, *icaD* and control