

## ANTIMICROBIAL ACTIVITY of *SPIRULINA PLATENSIS* AGAINST AQUATIC BACTERIAL ISOLATES

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**ABSTRACT**

Aquatic organisms are a rich source of novel and bioactive compounds. Cyanobacteria and microalgae being a rich source of bioactive compounds have recently found immense application in human and animal medicine. The present study was attempted to find out the effect of the various extracts of *Spirulina platensis*, *Chlorella vulgaris*, *Saragassum wightii* and *Saragassum latifolium* using different solvents (methanol, ethanol, ethyl acetate and chloroform) as antimicrobial agents against five bacterial pathogens; *S. aureus*, *E. coli*, *P. aeruginosa*, *Salmonella* sp, *Shigella* sp. Results indicate that among the various extracts used, methanol extracts of tested cyanobacterial and algal species appeared to be the most effective ones showing maximum antibacterial activity against the selected bacterial pathogens. *Spirulina platensis* appeared to be the most effective against all the pathogens studied. The antibacterial substance was purified using column chromatography. The nature of the purified active fractions was detected using different chemical analyses (UV, FT-IR, <sup>1</sup>H NMR and GC-MS) which indicated that it is an aliphatic compound and has different active groups (-OH, -C=O, -CH<sub>2</sub> and -CH<sub>3</sub>). The results of this investigation proved that the tested cyanobacterium could be a good source for the production of promising antimicrobial agents.

**Keywords:** *Spirulina platensis*, Antimicrobial activity, H-NMR, FT-IR, GC-MS

**INTRODUCTION**

Bacterial infection causes high rate of mortality in human population and aquaculture organisms (Kandhasamy and Arunachalam, 2008). Preventing disease outbreaks or treating the disease with drugs or the screening programs for selecting therapeutic chemicals tackles this problem. The search for natural compounds with antimicrobial activity has gained importance in recent years due to growing worldwide concern about alarming increase in the rate of infection by antibiotic resistant microorganisms (Kaushik and Chauhan, 2008). Various strains of cyanobacteria and algae are known to produce intracellular and extracellular metabolites with diverse biological activities such as antifungal, antibacterial, antifungal and antiviral activities (Noaman et al., 2004; Kumar et al., 2011; Al-Wathnani et al., 2012). Numerous substances were identified as antimicrobial agents from algae such as Chlorellin derivatives, acrylic acid, halogenated aliphatic compounds, terpenes, sulphur containing heterocyclic compounds, phenolic inhibitors etc. (Lavanya and Veerappan, 2011). It is generally considered that compounds produced naturally, rather than synthetically, will be biodegraded more easily and will therefore be environmentally acceptable (Ozdemir et al., 2004; Colla et al., 2007).

Attention is now being focused on the natural components produced by aquatic organisms. Cyanobacteria are potential sources of high value chemicals and pharmaceuticals (Tan, 2007). The cyanobacterium, *Spirulina platensis* has emerged as one of the most promising agents to synthesize potentially new therapeutic compounds. It is known to produce intracellular and extracellular metabolites with diverse biological activities such as antifungal (MacMillan et al., 2002), antiviral (Hayashi and Hayashi, 1996) and antibacterial activities (Kaushik and Chauhan 2008; Kumar et al., 2011).

The aim of the study is to: (1) measure *in vitro* the antibacterial activity of different extracts of some cyanobacterial and algal extracts against different aquatic microbial isolates collected from surface water of Al-Bahr El-Pherony, Menoufia, Egypt and (2) characterize the structure of active compound using different methods including; H-NMR, FT-IR, UV and GC-MS analysis.

**MATERIALS AND METHODS**

**Bacterial isolation**

The microorganisms used in the antibacterial assay were isolated from surface water of Al-Bahr El-Pherony, Menoufia, Egypt. The obtained isolates were identified as previously explained by Sabae et al. (2014). One Gram-positive bacterium namely *Staphylococcus aureus*, four Gram-negative bacteria namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* sp, *Shigella* sp. were tested for the antibacterial activity.

**Inoculum preparation**

The bacterial isolates were inoculated on Müller Hinton broth and incubated for 24 h at 30°C then suspended in saline solution 0.85% NaCl, adjusted to yield approximately 1.0 x 10<sup>7</sup>-1.0 x 10<sup>8</sup> CFU/ml by using spectrophotometer (25% transmittance at 530 nm).

**Cultivation of tested species**

*Spirulina platensis* and *Chlorella vulgaris* were obtained from the culture collection of the Botany Department, Faculty of Science, Tanta University, Tanta, Egypt. Zarrouk's medium (Zarrouk, 1966) was used for cultivation of *S. platensis* while, Kuhl's medium was used for cultivation of *Chlorella vulgaris* (Kuhl, 1962). Culture temperature was maintained at 30 ± 1°C. They were grown until the late exponential phase of the growth at which the cultures were harvested. The collected biomass was dried in a hot air oven at 60°C for 1 h.

**Collection of macroalgae**

*Saragassum wightii* and *Saragassum latifolium* samples were collected from the rocky areas surface in Red sea beach, Seuz, Egypt during spring 2013. After collection, samples were washed with seawater to remove epiphytes and other marine organisms. The seaweeds were transported to the laboratory in sterile

plastic bags. In the laboratory, samples were rinsed with tap water, identified following Abbott and Hollenberg (1976); Aleem (1993) and Taylor (1985) and dried on shadow at room temperature (25 to 30°C). Dried sample were cut into small pieces and powdered in a mixer grinder to get fine powder. Obtained powdered samples were stored in tight plastic bags.

**Preparation of various extracts**

Antibacterial extracts were prepared according to the method adopted by Kaushik and Chanhan (2008) by mixing 10 g of dried cyanobacterial and microalgae biomass to 150 ml of solvents (methanol, ethanol, chloroform and ethyl acetate) for 5 h at room temperature and sonicated for 15 min, for sea weeds the air dried samples were mixed with the respective solvent (1:15 w/v) for 72 h at room temperature with occasional shaking (Osman et al., 2013) and then filtered through Whatman filter paper No.1. The obtained extract was freed from solvent by evaporation under reduced pressure and then resuspended in the appropriate solvent to make the solution of known concentration of 50 mg/ml. The extract was stored at 4°C in airtight glass bottle for the antibacterial assay.

**Antibiotic susceptibility testing**

The susceptibility of the recovered bacterial isolates to 24 different antibiotics representing 14 different classes was performed by modified Kirby-Bauer single-disk diffusion technique on Müller Hinton agar (Robert et al., 2003). Ampicillin, oxacillin, carbenicillin, azteronam, ampicillin-sulbactam, piperacillin-tazobactam, cephalothin, cefatizidime, cefotixin, impenim, tobramycin, gentamicin, ciprofloxacin, chloramphenicol, tetracycline, erythromycin, rifampin, erythromycin, streptomycin, norfloxacin, ofloxacin, trimethoprim/sulfamethoxazole, nitrofurantoin and clindamycin were used for determination of antibiotic resistance profiles of the isolates. The results of the susceptibility tests were interpreted according to the criteria established by the Clinical and Laboratory Standards Institute (CLSI, 2010). Selected multidrug resistant bacteria were used in antibacterial assay.

**Antibacterial assay**

Antibacterial activity of the extracts was determined by microplate reader assay method according to Bechert et al. (2000) with some modifications. Aliquot of 100 µl of bacterial isolate (10<sup>6</sup> CFU/ml) in Müller Hinton broth medium was transferred to each well of 96 well plate. Volumes of 50 µl of extracts were added to each well in triplicate. The plates were incubated at 37°C for 24 h. After incubation, the absorbances of the plates were determined using automated ELIZA microplate reader adjusted at 620 nm. In every microtiter plate, one row was set for positive control (A0) without tested extract against DMSO and fresh Müller Hinton broth medium as negative controls (A2). While, the used extracts mixed with fresh medium was used as a blank group (A1). The inhibition percentage of tested extracts was calculated according to the following equation (Mulyono et al., 2012).

$$\text{Inhibition percentage} = 100 - \left( \frac{A - A_1}{A_2 - A_0} \times 100 \right) \text{ Where,}$$

- A : The absorbance of the treatment group
- A1 : The absorbance of the blank.
- A2 : The absorbance of the negative control group.
- A0 : The absorbance of the positive control group.

**Determination of the chemical structure of antagonistic material**

**Column chromatography**

The methanol extract of one gram of *Spirulina platensis* was applied in a silica gel column (60-120 mesh), and eluted with a mixture of toluene and ethyl acetate (10:1 to 1:10). The collected fractions were freed from solvents by evaporation until complete dryness in a rotary evaporator. The dried samples were dissolved in pure methanol to a final concentration of 5% and assayed for their antibacterial activity against *P. aeruginosa* and *S. aureus* using agar well diffusion assay.

**Ultra-Violet spectra (UV)**

The UV-spectra of the tested material were determined using UV2101/pc spectrophotometer. The wavelength ranged from 200 to 800 nm.

**Fourier transform-infrared spectroscopy (FT-IR) analysis**

The active fractions were analyzed using FT-IR spectroscopy. The unutilized balance fraction samples were encapsulated in KBr at a ratio of 1:100. The IR spectra were collected using a Shimadzu spectrometer within the range of 500-5000 cm<sup>-1</sup>.

**Proton Nuclear Magnetic Resonance (<sup>1</sup>H NMR) spectra**

The sample was dissolved in deuterated chloroform. The different functional groups were identified using NMR (Varian Mercury VX-300).

**Gas Chromatography -Mass Spectroscopy (GC-MS) analysis**

A sample of the extracted fraction was subjected to GC-MS (Perkin Elmer) analysis. Phytoconstituents of the sample were analyzed using Perkin Elmer Clarus 580 series gas chromatographic system and capillary column. Rtx-5ms (5% phenyl, 95% dimethylpolysiloxane-Column length: 30m Column id: 250 µm) was used with helium at a 1.2 ml/min as the carrier gas and the GC oven temperature was programmed at 270-280°C. Identification of the individual components was performed by comparison of mass spectra fragmentation pattern with the profiles from the Wiley GC-MS 275 libraries.

**Statistical analyses**

The results are presented as mean ± standard deviation of the mean (n = 3). The statistical analyses were carried out using SPSS program version 15. Data obtained were analyzed statistically to determine the degree of significance between treatments using one and three way analysis of variance (ANOVA) at P ≤ 0.01 and P ≤ 0.05 levels of significance.

**RESULTS**

**Screening for antibacterial activity**

Different recovered bacterial isolates possessed multi-drug resistant (MDR) pattern to different used antibiotics Table (1). Hence, the MDR isolates were selected for the antibacterial assay against tested extracts. Antibacterial activities of crude extracts of the tested cyanobacterium and algae were determined by ELISA microtiter plate reader and the results are summarized in Table (2). The extracts showed varying degrees of antibacterial activity against all five pathogenic bacteria tested. On a general note, methanolic extracts exhibited higher degree of inhibitory activity than other used solvents. The stated results indicated that the most promising organism for the production of the antibacterial agent was *S. platensis* against all tested bacteria. Therefore, it was selected for further investigations.

**Table 1** Percentage rates of resistance of different bacterial isolates to different antimicrobial agents.

Antimicrobial agent	No. (%) of resistant isolates				
	<i>E. coli</i> (n=7)	<i>Salmonella sp.</i> (n=7)	<i>Shigella sp.</i> (n=7)	<i>S. aureus</i> (n=7)	<i>P. aeruginosa</i> (n=7)
Ampicillin	6 (85.7 %)	7 (100 %)	6 (85.7 %)	-	-
Carbincillin	7 (100 %)	7 (100%)	4 (57.14 %)	-	7 (100 %)
Oxacillin	-	-	-	7 (100 %)	-
Amoxacillin	-	-	-	5 (71.4 %)	-
Azteronam	-	-	-	-	7 (100%)
Ampicillin/ Sulbactam	0 (0 %)	3 (42.86%)	1 (14.3 %)	0 (0 %)	-
Piperacillin/Tazobactam	-	-	-	-	0 (0 %)
Cephalothin	4 (57.1%)	4 (57.14%)	5(71.4 %)	6 (85.7 %)	-
Cefatizidime	7 (100%)	7 (100%)	7 (100%)	-	6 (85.7%)
Cefotixin	7 (100%)	7 (100%)	6 (85.7 %)	-	6 (85.7%)
Impenim	0 (0%)	2 (28.6%)	1 (14.3%)	0 (0 %)	0 (0%)

Gentamycin	0 (0%)	0 (0%)	0 (0%)	-	0 (0%)
Tobramycin	0 (0%)	2 (28.6%)	2 (28.6 %)	-	0 (0%)
Streptomycin	-	-	-	3 (42.7%)	-
Rifampin	-	-	-	6(85.7%)	-
Ciprofloxacin	1 (14.3%)	1 (14.3%)	2 (28.6 %)	0 (0 %)	1 (14.3%)
Ofloxacin	0 (0%)	0 (0%)	0 (0 %)	-	1 (14.3%)
Norfloxacin	0 (0%)	1 (14.3%)	2 (28.6 %)	-	0 (0%)
Co-trimoxazole	0 (0%)	2 (28.6 %)	2 (28.6 %)	2(28.6%)	3 (42.86%)
Clindamycin	-	-	-	7(100%)	-
Erythromycin	-	-	-	7(100%)	-
Nitrofurantoin	6 (85.7 %)	6 (85.7 %)	3 (42.86 %)	4(57.14%)	-
Chloroamphenicol	0 (0%)	3 (42.9 %)	5 (71.4%)	6(85.7%)	5 (71.4%)
Doxycycline	0 (0%)	0 (0%)	1 (14.3 %)	1(14.3%)	2 (28.6 %)

n: number of bacterial isolates

**Table 2** The mean inhibition percentage of different cyanobacterial and algal extracts against bacterial isolates recovered during summer from study area.

Tested extracts	Bacterial isolates				
	<i>E. coli</i>	<i>Salmonella sp.</i>	<i>Shigella sp.</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
	Inhibition percentage (%)				
<i>Spirulina platensis</i>					
Methanol	97.87±4.1	98.07±9.0	95.84±4.5	93.67±8.7	95.17±4.6
Ethanol	91.07±5.1	75.36±6.7	83.11±2.7	90.7±7.0	86.39± 6.3
Ethyl acetate	77.79±4.8	81.94±8.0	91.37±5.3	88.74±6.2	81.18±7.6
Chloroform	74.33±6.6	82.53±6.4	87.09±4.5	90.83±5.6	66.97±6.2
<i>Chlorella vulgaris</i>					
Methanol	92.27±6.8	92.61±5.0	90.29±5.2	85.79±7.4	82.01±5.3
Ethanol	87.51±7.5	90.64±5.1	86.43±7.2	81.96±5.5	71.89±6.4
Ethyl acetate	67.36±5.5	86.37±2.8	66.71±6.7	55.89±3.4	81.19±6.8
Chloroform	65.84±6.3	87.51±9.1	66.21±5.4	85.31±8.4	78.36±5.3
<i>Saragassum wightii</i>					
Methanol	89.38±5.6	84.93±6.5	84.56±4.5	92.61±8.6	88.03±4.7
Ethanol	81.43±6.5	87.1±5.1	74.13±5.7	67.7±5.5	83.57±6.3
Ethyl acetate	75.01±6.7	81.64±7.7	79.04±4.1	71.71±6.1	87.14±4.7
Chloroform	73.21±5.9	77.26±7.4	78.87±4.1	89.1±7.8	75±5.4
<i>Saragassum latifolium</i>					
Methanol	88.89±5.7	87.94±5.1	89.83±10.3	92.86±8.2	85.39±5.1
Ethanol	79.84±6.6	68.74±5.3	88.74±4.5	90.94±7.2	83.67±8.0
Ethyl acetate	81.44±7.6	86.99±7.3	83.04±6.5	83.56±4.4	70.57±6.3
Chloroform	73.91±5.8	67.31±6.1	81.51±27.1	84.57±8.1	84.1±5.2

Each value is the mean of three readings ± Standard Deviation Purification and characterization of the highly active crude extract of *Spirulina platensis*

**Column chromatography**

The methanol extract of *Spirulina platensis* was applied in a silica gel column and it was eluted with a mixture of toluene and ethyl acetate (10:1 to 1:10). The obtained 45 fractions were collected, tested for their antibacterial activity against *P. aeruginosa* and *S. aureus* using agar well diffusion. Only four fractions had antimicrobial activity as shown in Table (3). The UV absorption spectra of these fractions were determined using spectrophotometer (UV 2101/ pc) at range of 200 to 800 nm. The obtained results are shown in Figure 1. The results indicated that the four fractions had the same absorption peaks (three absorption peaks at 285, 365 and 510 nm). Therefore, they were pooled together and subjected to various chemical analyses to reveal its structure as far as possible.

**Table 3** The antimicrobial activities of the different fractions obtained from the silica gel column chromatography against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

No. of active fractions	Diameter of inhibition zone (mm)	
	<i>S. aureus</i>	<i>P. aeruginosa</i>
10	13±0.04	11±0.03
11	20±0.03	11.5±0.02
12	12±0.06	None
13	14±0.06	12±0.03

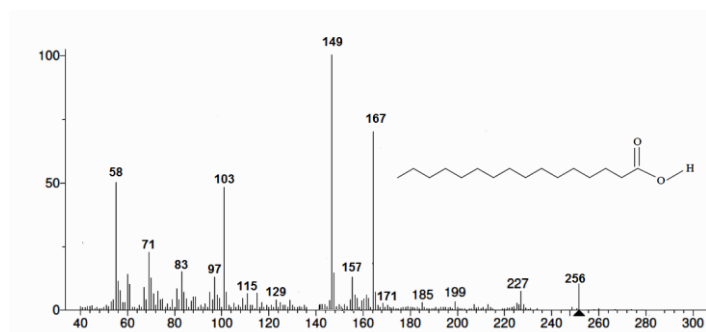
Total number of collected fractions= 45



**Table 4** GC-MS analysis of different compounds in active fractions of *S. platensis* methanolic extract.

Peak	Rt (min)	Area (%)	Name
1	5.929	2.798	Butane, 1-ethoxy-
2	7.589	1.419	2,2-Dimethoxybutane
3	15.162	0.515	Oxalic acid, isobutyl ester
4	32.624	0.817	5-Isopropyl-6-methyl-hepta-3,5-dien-2-ol
5	33.056	1.438	2-Pentadecanone, 6,10,14-trimethyl-
6	33.905	0.961	Pentadecanoic acid, 14-methyl-, methyl ester
7	34.105	1.948	cis-9-Hexadecenoic acid
<b>8</b>	<b>34.275</b>	<b>30.604</b>	<b>n-Hexadecanoic acid</b>
9	34.590	1.993	Octanoic acid, 4-methyl-, ethyl ester, (n̄)-
10	35.861	1.030	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
11	36.051	1.179	Ethanol, 2-(9-octadecenyl-), (Z)-
12	36.601	1.008	11,13-Dimethyl-12-tetradecen-1-ol acetate
13	38.807	0.684	1,2-Cinnolinedicarboxylic acid, 1,2,3,5,6,7,8,8a-octahydro-4-trimethylsilyloxy-, diethyl ester

RT: retention time

**Figure 4** Mass spectrum of the antibacterial substance obtained from *S. platensis* (n-Hexadecanoic acid)

## DISCUSSION

Al-Bahr El-Pheroany is an important watershed and a crucial source of irrigation water. It is considered one of the important sources of fisheries in Menoufia Government, Egypt. Plants and fishes living in these water bodies, when poisoned with harmful chemicals and metals can't survive (Ghannam et al., 2014). The crops and vegetables irrigated with such polluted water become harmful for human beings. The level of pollution is swelling day by day due to non-availability of proper drainage system for industrial units and housing societies established along the banks. The ever-growing level of pollution in water is posing serious threats to human health besides making the water harmful for irrigation and fisheries purposes. Microbiological assessment of the water stream revealed the presence of rich communities of both indicator and human pathogenic bacteria (Sabae et al., 2014).

Microorganisms have developed adaptation mechanisms against the action of antimicrobial drugs (Al-Haj et al., 2009). This problem is one of the main reasons for continued research into antimicrobial compounds, including molecules from cyanobacteria and marine algae (Kim et al., 2007; Al-Wathnani et al., 2012). Much attention is being paid towards plant extracts and biologically active compounds isolated from natural resources in the present. Aquatic organisms are a rich source of structurally novel and biologically active metabolites (Ely et al., 2004). Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry (Prakash et al., 2011). Most of the secondary metabolites produced by seaweeds have bactericidal or the antimicrobial compounds derived from seaweeds consist of diverse groups of bacteriostatic properties terpenols, sterols, polysaccharides, dibutenolides peptides and proteins metabolites. Compounds with antibacterial activity have been detected in green, brown and red algae (Yuan et al., 2005; Bansemir et al., 2006; Chew et al., 2008). Lipid soluble extracts from marine macroalgae have been investigated for their antibacterial properties.

In the present investigation, a high antibacterial activity of the studied cyanobacterium, namely; *Spirulina platensis* and algal species namely *Saragassum wightii*, *Saragassum latifolium* and *Chlorella vulgaris* have been reported against both Gram positive and Gram negative bacteria. These results were in accordance with the data obtained by several workers (Sastry and Rao, 1995; Priya, 2012; Kumar et al., 2011; Al-Wathnani et al., 2012).

Several different organic solvents have been used to screen algae for antibacterial activity (Jeyanthi Rebecca et al., 2012). Similar to the results found in the

current study, methanol seemed to be the best solvent for extracting the bioactive compounds.

In this study, the cyanobacterium *S. platensis* had the most effective antibacterial activity against both Gram positive and Gram negative bacteria compared with other screened algae and these results are in agreement with the findings by Abdo et al. (2012) and Kaushik and Chauhan (2008). The collected fractions of the methanol extract of *S. platensis* using silica gel chromatography showed a high inhibitory activity against *S. aureus* and *P. aeruginosa*. Physical and chemical characterizations of the most active fractions were applied. From UV analysis, maximum absorption spectrum at 285 nm was observed. Accordingly, the composition of the active antimicrobial material was suggested to contain an aliphatic chain. The FT-IR spectroscopy indicated the presence of many functional groups: the first band appeared at 3424 cm<sup>-1</sup> due to OH group, the second band appeared at 2958 cm<sup>-1</sup> due to the C-H aliphatic and the third band appeared at 1729 cm<sup>-1</sup> due to the carbonyl group (C=O). The <sup>1</sup>H NMR spectrum signals (ppm) at: δ0.9 (t, 3H, CH<sub>3</sub>), δ1.3 (t, 2H, CH<sub>2</sub>), δ 2.3 (s, 2H, CH<sub>2</sub>) and δ7.2 (COOH group).

According to the results of UV, IR, <sup>1</sup>H-NMR and mass spectral data, it was concluded that the compound was n-hexadecanoic acid. The results obtained herein are supported by Colla et al. (2007) who reported major fatty acids extracted from the *S. platensis* as palmitic acid (C16:1), stearic acid (C18:1), oleic acid, linoleic acid etc. Al-Wathnani et al. (2012) observed that GC-MS analysis of the volatile components of *S. platensis* resulted in the identification of 15 compounds which constituted 96.45% of the total compounds. The volatile components of *S. platensis* consisted of heptadecane (39.70%) and tetradecane (34.61%) as major components.

Antimicrobially active lipids and active fatty acids are present in a high concentration in *Skeletonema costatum* (Lampe et al., 1998). It was hypothesized that lipids kill microorganisms by leading to disruption of the cellular membrane e.g. bacteria, fungi and yeasts (Bergsson, 2005). They can also penetrate the extensive meshwork of peptidoglycan in the bacterial cell wall without visible changes and reach the bacterial membrane leading to its disintegration. This can probably be explained by the strong fabric of the cell wall of Gram-positive bacteria, which maintain their structure in spite of substantial hydrostatic turgor pressure within the bacteria (Bergsson et al., 2002; Shanmugapriya and Ramanathan, 2011).

## CONCLUSIONS

From the presented results it can be concluded that the extract of cyanobacterium, *S. platensis*, contains potential bioactive compound with an effective antibacterial activity. This compound can be utilized for the development of natural antibiotics against multi drug resistant bacteria.

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