ABSTRACT

The present investigation focused on screening of antibacterial and antioxidant potential of phytochemicals extracted from a corticolous cyanobacterium *Hassalia byssoida* isolated from Similipal Biosphere Reserve situated in the Mayurbhanj district of Odisha. Different phytochemicals were extracted with petroleum ether, chloroform, acetone, methanol and aqueous. Antimicrobial activity performed against pathogenic bacteria viz. *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Staphylococcus aureus*, *S. epidermidis*, *Bacillus licheniformis* and *B. brevis* following agar cup diffusion method. Antioxidant activity of the solvent extracts was investigated in terms of total phenolic contents, total flavonoid contents, total antioxidant activity and DPPH (diphenyl-1-picrylhydrazyl) radical scavenging activity. The results revealed the presence of flavonoids, terpenoids, sterols, glycosides, alkaloids, saponins and phenols in the extracts. All the solvent extracts have the potential to inhibit at least two of the pathogens except aqueous which fails to inhibit the test pathogens. The extract of petroleum ether showed maximum activity...
against *P. aeruginosa* followed by methanol against *S. dysenteriae*, chloroform against both the *Bacillus* sp. and acetone against *B. licheniformis*. *E. coli* and *S. typhimurium* were found resistant to all solvent extracts. Total phenolic and flavonoid contents of acetone extract were found higher than other extracts. However, the DPPH radical scavenging activity was found maximum in methanol extract and the highest antioxidant activity was observed in aqueous extract.

**KEY WORDS:** Antibacterial, Antioxidant, Corticolous Cyanobacterium, Phytochemicals, *Hassalia byssoida*.

**INTRODUCTION**

The search for the natural alternatives to replace the use of synthetic compounds resulted in the exploration of potential organisms for their bioprospecting. In recent years, Cyanobacteria, a unique group of photosynthetic bacteria, have been recognized as the most promising microalgae which produce variety of bioactive compounds with prospective pharmacological and therapeutic value. These are excellent source of natural metabolites with antibacterial, antifungal, antiviral, antioxidant and cytotoxic activities.\(^1\),\(^2\),\(^3\) With increasing resistance of pathogenic microbes to commercial antibiotics and its derivatives, the cyanobacteria hold great promise for novel medicines in modern times as they are found to be rich source of structurally novel and biologically active metabolites.\(^4\),\(^5\) The primary and secondary metabolites produced by these microorganisms may even prove to be potential bioactive compounds of interest in the pharmaceutical industry.\(^6\),\(^7\) Moreover, the biologically and pharmacologically active substances produced by them are difficult to be synthesized chemically.\(^8\),\(^9\) They represent almost an untapped resource of natural antimicrobials and antioxidants, due to their enormous biodiversity, much more diverse than higher plants.

Cyanobacteria have a well developed antioxidant system to catalyze the free oxygen radicals producing during photosynthesis. Synthesis and accumulation of minerals, polysaccharides, amino acid derivatives, carotenoids, flavonoids and phenolic compounds can display antioxidant properties at very low concentrations\(^10\),\(^11\) and play an important role in inhibiting and scavenging radicals, thus providing protection to human against infections and degenerative diseases. Miranda *et al.*, 1998\(^12\) extracted carotenoids, phenolics and tocopherols from *Spirulina maxima* found that phenolic compounds are responsible for the antioxidant activity. Antioxidant activity of *Spirulina* sp. also studied from the crude extracts.\(^13\) Similarly flavonoids which are a group of polyphenolic compound and
phycocyanins, a water soluble pigment is known to exhibit antioxidant, anti-inflammatory, hepato protective effects.\textsuperscript{[14,15]} For this, pharmaceutical industries are giving importance to the compound derived from traditional sources such as plants and microorganisms, like cyanobacteria\textsuperscript{[16]} and use of phytochemicals as natural antimicrobial and antioxidants, is gaining popularity. Keeping this in view, the present work aims to evaluate the antibacterial and antioxidant activity of a corticolous Cyanobacterium \textit{Hassalia byssoidea}.

**MATERIALS AND METHODS**

**Sample collection**
The sampling site Similipal Biosphere Reserve (21°28' - 22°08' North latitude and 86°04' - 86°37' East longitude) is located in the central part of the Mayurbhanj district of Odisha, India. The desiccated cyanobacterial samples were collected from the tree barks in clean, air tight sample bottles (Tarson-25x50 mm) with the help of forceps and scalpels. The samples were brought to the laboratory for culture, identification and germplasm conservation.

**Culture, identification and maintenance**
The desiccated algal samples were soaked in distilled water for 4-5 days and observed under microscope intermittently to observe the morphometrics of the particular species. The sprouted filaments were streaked on agar plates containing BG-11 (-N) medium and incubated under white fluorescence light to obtain unialgal culture. The cultured cyanobacterium was identified following monograph\textsuperscript{[17]} and www.algaebase.org for current accepted name. The unialgal culture was maintained in agar slant in a screw cap culture tube and was deposited at Department of Botany, North Orissa University, Odisha assigned with a voucher number. For getting sufficient biomass, pure cultures were grown in 1000 ml capacity conical flasks containing 500 ml medium in a temperature-controlled culture room at 25±2°C under continuous light intensity of 7.5 W/m² from white fluorescent tube.

**Preparation of crude extracts**
Fresh algal biomass was harvested through filtration from the late exponential phase to stationary phase culture and shade dried. The dried biomass was blended in to coarse powder. For the active metabolites, 1gm of powdered cyanobacterial biomass was extracted fractionally using petroleum ether, chloroform, acetone, methanol, aqueous for 24-48 hours each successively. The crude extracts of known amount were stored for further use.
Phytochemical analysis of crude extracts

The qualitative phytochemical screening of cyanobacterial extracts was carried out following standard methods such as: test for Phenols (Ferric chloride test), Tannins (Braymer’s test), Flavonoids (Alkaline reagent test), Saponins (Foam test), Terpenoids & Sterols (Salkowski’s test), Glycosides (Legal’s test) and Alkaloides by Wagner’s Test\[18\] and through Spectrophotometric analysis using a UV-VIS Spectrophotometer (Shimadzu, model UV-2450) capable of producing monochromatic light in the range between 200- 800 nm for measuring the absorbance.

Antimicrobial activities

Antimicrobial activity of petroleum ether, chloroform, acetone, methanol and aqueous extracts of *Hassalia byssoidea* was screened by agar cup diffusion method. 50 µl crude extract dissolved in Dimethyl sulfoxide (DMSO) was inoculated in agar cup plate against test pathogen with standard inoculum size $10^5$ CFU/ml cells. 50 µl of (DMSO) was taken as negative control and antibiotic Vancomycin (10µg) as positive control. Test organisms i.e. 4 Gram negative bacteria viz. *Pseudomonas aeruginosa* (S1), *Escherichia coli* (S2), *Salmonella typhimurium* (S5), *Shigella dysenteriae* (S21) and 4 Gram positive bacteria such as *Staphylococcus aureus* (S3), *Staphylococcus epidermidis* (S4), *Bacillus licheniformis* (S6) and *Bacillus brevis* (S22) collected from Microbial Type Culture Collection (MTCC), Chandigarh were selected for screening of antibacterial activity of the crude extracts.

Antioxidant activities

Antioxidant activity of solvent extracts of the test organism was determined in terms of total phenolic content by Folin-Ciocalteau reagent method\[19\], total flavonoid content by Aluminum chloride method\[20\], free radical scavenging assay by DPPH method\[21\] and total antioxidant activity.\[22\]

RESULTS

Enumeration and identification of the test cyanobacterium

*Hassalia byssoidea* Hass. Ex Born. et Flah. (Fig 1)

Synonym: *Tolypothrix byssoidea* (Berk.) Kirchner, Desikachary, 1959 (P. 502, Pl. 103, Figs. 3, 4 & 7). Holotype - Voucher No. –30

Thallus pale brown to bluish green in color; filamentous, filament pseudo branched; heterocystous with intermediate necridia, heterocyst intercalary as well as basal to false
branch, oval, elliptical or rectangular, 6.6 - 8μm long, 10 - 12μm wide; cells barrel shaped to elliptical or rectangular, granulated, vacuolated, 12.0-16.5μm broad, 10.0-13.2μm long.

Fig 1. Microscopic photograph of the test cyanobacterium *Hassalia byssoidea*. Scale bar = 10 μm.

**Phytochemical analysis:** The results on phytochemical screening of *Hassalia byssoidea* using different solvents e.g. petroleum ether, chloroform, acetone, methanol and aqueous showed the detection of phenols, flavonoids, saponins, terpenoids, sterols, glycosides, alkaloids (Table 1; Figs 2). Saponin is the most predominant phytochemical found in all solvent extracts, Phenols in all extracts except petroleum ether whereas, Flavonoids more in acetone but not detected in petroleum ether and aqueous extract. Terpenoids and Sterols were absent in almost all the extracts except acetone. Glycosides were found positive in petroleum ether, acetone and aqueous extract. Alkaloids were found to be present in all the solvent except aqueous. Tannins were nowhere detected in all solvent extracts. The UV-VIS spectra of crude extracts within 200 nm to 800 nm also determine the presence of active metabolites. Peaks were observed mostly in UV regions. Peaks were also detected in visible region in the extracts of chloroform (406nm & 671nm), acetone (675nm) and methanol (403nm & 671nm).

**Table 1. Phytochemical screening of Hassalia byssoidea using different solvents.**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Phenols</th>
<th>Tannins</th>
<th>Flavonoids</th>
<th>Saponins</th>
<th>Terpenoids</th>
<th>Sterols</th>
<th>Glycosides</th>
<th>Alkaloids</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. Ether</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chloroform</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Acetone</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Methanol</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Aqueous</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

High concentration (++), Low concentration (+), and absent (-).

**Antibacterial activity:** All the pathogens were inhibited at least by one of the solvent extracts of the test organism except *E. coli* and *S. typhimurium* (Figs 3; Table-2). The two were resistant to all the solvent extracts and did not show any zone of inhibition. The maximum zone of inhibition was shown by petroleum ether against *P. aeruginosa* followed by methanol extract against *S. dysenteriae*. The petroleum ether extract showed inhibitory activity against *B. licheniformis*, *S. dysenteriae* and *B. brevis*. The methanol extract also showed activity against *B. brevis*. The chloroform extracts inhibited significantly against *S. epidermidis* and *B. brevis*. The acetone extract also found active against *P. aeruginosa*, *S. aureus*, *S. epidermidis* and *B. licheniformis*. The aqueous extract of the test sample failed to inhibit any of the pathogens.


<table>
<thead>
<tr>
<th></th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S21</th>
<th>S22</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. Ether</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Chloroform</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>Acetone</td>
<td>9</td>
<td>-</td>
<td>9</td>
<td>9</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>9</td>
<td>-</td>
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<tr>
<td>Aqueous</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>
Fig. 3: Nutrient agar plate showing antibacterial activities of crude extract of *Tolypothrix byssoidea* against a- *Pseudomonas aeruginosa* (S1), b- *Escherichia coli* (S2), c- *Staphylococcus aureus* (S3), d- *Staphylococcus epidermidis* (S4), e- *Salmonella typhemurium* (S5), f- *Bacillus licheniformis* (S6), g- *Shigella dysentriae* (S21) & h- *Bacillus brevis* (S22). PE= petroleum ether, Chl = Chloroform, Ac = Acetone, Met = Methanol, Aq = Aqueous, DM = DMSO.

**Antioxidant activity:** The antioxidant efficacies of different solvent extracts were determined in terms of Phenolic, flavonoid content, DPPH (%) scavenging activity and total antioxidant activity (Table-3). The results showed that the acetone extract had a maximum phenolic and flavonoid contents followed by methanol extract (Fig 4). Chloroform extract possessed low phenolics but high flavonoids as reverse to the aqueous extract. As compare to others Petroleum ether showed a low concentration of both phenolic and flavonoid content. Free radical scavenging % of methanol extract was found maximum followed by aqueous and petroleum ether and minimum in acetone extract. The total antioxidant activity was found maximum in methanol extract followed by acetone, aqueous and chloroform. Petroleum ether had a low total antioxidant potential.

<table>
<thead>
<tr>
<th></th>
<th>Total phenols mg/1gm dry biomass</th>
<th>Total flavonoids mg/1gm dry biomass</th>
<th>DPPH Scavenging %</th>
<th>Total Antioxidant mg/1gm dry biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. Ether</td>
<td>0.796± 0.002</td>
<td>0.413± 0.004</td>
<td>60.404± 0.058</td>
<td>0.975± 0.009</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.523± 0.033</td>
<td>7.313± 0.042</td>
<td>31.266± 0.043</td>
<td>11.61± 0.06</td>
</tr>
<tr>
<td>Acetone</td>
<td>4.108± 0.038</td>
<td>9.488± 0.038</td>
<td>6.593± 0.187</td>
<td>18.69± 0.179</td>
</tr>
<tr>
<td>Methanol</td>
<td>2.977± 0.015</td>
<td>6.956± 0.02</td>
<td>73.555± 0.038</td>
<td>27.105± 0.079</td>
</tr>
<tr>
<td>Aqueous</td>
<td>2.931± 0.011</td>
<td>0.694± 0.004</td>
<td>66.785± 0.185</td>
<td>15.03± 0.072</td>
</tr>
</tbody>
</table>

DISCUSSION

Cyanobacteria can withstand extreme condition by modifying their morphology, physiology and molecular mechanism. It is reported that *Hassalia scytonemoides* subjected to desiccation exhibit increased antioxidant enzyme activities when compared to fresh cells.[23] The present study reveals that *Hassalia byssoida*, a naturally desiccated corticolous cyanobacteria, is a potent organism with phyto-constituents having antibacterial and antioxidant activity. It is also evident from this work that the most efficient solvent for phytochemical extraction is acetone followed by methanol, chloroform, petroleum ether and aqueous. Earlier reports established that the range of potential activity of solvent extracts varies from cyanobacterial species to species and test pathogens.[24, 25] Moreover, certain studies reveaed the presence of sterols and terpenoids in acetone extract where the amounts of terpenoids are rare in cyanobacteria.[26] However, the major phytochemicals found in our work were saponins, phenols, flavonoids, alkaloids and glycosides supported by the result of UV-VIS spectroscopic analysis that confirmed the presence of phenolics, flavonoids and antioxidant pigments like carotenoids. The spectra of phenolic compounds and flavonoids typically lie in the wavelength range of 225-295 nm with minor bands for flavonols and flavonons within the range of 250-390 nm and carotenoids within 400-450 nm.[27] In this study, the crude extracts of *H. byssoida* were found effective against six pathogenic bacteria out of eight. The antimicrobial property of the extracts may be due to presence of phenolic
compounds/terpenes/alkaloids or other constituents. Similar studies also revealed antimicrobial effects of different organic solvent extracts from many cyanobacteria using selected pathogens as test organisms.[28, 29, 30] Both polar and non-polar solvents showed their potential against all these pathogens. The gram negative bacteria E. coli and S. typhimurium are found to become resistant, may be due to impermeability of their outer membrane of the cell wall to many hydrophobic antibacterial compounds and supports the earlier works of Sheu and Freese, 1973.[31]

It is studied that the potent antioxidant activity of the extract of different cyanobacteria is due to total phenolics, phycocyanin, triterpenoids present in the crude extracts.[32] Present findings on antioxidant activity revealed that the acetone and methanol were found efficient to extract phenolics and flavonoids in comparison to other solvents. This result is most significantly correlated with the results carried out earlier.[13, 33] Methanolic extracts also exhibit maximum total antioxidant activity. It is clear that DPPH radical scavenging activity is due to the hydrogen donating ability of the antioxidant compounds.[34, 35] The petroleum ether extracts possess more scavenging activity with less phenolics and flavonoid compounds. In spite of having high phenolics and flavonoids in acetone extracts, probably the compounds present were unable to donate hydrogen for which minimum scavenging activity is seen. However, the mechanism of action is not clearly understood.

CONCLUSION

The present study proved that the cyanobacterium Hassalia byssoidea is a potential source of bioactive metabolites that possesses both antibacterial and antioxidant activity. Extraction with different solvents could affect the yield of different phytoconstituents, their antioxidant and antibacterial potentials. The most efficient solvent for phenolic extraction is acetone when compared to others. The existing data is not sufficient to explain the mechanism of action; however, it may enrich the strength of the comprehensive data in support of antibacterial and antioxidant activity. Further work is needed to isolate and identify the compounds present in the cyanobacterial extract which might be useful for therapeutic purposes.

ACKNOWLEDGMENT

The authors are thankful to the Head, Dept. of Botany, North Orissa University for providing necessary facilities to carry out the research work.
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