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GC-MS Analysis Of Bioactive Compounds In Spirogyra Extract And Its Antibacterial Activity

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Abstract
Methanolic extracts of Spirogyra varians are investigated for their effectiveness against Pseudomonas aeruginosa, Escherichia coli, Acinetobacter and Staphylococcus aureus. The algae used in my experiment were collected in the summer from the Al-Baietha area in Iraq. Antibacterial effectiveness was tested using the agar well diffusion method at different concentrations of the extracts (25, 50, 100 and 200 µg/mL). High extract amounts were shown to strongly inhibit the bacteria tested this result obtain in this study while increasing the concentration of an extract can enhance its antibacterial efficacy, this relationship is not absolute and depends on various factors, including the type of bacteria, extraction method, and the chemical composition of the extract. Therefore, specific studies are necessary to determine the optimal concentration for each case. "Furthermore, GC-MS analysis of the extract showed that it contains fatty acids, hydrocarbons and esters as active compounds. In this final phase of the study, it was demonstrated that the extract decreased the expression of icaA and icaD, genes involved with making the biofilm the study revealed that the extract exerted a more significant influence on the icaD gene compared to the icaA. These findings suggest that Spirogyra possesses significant potential as a natural source of antimicrobial agents. Further studies focusing on the isolation of individual compounds and the elucidation of their mechanisms of action are recommended to fully explore the therapeutic applications of Spirogyra extracts in combating bacterial infections and biofilm-related diseases.
Keyword: *Acinetobacter, Antibacterial,GC-MS, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Spirogyra.*

Running title: Chemical Composition and Antibacterial Properties of Spirogyra Extract

INTRODUCTION

Algae are photosynthetic organisms found in a wide range of aquatic and terrestrial environments. They range from microscopic unicellular forms (microalgae) to large multicellular seaweeds (macroalgae), playing essential ecological roles such as oxygen production, carbon fixation, and serving as the base of aquatic food chains. It is estimated that algae are responsible for producing up to 50% of the atmospheric oxygen, highlighting their crucial role in maintaining ecological balance (Field et al., 1998).

In addition to their environmental importance, algae are gaining significant interest in biotechnology due to their capacity to produce a diverse array of bioactive metabolites. These include pigments (e.g., chlorophylls, carotenoids), polyphenols, polysaccharides, fatty acids, alkaloids, sterols, and terpenoids. Such compounds have shown various therapeutic potentials, including anti-inflammatory, antitumor, hepatoprotective, immunomodulatory, and antimicrobial activities (Chojnacka et al., 2020).Macro algae are known for being a rich source of bioactive compounds, producing a wide range of secondary metabolites with various biological activities. Compounds with antioxidant, antiviral, antifungal, and antimicrobial properties have been identified in brown, red, and green algae (Hussein, 2021).

Green algae, one of the most widespread types of algae globally, thrive in various environments, including freshwater. They can also be found in saline waters, which constitute about 10% of their habitats. These algae grow rapidly and can be found adhering to rocks (endolithic), wet soil (endaphic), or plants (epiphytic) (Lee, 2019).

Green algae are recognized for their antimicrobial properties, effective against both Gram-positive and Gram-negative bacteria (Michalak et al., 2022).

It has also been reported that algae synthesize compounds such as antibiotics that can combat pathogens in both humans and fish (Anisha et al., 2022).

Macroalgae, particularly green algae (phylum Chlorophyta), are easily cultivated and harvested, which makes them economically viable for large-scale applications. Among green algae, species of the genus Spirogyra are commonly found in freshwater bodies across the globe. They are filamentous, unbranched

algae known for their characteristic spiral chloroplasts and their ability to form dense mats on the surface of ponds and slow-moving rivers.

Spirogyra is particularly rich in bioactive compounds that are believed to serve as defense mechanisms against microbial invasion and environmental stressors. These compounds, including phenolic acids, flavonoids, sterols, and unsaturated fatty acids, have been demonstrated to possess antimicrobial properties against a broad spectrum of microorganisms.The antimicrobial action may involve disruption of microbial membranes, inhibition of protein synthesis, or interference with nucleic acid replication.

With the alarming rise in antimicrobial resistance (AMR), which the World Health Organization considers one of the top 10 global public health threats, there is a pressing need to identify novel and effective antimicrobial agents. Traditional antibiotics are increasingly losing their efficacy due to overuse and misuse, leading to the emergence of multidrug-resistant strains such as Methicillin-resistant Staphylococcus aureus (MRSA), Escherichia coli, and Pseudomonas aeruginosa (Sitthiwong et al.,2014).

Natural products from algae are now being explored as potential alternatives to synthetic antibiotics. Several studies have reported that algal extracts not only inhibit planktonic bacterial growth but also reduce biofilm formation—an important virulence factor for many pathogens. This is especially relevant for pathogens like MRSA, which can form robust biofilms that protect them from host defenses and antibiotics (Behzadnia et al., 2024).

Moreover, the use of advanced analytical techniques such as Gas Chromatography-Mass Spectrometry (GC-MS) has made it possible to identify the precise chemical constituents in algal extracts. GC-MS allows the detection and characterization of volatile and semi-volatile compounds, providing insights into the functional components responsible for the biological activities of algae.

Therefore, this study aims to analyze the chemical composition of e extracts from the freshwater green alga Spirogyra varians using GC-MS, and to evaluate its antibacterial efficacy against selected pathogenic bacteria. The findings may contribute to the discovery of novel, eco-friendly antimicrobial agents and support the development of algal-based applications in pharmaceuticals, food preservation, and biomedical materials.

MATERIALS AND METHODS:

1. **Collection of Samples:** Spirogyra algae were collected from the Al-Baietha region during the summer, the harvested macro algae were stored in plasticbags and transported to the laboratory. Voucher specimen of species were pressed and stored in 5%formalin for identification according, the algae were then washed well several times to ensure that all impurities were removed and left at room temperature to dry (Salman et al., 2024).
2. **Extraction Procedure:** The dried algae were subjected to methanol extraction using the Soxhlet apparatus. The resulting extract was concentrated and stored at -4°C until further use.
3. **Bacterial Strains:** The following bacterial strains, isolated from human skin, were used in the study:

- ❖ Pseudomonas aeruginosa
- ❖ Escherichia coli
- ❖ Acinetobacter
- ❖ Staphylococcus aureus

4. **GC-MS Analysis:** The chemical constituents of the Spirogyra extract were characterized using Gas Chromatography-Mass Spectrometry (GC-MS). This analytical technique enabled the identification of various bioactive compounds by determining their retention times (Rt) and corresponding peak areas, providing insights into the extract's potential antimicrobial properties (Wang & Zhang, 2019).
5. **Antibacterial Activity Testing:** The antibacterial activity of the Spirogyra extract was evaluated using the **agar well diffusion method**. Different concentrations of the extract (25, 50, 100, 200 µg/mL) were applied to the agar surface, and the diameter of inhibition zones was measured after 24 hours of incubation at 37°C (Nisar & Jabeen, 2015).
6. **Gene Expression Analysis in Biofilms**

To assess the impact of algal extract treatment on biofilm-associated gene expression in methicillin-resistant Staphylococcus aureus (MRSA) isolates, quantitative real-time PCR (qPCR) was employed. This technique measured the expression levels of the icaA and icaD genes and gyrB as housing gene, providing insights into how the algal extract influences biofilm formation at the molecular level.

The methodology involved several key steps:

• **RNA Extraction**

Total RNA was isolated from two groups of MRSA cultures: one treated with the algal extract and the other untreated (control). Total RNA was isolated using the TransZol Up Kit (TransGen Biotech, China). Initially, bacterial cultures were centrifuged at 8000 rpm for 2 minutes to pellet the cells. The supernatant was discarded, and the cell pellet was resuspended in 1000 µL of TransZol reagent. RNA extraction proceeded according to the manufacturer's instructions. The extracted RNA samples were stored at -80°C until further analysis. The concentration and purity of RNA were assessed using a NanoDrop spectrophotometer. Purity was evaluated by measuring absorbance ratios at 260/280 nm and 260/230 nm, with ratios of approximately 2.0 and 2.0–2.2 indicating high-quality RNA.

• **Genomic DNA Removal and cDNA Synthesis**

Genomic DNA was removed, and complementary DNA (cDNA) was synthesized using the Easy Script® One-Step gDNA Removal and cDNA Synthesis Super Mix (Trans, China), according to the manufacturer's instructions.

• **Real-Time PCR Conditions and Primer Sequences**

The qPCR reactions were conducted using primers listed in Table 1. Each reaction contained 10 µL of Syper Green Master Mix, 3 µL of cDNA, 1 µL of each primer (forward and reverse), and nuclease-free water to a final volume of 20 µL. The amplification process was performed on a CFX96 Real-Time PCR System (Bio-Rad, USA) with the following thermal cycling conditions: initial denaturation at 94°C for 1 minute, followed by 40 cycles of denaturation at 94°C for 10 seconds, annealing at 58°C for 15 seconds, and extension at 72°C for 20 seconds. The fluorescence signals corresponding to the amount of amplified product were recorded throughout the cycles, with cycle numbers represented on the X-axis and fluorescence intensity on the Y-axis.

Table 1: Primer Sequences Used in Real-Time PCR

Gene	Direction	Sequence (5′→3′)
icaA	Forward	GAGGTAAAGCCAACGCACTC
	Reverse	CCTGTAACCGCACCAAGTTT
icaD	Forward	ACCCAACGCTAAAATCATCG
	Reverse	GCGAAAATGCCCATAGTTTC
gyrB	Forward	GTCGAAGGGGACTCTG
	Reverse	GCTCCATCCACATCGG

RESULTS

Spirogyra varians are filamentous, unbranched green algae develops in free-floating mats in shallow seas which collected from Al-Baietha region in Baghadad- Iraq at the morning as shown in figure(1).

Figure1: Spirogyra varians under microscope 40 x

The methanol extract of Spirogyra demonstrated varying degrees of antibacterial activity against the tested bacterial strains. The inhibition zones increased with higher concentrations of the extract (200 µg/mL).

1. Result GC-MS analysis

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The GC-MS analysis of the Spirogyra extract revealed a rich composition of bioactive compounds, primarily fatty acid methyl esters and long-chain hydrocarbons, which are known for their potent antibacterial properties. The high abundance of Hexadecanoic acid, methyl ester and Methyl stearate, among others, likely contributed to the strong antibacterial activity observed against the tested bacterial strains. A total of nine major compounds were identified, as shown in Table 2 and figure (2).

Table 2: GC-MS data table

No.	Rt (min)	Area (%)	Compound
1	7.362	2.88	Benzene, methyl-Toluene
2	53.067	1.62	1-Nonadecane
3	53.919	12.15	Nonadecane
4	54.748	27.47	Hexadecanoic acid, methyl ester
5	60.309	14.73	Heneicosane
6	60.514	5.09	10-Octadecenoic acid, methyl ester
7	60.657	7.45	Heptadecene-(8)-carbonic acid
8	61.109	16.95	Methyl stearate
9	62.538	7.20	Eicosane

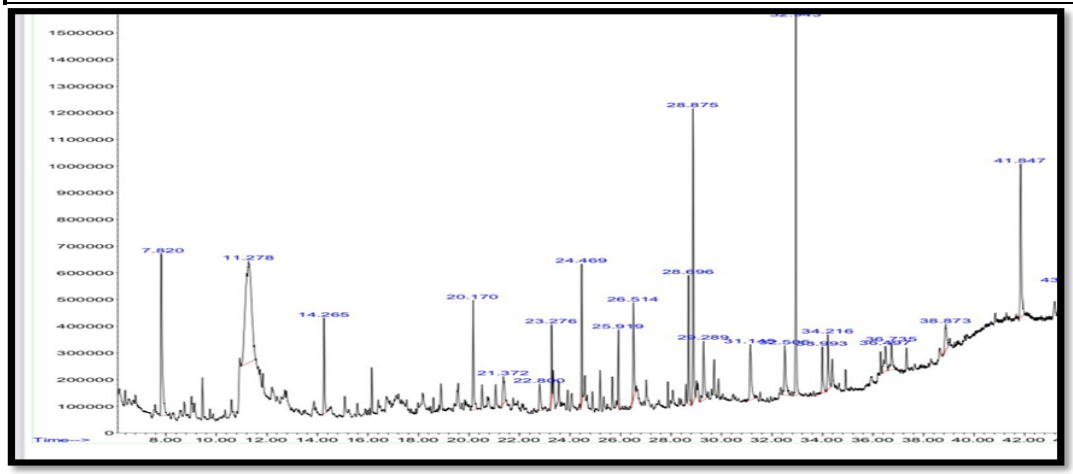

Figure1: Refer to Chromatogram of GC-Mass Spectrophotometry Chromatogram of major compound in the Spirogyra varians Methanol Extract

The most abundant compound was **hexadecanoic acid, methyl ester** (27.47%), commonly known as methyl palmitate this compound is widely recognized for its **antimicrobial, antioxidant, and anti-inflammatory** properties, which may contribute significantly to the bioactivity observed in the Spirogyra extract. Similarly, **methyl stearate** (16.95%), a methyl ester of stearic acid, also possesses known antibacterial and antifungal effects, often associated with membrane disruption in microbial cells.

Long-chain alkanes such as **heneicosane** (14.73%), **nonadecane** (12.15%), and **eicosane** (7.20%) were also detected in considerable amounts. These hydrocarbons have been reported to exert **bacteriostatic effects**, likely due to their ability to integrate into and destabilize bacterial membranes Guleria et al. (2024). Their hydrophobic nature enhances their interaction with lipid bilayers, disrupting membrane integrity and functionality.

The presence of **unsaturated fatty acid esters**, such as **10-octadecenoic acid, methyl ester** (5.09%) and **heptadecene-(8)-carbonic acid** (7.45%), adds further evidence to the antimicrobial potential of the extract. Unsaturated chains are known to create greater membrane fluidity in microbial cells, potentially leading to increased permeability and eventual lysis.

In addition, a small proportion of **methylbenzene (toluene)** (2.88%) was detected. Although found in low concentration, aromatic hydrocarbons such as toluene have shown **mild antimicrobial activity** and may enhance the overall synergistic effect of the compound mixture (Michalak et al., 2022).

These results are consistent with previous studies which reported that Spirogyra species produce a range of biologically active compounds, particularly **fatty acid methyl esters (FAMEs)**, that have been linked to antimicrobial and antioxidant activities (Sitthiwong, et al., 2024). The diversity and relative abundance of these compounds strongly support the potential of Spirogyra as a natural source of antimicrobial agents.

2. The effect of Spirogyra extract on gene expression

The effect of Spirogyra extract on the expression of the icaA gene in Staphylococcus aureus was evaluated using Real-Time PCR. As shown in Table 3, treatment with increasing concentrations of the extract resulted in a progressive down regulation of icaA gene expression compared to the control group.

Table3. Effect of Spirogyra extract on icaA gene expression in Staphylococcus aureus

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

(Different letters indicate significant differences between groups at $p < 0.01$.)

The expression of icaA significantly decreased at all tested concentrations compared to the control. The highest suppression was observed at 1000 µl concentration (0.4760), indicating a strong inhibitory effect on biofilm-related gene expression.

The findings of this study demonstrate that Spirogyra extract significantly down-regulates the expression of the icaA gene in Staphylococcus aureus. The icaA gene encodes N-acetylglucosaminyltransferase, a key enzyme in the biosynthesis of polysaccharide intercellular adhesin (PIA), which is essential for biofilm accumulation and structural integrity (Schilcher &Horswill, 2020).

The observed decrease in icaA expression correlates with increasing concentrations of the Spirogyra extract, indicating a dose-dependent antibiofilm activity. Similar inhibitory effects on icaA expression and biofilm formation have been reported for Spirulina platensis extracts against MRSA strains .

The active constituents identified by GC-MS—particularly fatty acid derivatives such as methyl palmitate and methyl stearate—are known to attenuate bacterial quorum sensing and biofilm gene regulation (Mun, Kwon, and Seo, 2022). Such fatty acids may alter membrane fluidity, disrupt signal transduction pathways involved in biofilm maturation, and directly repress transcription of biofilm-associated operons. Moreover, unsaturated fatty acids have been shown to inhibit the ica operon, thereby reducing both biofilm biomass and bacterial adhesion (Sheehan & Gilmore, 2021).

Thus, the current results support the potential of Spirogyra extract as an effective natural antibiofilm agent against Staphylococcus aureus, offering new strategies to manage biofilm-associated infections in the era of rising antibiotic resistance.

The expression of the icaD gene in S. aureus was also evaluated under the same experimental conditions using real-time PCR, with gyrB as the housekeeping gene for normalization. The results, summarized in Table 4, demonstrate a significant alteration in icaD expression across the different extract concentrations.

Table4: Effect of Spirogyra extract on icaD gene expression in Staphylococcus aureus

Group	Mean Expression	Std. Deviation	Std. Error
Control	0.9900 d	0.01414	0.01000
200 µl	6.3880 a	0.15839	0.11200
400 µl	4.4560 b	0.48649	0.34400
600 µl	5.9485 a	0.07283	0.05150
1000 µl	2.2220 c	0.11031	0.07800
1200 µl	0.6490 d	0.05515	0.03900

(Different letters indicate significant differences between groups at $p < 0.01$.)

Interestingly, at lower concentrations (200 µl and 600 µl), the expression of icaD increased significantly compared to the control. However, at higher concentrations (1000 µl and 1200 µl), a substantial down-regulation of icaD was observed, with the 1200 µl group showing the lowest expression (0.6490), even lower than the control group.

The differential expression pattern of the icaD gene following treatment with Spirogyra extract suggests a complex interaction between the extract's bioactive compounds and bacterial gene regulation mechanisms. While lower concentrations appeared to induce icaD expression, higher concentrations (≥1000 µl) resulted in significant repression. This biphasic response can be attributed to hormetic effects, whereby sub-inhibitory doses activate stress-response pathways, whereas higher doses directly inhibit transcriptional machinery (Calabrese & Mattson, 2017).

Consistent with previous findings, natural extracts rich in unsaturated fatty acids have been shown to suppress the ica operon, leading to reduced biofilm biomass and bacterial adhesion (Sheehan & Gilmore, 2021). Given that icaA and icaD work synergistically to produce the polysaccharide intercellular adhesin (PIA) matrix (Montanaro et al., 2021), the simultaneous down-regulation of both genes at higher extract concentrations underscores the strong antibiofilm potential of Spirogyra metabolites. • Higher concentrations of the extract (400 µl, 600 µl, 1000 µl, and 1200 µl) show progressively lower inhibition, suggesting that there might be an optimal dose where the extract is most effective in reducing biofilm formation.

DISCUSSION:

The identification of various bioactive compounds suggests a strong antibacterial potential of the Spirogyra extract. Hexadecanoic acid, methyl ester (methyl palmitate) is a fatty acid methyl ester known for its antimicrobial, antioxidant, and anti-inflammatory activities (Alves et al., 2020).

Its high concentration likely contributes significantly to the observed antibacterial activity by disrupting bacterial membrane integrity and inhibiting essential metabolic processes.

Methyl stearate, another abundant component, has been reported to possess antibacterial effects by integrating into and destabilizing bacterial membranes, resulting in increased permeability and cell death this result agree with (Shanmugapriya et al., 2012).

Long-chain alkanes such as Heneicosane and Eicosane have demonstrated moderate antibacterial properties, possibly through mechanisms involving alteration of membrane fluidity and inhibition of bacterial growth (El-Khateeb et al., 2016).

Additionally, the presence of unsaturated fatty acid derivatives such as 10-Octadecenoic acid, methyl ester (methyl oleate) and Heptadecene-(8)-carbonic acid emphasizes their crucial role in inhibiting microbial growth and biofilm formation by interacting with the bacterial cell wall structure (Desbois & Smith, 2010).

Although present in lower concentration, Toluene (methyl-benzene) may enhance bacterial membrane permeability and contribute synergistically to the overall antibacterial effect (Benedetti et al., 2014).

The study revealed that the extract exerted a more significant influence on the **icaD** gene compared to the other gene analyzed. This heightened effect can be attributed to several factors:

1. **Essential Role of icaD**: The **icaD** gene is pivotal in the biosynthesis of polysaccharide intercellular adhesin (PIA), a key component in the formation of biofilms by Staphylococcus aureus. Disruption of **icaD** hampers PIA production, thereby inhibiting biofilm development (Khaleghi and Khorrami 2021).

2. **Sensitivity to Plant Extracts**: Research indicates that **icaD** expression is particularly susceptible to down regulation by certain plant-derived compounds. For instance, treatments with curcumin have demonstrated a marked decrease in **icaD** expression, leading to reduced biofilm formation (Kashi et al., 2021).
3. **Molecular Binding Affinity**: Molecular docking studies suggest that specific compounds exhibit a higher binding affinity to the IcaD protein, enhancing their inhibitory effects on its function (Khaleghian 2023).

Consequently, the pronounced impact of the extract on **icaD** expression may stem from its targeted interaction with the gene's regulatory mechanisms or the protein's structure, resulting in more effective suppression of biofilm formation.

Overall, the potent antibacterial activity observed in the Spirogyra extract can be attributed to the synergistic action of these bioactive compounds, resulting in membrane disruption, enzymatic inhibition, and prevention of biofilm development. These findings highlight Spirogyra as a promising natural source of antibacterial agents for potential pharmaceutical and biomedical applications.

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