Antimicrobial activity of *Spirulina platensis* and *Aphanothece* sp. on selected clinical bacterial isolates and its Antioxidant activity

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Abstract

The screening of antioxidant and antimicrobial potentiality of *Spirulina platensis* and *Aphanothece* sp against multidrug resistant pathogens were investigated using standard microbiological techniques and antioxidant is determined by FRAP and DPPH. The intracellular and extra cellular extracts were tested by agar well diffusion method for activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* sp, *Proteus* sp and *Embedobacter* sp. isolated from clinical samples. The susceptibility patterns of the test isolates against the extract intracellular and extra cellular was determined. The result showed that no significant antibacterial activity by *Aphanothece* sp, in contrast intracellular extracts of *S.platensis* produce antibacterial activity against above mentioned strains and *S.platensis* have more antioxidant activity than *Aphanothece* sp. The observed antibacterial effects were believed to be due to the presence of alkaloids, tannins, and flavonoids identified in the extracts. The results apparently justified their use in the treatment of infections.

Key words: *Spirulina platensis*, *Aphanothece* sp, alkaloids, tannins, and flavonoids.
Introduction

Several studies have focused on physiological properties of some valuable antiviral or antioxidant compounds in blue green alga *Spirulina*\textsuperscript{12}. The occurrence of many compounds possess antioxidant activity in biological systems in higher plants is well documented, while in microalgae are less documented\textsuperscript{3}. The presence of certain substances act as antioxidant or free radical scavenger may protect the body from the consequences of oxidative stress. Thus, antioxidants play an important role in the protection of cells against oxidative damage caused by ROS\textsuperscript{4,5}. Among algal species, *Spirulina* has been reported to prevent oxidative damage by scavenging free radicals and active oxygen, and hence can indirectly reduce cancer formation in human body. In this respect, the increased consumption of foods characterized by free radical scavenging activity, leads up to a doubling of protection against many common types of cancer formulation\textsuperscript{6}. *Spirulina platensis* or its extracts show therapeutic properties, such as the ability to prevent the incidence of cancers, decrease blood cholesterol levels, stimulate the immunological system, reduce the nephrotoxicity of pharmaceuticals and toxic metals and provide protection against the harmful effect of radiation\textsuperscript{7,8}. Although extremely effective, antibiotics are able to induce resistance in bacteria. For 450 years, bacterial resistance has been the main factor responsible for the increase of morbidity, mortality and health care costs of bacterial infections. The defense mechanism against antibiotics is widely present in bacteria (e.g. *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Acinetobacter*, *Salmonella*, *Staphylococcus*, *Enterococcus* and *Streptococcus*) and became a world health problem\textsuperscript{9}. Some *Spirulina* species exhibit antibacterial activity\textsuperscript{10}. *Spirulina* as many other cyanobacteria species have the potential to produce a large number of antimicrobial substances, so they are considered as suitable organisms for exploitation as biocontrol agents of plant pathogenic bacteria and fungi\textsuperscript{11}. Other studies have found a significant coupling of cyanobacterial primary production and bacterial secondary production, particularly during the development and cessation of blooms including *Aphanothece* sp\textsuperscript{12,13}. According to the authors, the cyanobacterial exudates represented a striking proportion of bacterial organic carbon supply. Antimicrobial compounds found in cyanobacterial exudates include polyphenols, fatty acids, glycolipids, terpenoids, alkaloids, and a variety of yet to be described bacteriocins\textsuperscript{14}. The present work was conducted to study the antioxidant and antimicrobial activities as well as the phytonutrients of *Spirulina platensis* and *Aphanothece* sp.
**Materials and methods**

**Collection of cyanobacterial samples**

Cyanobacteria found as mat in the water, flakes on the dried sediment and on pneumatophores were collected and transported to the laboratory in sterile polythene bags as well as in plastic vials containing sterile ASN III medium. The samples were transferred to Erlenmeyer flasks containing the ASN III medium for further identification.

**Cyanobacterial identification**

The collected samples were observed by microscopic and macroscopic view and identification of cyanobacteria was made by using the standard taxonomic manuals of Geitler and Desikachary.

**Spirulina platensis**

The blue green algae, *Spirulina platensis* was obtained from the OFERR Nallayan Research Centre, Chennai and the cultures are grown in ASN III medium had kept in room temperature near north facing window in natural light.

**Growth conditions**

Large-scale cultivation on ASN III medium containing different concentration of Nacl, Mgcl₂, Kcl, NaNO₃, K₂HPO₄.3H₂O, MgSO₄.7H₂O, CaCl₂.2H₂O, Citric acid, Ferric ammonium citrate, EDTA, Na₂CO₃.H₂O, Trace elements at pH 7.5 were done in five aquariums (16 L, each). The cultures were gassed with air containing 0.3% CO₂ (v/v), and continuously illuminated with two cool white fluorescent lamps (40 W each, Philips). The culture temperature was maintained at 28º ± 2ºC.

**Growth measurements**

Algal growth was spectrophotometrically measured as described by Payer (22). The calculated biomass (the average of three experiments) was used to obtain maximum specific growth rates (m<sub>max</sub>) from the log phase of the growth curves by exponential regression. Productivities was calculated from the equation P=(Xi - X₀) / ti, where P =productivity (mg L⁻¹ day⁻¹), X₀ =initial biomass density (mg L⁻¹), Xi =biomass density at time i (mg L⁻¹) and ti =time interval (h) between X₀ and Xi.
Drying

The harvested algal biomass were collected in aseptic plastic bags and subjected to drying under direct sunlight. After complete drying, the dried algal biomass was placed in hot air oven at 60°C for one hour. Until further use, the algal biomass was stored under refrigerated condition (4°C).

Preparation of algal extracts

Freshly dried *Spirulina platensis* and *Aphanthece sp* was mixed with ethanol (150ml solvent/100g of Algae) in soxhlet apparatus and extracted for 60 minutes. The extracts were filtered and the solvent was removed by air drying. The extracts were stored in an airtight glass bottles in a refrigerator for the analysis of phytonutrients and other findings.

Phytochemical Analysis

**Qualitative analysis of phytonutrients of algal extracts:** Qualitative analysis of phytonutrients was done for ethanolic extract of *Aphanthece sp* and *Spirulina platensis*.

**Test for carbohydrates**

A small quantity of extract was dissolved separately in 5 ml of distilled water and filtered. The filtrate was tested to detect the presence of carbohydrates.

**Molisch’s test:** To 2ml of algal extract, 2 ml of Molisch’s reagent was added. Then, 2 ml of concentrated sulfuric acid was added along the sides of the test tubes. Disappearance in color on the addition of excess solution indicated the presence of carbohydrates.

**Benedict’s test:** To 0.5 ml of extract, 5 ml of Benedict’s reagent was added. The mixture is then boiled for 5 minutes. Presence of a bluish green precipitate indicated the presence of carbohydrates.

**Test for Glycosides:** To 2ml of algal extract 1ml of aqueous NaOH solution was added. The appearance of a yellow color indicated the presence of glycosides.
Test for Proteins and Amino acids

Ninhydrin test: A small quantity extract solution was boiled with 0.2% solution of ninhydrin. Purple color indicated the presence of free amino acids.

Test for Phytosterols and Triterpenoids

Salkowski test: To 2 ml of the algal extract, 1 ml of concentrated sulfuric acid added. Chloroform was added along the sides of the test tube. A red color produced in the chloroform layer indicated the presence of Phytosterols or if it is yellow in color at the lower layer indicated the presence of triterpenoids.

Test for Flavanoids

Zinc hydrochloride reduction test: The extract was treated with mixture of zinc dust and concentrated hydrochloric acid. Red color indicated the presence of flavanoids.

Test for Alkaloids: A small portion of the solvent free extract was stirred separately with a few drops of dilute hydrochloric acid and filtered. The filtrate was tested with Mayer’s reagent (Potassium mercuric iodide solution). The cream precipitate indicates the presence of alkaloids.

Test for Tannins

Gelatin test: To 5 ml of algal extract, few drops of 1% lead acetate were added. Absence of a yellow or red precipitate indicated the absence of tannins.

Test for Saponins: To 5 ml of the algal extract, a drop of sodium bicarbonate was added. It was then shaken vigorously and kept undisturbed for 3 minutes. Appearance of a honey comb like froth indicated the presence of saponins.

Estimation of In-vitro antioxidant activity

In these experiment, two complementary methods of free scavenging activity; DPPH radical scavenging test and FRAP were used.

DPPH assay

1 mg extract powder was dissolved in 1 ml of 50% ethanol solution to obtain 1000 µg/ml sample solution. 1000 µg/ml solutions were series diluted into 1 µg/ml, 5 µg/ml, 10 µg/ml, 20
µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 500 µg/ml, and 1000 µg/ml with 50% ethanol. In each reaction, the solutions were mixed with 1 ml of 0.1 mM 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 0.45 ml of 50 mM Tris-HCl buffer (pH 7.4), and 0.05 ml samples at room temperature for 30 min. 50% ethanol solution was used as control. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm. DPPH, a purple-colored, stable free radical is reduced to the yellow-colored diphenyl picrylhydrazine when antioxidants are added. L-ascorbic acid and (+)-catechin were used as positive controls. The inhibition ratio (percent) was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of test sample)/absorbance of control] x 100%. The antioxidant activity of each sample was expressed in terms of IC50 (micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the inhibition curve.

**FRAP assay**

The procedure described by Benzie and Strain was followed\textsuperscript{17}. The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants. The FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored FeII-tripyridyltriazine compound from the colorless oxidized FeIII form by the action of electron donating antioxidants. The FRAP reagent consist of 300 mM acetate buffer (3.1 g sodium acetate + 16 mL glacial acetic acid, made up to 1 L with distilled water; pH =3.6), 10 mM TPTZ in 40 mM HCl and 20 mM FeCl₃·6H₂O in the ratio of 10:1:1. Briefly 50 µL of sample supernatant was added to 1.5 mL freshly prepared and pre warmed (37°C) FRAP reagent in a test tube and incubated at 37°C for 10 min. The absorbance of the blue colored complex was read against reagent blank (1.5 mL FRAP reagent+50 µL distilled water) at 593 nm. For construction of the calibration curve, five concentrations of FeSO₄·7H₂O (1000, 750, 500, 250 and 125 µmol L⁻¹) were used and the absorbencies were measured as sample solution. The data was expressed as mole ferric ions reduced to ferrous form per liter (FRAP value).

**Antimicrobial activity of crude algal extract**

**Test organisms**

18 hours incubated peptone broth of clinical isolated cultures (equilibrated to Mcfarland standard turbidity scale 10⁵ -10⁶cfu/ml for bacteria) of *Staphylococcus aureus*, *Escherichia coli*,
Pseudomonas aeruginosa, Klebsiella sp, Proteus sp and Embedobacter sp, were used in the antibacterial studies. The test strains are collected from KMCH of Kovai Medical Centre.

**Preparation of the crude algal extract**

The algal culture was filtered and the filtrate was used as the extracellular extract. The biomass obtained was grinded and used as the intracellular extract.

**Agar well diffusion technique**

Sterile Mueller Hinton agar plates were prepared and swabbed with the clinical isolated cultures. Using a sterile cork borer, wells were cut in the Mueller Hinton agar plates. Intracellular extract, extracellular extract were added into their respective wells. The appropriate standard antibiotic discs for each organism were placed on the plates. The plates were subjected to incubation at 37°C for 24 hours. After incubation, the zones of inhibition were examined.

**Result and Discussion**

Spirulina is a microscopic blue-green alga in the shape of a spiral coil, living both in sea and fresh water. Spirulina is the common name for human and animal food supplements produced primarily from two species of cyanobacteria: Arthrospira platensis, and Arthrospira maxima. Antimicrobially active lipids and active fatty acids are present in a high concentration in this alga. It was hypothesised that lipids kill microorganisms by leading to disruption of the cellular membrane as well as bacteria, fungi and yeasts because they can penetrate the extensive meshwork of peptidoglycan in the 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. Among the collected cyanobacterial culture sample from muthupet mangrove, Aphanothece sp plays an predominant role. It is under the 45X of the microscope, Aphanothece were visualized as green to olive green colored ovoid cells. The cells were embeded in mucilage and there was no sheath around the cell and the cells of the Aphanothece plated on the ASN III medium containing 0.5% agar and incubated at room temperature showed asymmetric mucilaginous spreading growth. The color of the algae in nature as well as in the culture was
dirty brown due to Phycoerythrin pigment. So the Aphanothece sp chosen for examination of various parameters with S.platensis. The results obtained from the present study concerning the antimicrobial activity of Spirulina platensis and Aphanothece sp crude extract against different species of bacteria are recorded in table 1.

Table: 1 Antibacterial activity of Aphanothece and Spirulina

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Effect of algal crude extracts (Zone of inhibition diameter in mm)</th>
<th>Antibiotics (Inhibition zone diameter in mm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Spirulina platensis Crude extract</td>
<td>Aphanothece sp Crude extract</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>11 R</td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>7 R</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>9 R</td>
<td></td>
</tr>
<tr>
<td>Proteus sp</td>
<td>6 R</td>
<td></td>
</tr>
<tr>
<td>Empedobacter sp</td>
<td>10 R</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>8 R</td>
<td></td>
</tr>
</tbody>
</table>

It is clear from study that the diameter of the inhibition zone depends mainly on the crude extracts used and the tested bacteria. The phytochemical characters of the two algae (S.platensis and Aphanothece sp) were investigated are summerized in Table 2. Carbohydrates, alkaloids, flavonoids, glycosides, proteins and saponins were present in S.platensis and carbohydrates, terpenes, flavonoids, tannins were present in Aphanothece sp. The crude extract of Spirulina platensis showed maximum antimicrobial activity of 11.0 mm against Escherichia coli and a adequate activity of 6.0 mm against Proteus sp, 7mm against Klebsiella pneumonia, 9mm against Pseudomonas aeruginosa, 10 mm against Empedobacter sp and 8mm against Staphylococcus aureus. All the tested microorganisms were resistant to crude extracts of Aphanothece sp and the crude extracts of S.platensis was better antimicrobial than Aphanothece sp extracts.
Table 2. Preliminary phytochemical analysis of ethanolic extract of the cyanobacterium *Spirulina* and *Aphanothece*

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Test</th>
<th>Spirulina</th>
<th>Aphanothece</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>Molisch’s test</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Benedicts test</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Killer-killanis test</td>
<td>++</td>
<td>--</td>
</tr>
<tr>
<td>Amino acids/Protein</td>
<td>Ninhydrin test</td>
<td>++</td>
<td>--</td>
</tr>
<tr>
<td>Terpenes and steroids</td>
<td>Salkowski test</td>
<td>--</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Zinc hydrochloride reduction test</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Meyer’s test</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>Phenolics/Tannins</td>
<td>Gelatin Test</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>Frothing test</td>
<td>++</td>
<td>--</td>
</tr>
</tbody>
</table>

Key: - = Negative (absent); + = Positive (slightly present); ++ = Positive (moderately present)

Antioxidant activity says chemical assay based on the removal of stable DPPH free radical has been shown to indicate the presence of reducing compounds in terms of hydrogen donation capacity in the test system. Purple color of the freshly prepared DPPH solution fades when an antioxidant compound is present in the test solution, to quench DPPH; the antioxidant molecule provides a hydrogen atom or donates an electron, to DPPH. The table 2 shows the results of the DPPH free radical scavenging activity, the absorbance of DPPH solution changed when the algal extracts were added to the solution. In the present study the antioxidant activity of the *S. platensis* sample at concentration of 15mg/ml exhibited about 51.94% DPPH scavenging activity with IC$_{50}$ value is 14.43 and *Aphanothece* sp sample at a concentration of 20mg exhibited 16.31% DPPH scavenging activity with IC$_{50}$ Value is 61.31. The FRAP assay showing the result for *S. platensis* is 1400 (micromoles/mg) and *Aphanothece* sp is 720 (micromoles/mg).

It is intended that the present work will contribute to an understanding and determining the phytonutrients, antimicrobial and antioxidant factors of *S. platensis* and *Aphanothece* sp. Because antimicrobial metabolites of algae are of special interest in the development of new harmless environment.
Table 3: The DPPH and FRAP assay

<table>
<thead>
<tr>
<th>Algae species</th>
<th>Sample concentration</th>
<th>% of DPPH Scavenged</th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spirulina</td>
<td>0.3</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>3.75</td>
<td>1400 (micromoles/mg)</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>10.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>21.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>51.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Aphanothece</td>
<td>2.0</td>
<td>0.18</td>
<td>720 (micromoles/mg)</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>3.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>8.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>16.31</td>
<td></td>
</tr>
</tbody>
</table>

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