

## Research article

**Antibacterial activity of *Spirulina platensis* on some pathogenic bacteria**

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Corresponding author: **Israa N. H. Al-Asady**. Email: israa.nidhal@uomosul.edu.iq**ABSTRACT**

**Introduction and Aim:** *Spirulina platensis* is a planktonic filamentous cyanobacterium composed of discrete cellular units. Three types of *Spirulina* have garnered significant attention as possible medicinal agents. This study aimed to investigate the antibacterial properties of extract from *S. platensis* against various bacterial strains.

**Materials and Methods:** The identification of *S. platensis* was accomplished by employing both microscopic techniques and genetic investigation of phycocyanin using *cPCBA* genes. PCR was employed to identify *mcyE* gene, which is responsible for the production of microcystin, a toxin of *S. platensis*. The antibacterial efficacy of the crude extract was applied against various pathogenic bacteria. The bioactivity compounds were identified by GC-MS spectrophotometry.

**Results:** GC mass analysis established the presence of 11 active compounds (N-Methoxy-N-methyl-acetamide, n-Hexadecanoic acid, ethyl ester, Hexadecanoic acid, Octanoic acid, 2-Ethylhexyl ester, Tridecanoic acid ethyl ester, Tetradecanoic acid, Phytol 2-Hexadecen-1-ol, Tetracosane, and 8-Hexadecen-1-ol) in the methanolic crude extract. At varied doses, the hot methanolic crude extract exhibited antibacterial activity against all bacterial species tested with inhibition zones ranging from 5 to 20 mm.

**Conclusion:** The study findings demonstrates that the crude extract of *Spirulina platensis* to be a viable source for the synthesis of drugs that could be safely used as antimicrobials against pathogens.

**Keywords:** *Spirulina platensis*; antibacterial activity; GC mass spectrophotometry; *mcyE* gene.

**INTRODUCTION**

Throughout the past five decades, the advancement of pharmaceutical medications has primarily depended on the empirical screening of a significant number of pure compounds to discover new potential candidates. The medicinal potential of *Spirulina platensis*, or its extract, encompasses various areas including cancer prevention, lowering of blood cholesterol levels, mitigation of nephrotoxicity generated by pharmaceuticals and exposure to toxic metals, as well as protection against the harmful effects of radiation. The employment of cyanobacteria as a means of screening antibiotics and other pharmacologically active substances has attracted considerable interest in contemporary research, mostly due to their ancient lineage (1). The cultivation and consumption of three species of spirulina, namely *S. platensis*, *S. maxima*, and *S. fusiformis*, are prevalent on a global scale due to their substantial nutritional value and possible therapeutic properties (2). *S. platensis*, also known as *Arthrospira platensis*, has demonstrated the ability to synthesize a wide array of bioactive compounds, rendering it a valuable reservoir of various medicinal agents. These compounds exhibit medicinal properties such as antioxidants, immunomodulators, and anti-inflammatory agents (3). Consequently, *S. platensis* has found utility in numerous nutritional and medical contexts (4). Resistance to antibiotics and antifungal drugs has been on the rise in the past decade.

Health care expenditures, mortality rates, and the spread of infectious diseases worldwide are all affected by antibiotic resistance in hospitals and the general population. As a result of these changes and the subsequent rise in bacterial illnesses, the hunt for new, safer, and more effective treatments to address these infections has accelerated. The objective of this work was to identify the bioactive constituents present in the crude extract of *S. platensis* and evaluate its antibacterial efficacy against specific bacterial strains

**MATERIALS AND METHODS****Isolation and purification of *Spirulina platensis***

To obtain samples of *S. platensis*, water specimens were collected from receptacle regions of the Tigris River flowing near Mosul (longitude of 43° 06' 35"E and latitude of 36° 22' 14"N). The water samples were conveyed to the laboratory for further examination. To detect the presence of *S. platensis* microscopically, 1 mL of the water sample was transferred to 9 mL of sterilized distilled water in tubes. A drop of the sample was observed under a light microscope (40X magnification) for the presence of *S. platensis*. To isolate *S. platensis*, the alga was first grown on Zarrouk's feeding solution, the composition of which is provided in Table 1.

**Table 1:** Ingredients of selective medium (Zarrouck medium).

Ingredients	NaHCO <sub>3</sub>	NaCl	MgSO <sub>4</sub> .6H <sub>2</sub> O	FeSO <sub>4</sub> .6H <sub>2</sub> O	K <sub>2</sub> SO <sub>4</sub>	CaCl <sub>2</sub> .2H <sub>2</sub> O	NaNO <sub>3</sub>	K <sub>2</sub> HPO <sub>4</sub>	EDTA	Dist. water
Amount (g/L)	16.8	1.0	0.2	0.01	1.0	0.04	2.5	0.5	0.08	1000 ml

After growth, 1 mL of the solution was aseptically placed onto agar plates using a sterile spreader. Concurrently, a loopful of the water sample was also streaked onto agar plates. The plates were placed in an incubator set at a light intensity of approximately 200 E/m<sup>2</sup>/s and a temperature of 26.2°C for a duration of 10-12 days. The colonies that manifested on the plates were carefully selected and subsequently transferred onto additional plates to achieve a pure culture of the algae. To acquire a substantial quantity of the algae, a selection of uncontaminated *S. platensis* specimens was carefully chosen and thereafter put into 250ml flasks filled with Zarrouck nutrient solution. These specimens were then cultivated in the solution for a duration of 2-3 weeks, employing the methodologies previously outlined (5). To maintain the continuous growth of a single algal species, the cultures were periodically replenished every fortnight by the process of sub-culturing. This involved transferring the algal cultures into new flasks filled with fresh Zarrouck media.

#### Identification of *Spirulina platensis*

*S. platensis* isolated in this study, was identified based on its morphological features as described earlier (6). Molecular confirmation of identification of the algae was done using polymerase reaction chain (PCR) technique using specific sequence of primers PC-F: 5'-GGCTGACTTGTTCACGCGACA-3', while PC-R: 5' CCAGTACCACCAGCAACTAA-3' which amplified a 650bp fragment of the phycocyanin operon (7). A Cyanobacterial as well as a Chlorella spp. that lacked the phycocyanin operon were used as negative controls. DNA was extracted for PCR analysis and the PCR reaction carried out according to (8).

#### Detection of microcystin genes

To determine the ability of *S. platensis* to produce microcystins, the algal DNA was checked for the presence of mcyE gene, a component of the microcystin synthetase enzyme complex that encodes microcystin, a potent toxin. Amplification of a 472bp segment of the mcyE gene was carried out using the HEP forward 5'-TTTGGGGTTAACTTTTTTGGG CATAGTC-3' and reverse 5'-AATTCTTGAGGCTGT AAATCGGGTTT-3' (9). In general, the PCR protocols consisted of an initial denaturation step lasting 2 minutes at a temperature of 95°C. Subsequently, a total of 35 denaturation cycles were conducted, which were succeeded by the annealing step. The annealing process for the phycocyanin operon was carried out at a temperature of 52°C for a duration of 30 seconds, while for the mcyE gene, it was performed at a temperature of 44°C for a duration of 40 seconds. The extension step was at 72°C for 60 seconds,

followed by a final extension step lasting 5 minutes at 72°C. Ten microliters of PCR product were run across an electrophoresis gel containing 1.5% agarose. The gel was stained with ethidium bromide and examined using a UV transilluminator. After amplification, the products were checked against a 100-base DNA ladder to determine the accurate band size.

#### *Spirulina platensis* crude extract preparation

To achieve large densities of vegetative cells, the *S. platensis* isolate was grown in a bioreactor with Zarrouk's *Spirulina* media. The algal cells were harvested by centrifugation, dried and subjected to preparation of crude extract using the Soxhlet extraction method (10). Briefly, 1 g of fresh algal biomass was ground with 10 mL of solvent (70% methanol), and transferred to Soxhlet apparatus for extraction. After 24 h the solution was centrifuged at 10000 rpm for 15 mins, and the liquid solvent phase evaporated using a rotary evaporator maintained at 50°C to obtain a dry powder. After determining the dry extract's mass, two grams were dissolved in twenty milliliters of dimethyl sulfoxide solution to create a stock solution with a concentration 100 mg/ml. using a 0.21 mm sterile membrane, the liquid was filtered and stored at -80°C in dark until further use (11).

#### Evaluation of active compounds in *S. platensis* crude extract

Active compounds in *S. platensis* algal extract were studied using standard protocols (12)

#### Gas Chromatography-Mass spectrophotometry analysis

Agilent Technologies' (SHIMADZU-Japan) high-temperature column was used in the GC-MS spectrophotometry analysis. The column was run at high temperatures, with a constant starting temperature of 100°C and injector and detector temperatures of 280°C. The column was loaded with a 5 mL test sample in split (1:10) mode. After the initial minute, the oven temperature was raised to 225 °C at a ramp ratio of 12.5°C/min and maintained for 4 mins. Subsequently, the temperature of the oven was increased to 300°C with a ramp rate of 7.5°C per minute, and it was maintained at this temperature for a duration of 5 minutes. The carrier gas employed in this study was helium, with a flow rate of 17.5 mL/min.

**Table 2:** Bacterial isolates used in the study and their source of isolation

Bacterial isolates	Isolation source
<i>Shigella spp.</i>	Stool
<i>Clostridium spp.</i>	Wounds
<i>Micrococcus spp.</i>	Skin
<i>Enterobacter spp.</i>	Stool

**Table 3:** Biochemical test used in identification of pathogenic bacterial isolates

Biochemical test	<i>Shigella spp.</i>	<i>Clostridium spp.</i>	<i>Micrococcus spp.</i>	<i>Enterobacter spp.</i>
Indole	+	-	-	-
Methyl Red	+	+	-	-
Voges-proskauere	-	-	-	+
Simmon citrate	-	v	-	+
Oxidase	-	-	-	-
Catalase	+	-	+	+
Nitrate reduction	+	+	+	+
Mannitol Fermentation	+	v	-	+
Gelatinase	-	-	-	-
Urease	-	+	+	+
H <sub>2</sub> S production	-	+	v	-
Motility	-	+	-	+
Endospores	-	+	+	-

The acquisition and analysis of mass spectra were conducted utilizing two software platforms, namely Agilent GC-Mass Solution (SHIMADZU-Japan) and post-run software. The chemicals that were identified were determined by conducting a comparison of their masses with the NIST library search and an authentic criterion that was obtained from the reference (13, 14).

**Antibacterial activity of the crude extract**

The antibacterial activity of a crude extract of *S. platensis* was assessed using the well diffusion technique on petri plates. The activity was evaluated using pathogenic bacterial isolates as outlined in Table 2. The isolates were acquired from the laboratories of Ibn-Sina Hospital in Mosul, Iraq. They were diagnosed using API and biochemical assays, which were conducted in the laboratory to confirm their identification (Table 3). The bacterial isolates were streaked onto nutrient agar plates to facilitate growth. Wells were then created in the agar, and these wells were inoculated with 0.1 ml of activated bacterial growth. After a brief incubation period, the plates were covered and placed in an incubator at a temperature of 37°C for 24-48 hours. The widths of the inhibitory zones surrounding the bacterial growth were measured in millimeters, and the results were subsequently expressed. The negative control utilized in the experiment was dimethyl sulfoxide (15).

**RESULTS**

**Morphological and molecular identification of *S. platensis***

*Spirulina platensis* possesses various distinguishing traits when compared to other species. One distinguishing feature is the existence of a cylindrical, multicellular trichome that lacks heterocyst. This trichome is organized in an open helical pattern and

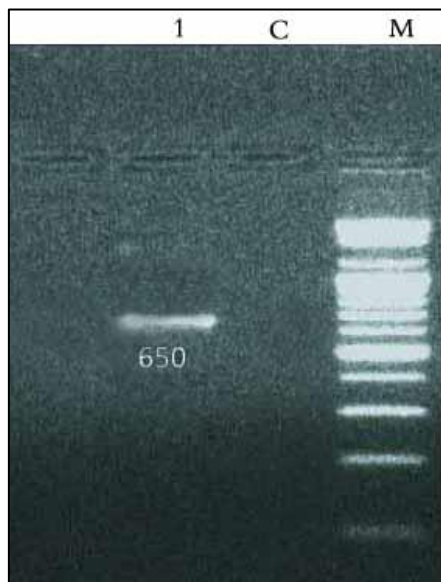
may be observed along the cross-walls of the trichome (Fig. 1).



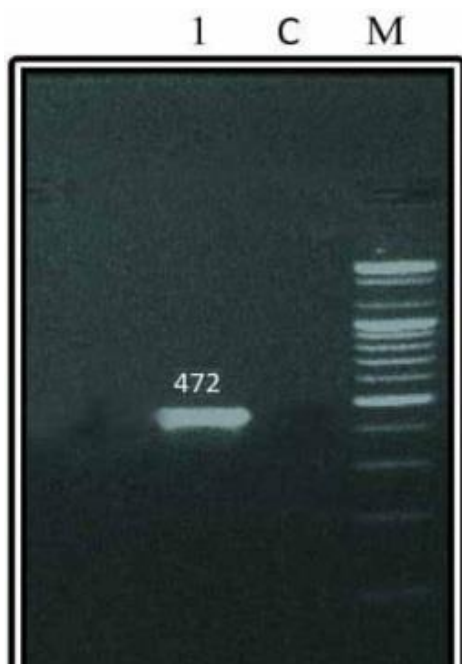
**Fig. 1:** The morphological appearance of *S. platensis* under (40X)

The polymerase chain reaction (PCR) study demonstrated the amplification of a specific fragment of the phycocyanin operon gene (*cpcBA-IGC*), which had a length of 650 base pairs. This result offered further validation that the specific algae species being investigated was truly *S. platensis*. The negative control refers to the lack of amplification seen for the chlorophyte *Chlorella sp.*, as illustrated in Fig. 2.

The molecular technique was utilized for the purpose of identifying the existence of cyanobacteria that possess the ability to produce microcystin. The HEP primers successfully amplified 472 base pair portions of the *mcy E* gene from all cyanobacterial isolates that produce microcystin, except for *Spirulina sp.* The primers utilized in this study demonstrated a notable degree of specificity towards microcystin-producing isolates, as evidenced by the absence of DNA amplification from the Chlorophyta utilized in the experimental analysis. *Microcystis aeruginosa* used as a positive control was seen as positive for the gene (Fig. 3).



**Fig. 2:** PCR gel showing the amplification of the 650 bp phycocyanin operon gene fragment. Lane 1: *S. platensis*, Lane C: *Chlorella sp.*, (negative control); M: 100 bp DNA ladder



**Fig. 3:** PCR amplification of *mycE* gene. Lane 1: *Microcystis aeruginosa* positive for *mycE* (472bp); Lane C: *Spirulina platensis*; M: 100 bp DNA ladder.

### Antibacterial activity of *S. platensis* extract

The methanolic crude extract yield obtained for *S. platensis* was 1.43 g. The presence of antibacterial activity in the crude extract was tested at concentrations of 12.5, 25, 50, and 100 mg/ml. The smallest diameter of inhibition zones was observed at a concentration of 12.5 mg/ml against both Gram negative bacteria and Gram-positive bacteria ranging 5-7 mm, while the highest biological inhibitor activity was observed at doses of 100 mg/ml. Among the microorganisms tested, *Micrococcus* spp exhibited the highest susceptibility to the treatment with 20 mm, followed by *Clostridium* spp, *Enterobacter* spp, and *Shigella* spp with 17, 16, and 13 mm respectively (Table 3). Statistical analysis at a significance level of  $P < 0.05$ , revealed no statistically significant disparity in antibacterial efficacy between the concentrations of 50 and 100 mg/ml (Table 4). However, the crude extract exhibited higher antibacterial effect on Gram positive bacteria.

### Evaluation of active compounds for *S. platensis*

To determine the specific constituents accountable for the observed biological inhibition, a chemical investigation was conducted on a hot methanolic crude extract of *S. platensis*. Additionally, the volatile chemicals present in isolated fractions of the methanolic crude extract, which demonstrated antibacterial activity, were assessed using gas chromatography-mass spectrometry (GC-MS) techniques. GC-MS analysis revealed eleven distinct active compounds in the hot alcoholic solution, as determined by their retention time (RT) and percentage of area which included: N\_Methyl\_N\_methoxyacetamide (RT-8.72; 13.75 %), ethyl ester, Hexadecanoic acid (RT-12.224; 10.56 %), n-Hexadecanoic acid (RT-15.133; 1.32 %), Octanoic acid, 2-ethylhexyl ester (RT-16.259; 14.09 %), Tetradecanoic acid (RT-17.310; 1.44 %), Tridecanoic acid ethyl ester (RT-17.752; 0.28 %), Phytol 2\_Hexadecen\_1\_ol (RT-24.196; 0.32 %), tetracosane (RT-28.789; 2.01 %), 8\_hexadecen-1-ol (RT-30.620; 21.87 %), Decanohydrazid (RT-32.989; 0.34 %), and 2H\_1\_Benzopyran\_6-sulfonamide (RT-35.731; 0.91 %) respectively (Fig. 4).

**Table 4.** Diameter  $\pm$  SE of inhibition zone (mm) of methanolic crude extract *S. platensis* against pathogenic bacteria isolates

Bacteria isolates	mg/ml concentration				LSD Value
	100	50	25	12.5	
<i>Micrococcus spp.</i>	20 $\pm$ 2	16 $\pm$ 2	10 $\pm$ 2	7 $\pm$ 1	0.543NS*
<i>Clostridium spp.</i>	17 $\pm$ 1	15 $\pm$ 0.5	11.5 $\pm$ 1	6 $\pm$ 1	0.509NS*
<i>Shigella spp.</i>	13 $\pm$ 2	11 $\pm$ 1	8 $\pm$ 2	5 $\pm$ 1	0.443NS*
<i>Enterobacter spp.</i>	16 $\pm$ 2	10 $\pm$ 2	9 $\pm$ 1	6 $\pm$ 1	0.45NS*

( $P < 0.05$ ), NS: Non-Significant \*

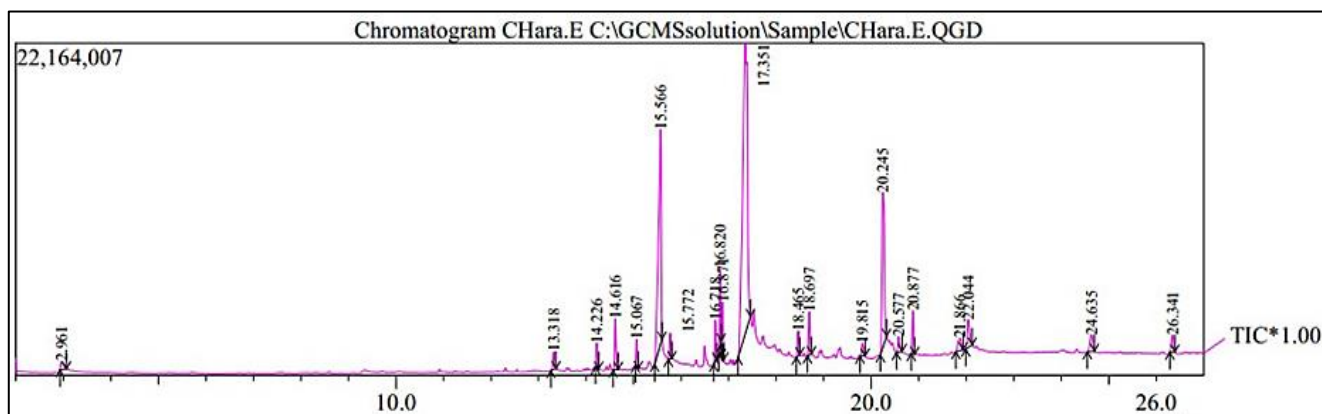


Fig. 4: GC-Mass spectrophotometry chromatogram of *S. platensis* hot methanolic crude extract

## DISCUSSION

A wide array of antibacterial compounds widely distributed in plant or algal extracts, alongside nanoparticles and other substances that have been employed as alternative therapeutic agents (16-19). Most cyanobacterial species possess the ability to produce chemical compounds with antibacterial, antifungal, and antiviral properties (20, 21). These properties are attributed to specific compounds, such as alkaloids, cyclic peptides, and lipopolysaccharides (22). The observed phenomenon could potentially be attributed to the toxins produced by the organism's cells, which have resemblance to the toxins synthesized by several cyanobacteria, known for their potential in therapeutic applications. The study conducted by Volk and Furkert revealed that certain microalgae had significant biological efficacy against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida tropicalis* (23). Organic solvents consistently yield superior extraction efficiency for antimicrobial chemicals in comparison to water-based methodologies. The active components extracted using ethane, ethyl acetate, and diethyl ether, are determined to be the most efficient solvents, both in terms of water miscibility and immiscibility; While all solvents tested demonstrated efficacy against the bacteria, it was observed that the ethanol extract exhibited antibacterial activity (24). The antibacterial action of *S. platensis* extracts has been linked to a number of different variables, including the presence of the active fatty acid -linolenic acid (25), as well as the synergistic effects of lauric and palmitoleic acid (26).

Gram positive bacteria exhibited significantly greater sensitivity levels compared to Gram-negative bacteria in this study. The observed disparity may be attributed to the distinct chemical composition of the bacterial cell walls present in each species. This elucidation would provide a rationale for the distinctiveness of the cell wall composition in each bacterial species. Gram positive bacteria are characterized by the presence of a single layer of cell wall, in contrast to Gram negative bacteria which have many layers (27). This architecture serves as a permeability barrier, preventing the accumulation of substances in the cell wall or

membrane (28). Nevertheless, it was observed that certain bacterial species did not exhibit any response when exposed to the extracts of *S. platensis*. However, the purified fractions of these extracts showed a diverse spectrum of activity against other examined organisms. Sastry and Rao proposed a theoretical framework to account for the observed paradox, and attributing the existence of inhibitory compounds within the extract to be responsible. The absence of these molecules would facilitate the observation of antibacterial action (29).

## CONCLUSION

In this study the hot methanolic crude extract of *S. platensis* was found to contain 11 active chemical components. At varying concentrations, *S. platensis* crude extract exhibited antibacterial activity being more effective against Gram positive bacteria than against Gram negative bacteria.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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