Total phenolics, flavonoids and antioxidant potential of organic extract of fresh water algal sample collected from a marine lake

Vijay Rahul¹, Pooja Agrawal², Manik Sharma¹, Shruti Shukla³,*

¹Bhoj College, Barkatullah University, Bhopal, MP, INDIA
²Madhya Pradesh Council of Science & Technology, Bhopal, MP, INDIA
³Department of Food Science and Technology, Gyeongsang University, Republic of Korea

*E-mail: shruti.shukla15@yahoo.com

Received 13 April 2015; revised 28 May 2015

In the present study, we carried out a research on fresh water algal sample of upper-lake of Bhopal, Madhya Pradesh, India. Several different species of fresh water mixture algal samples were collected such as cyrophyceae (46.17%), chlorophyceae (39.27%) and bavillaior phyceae (26.65%). Further, the collected algal material was extracted with 70% methanol and analyzed for its total phenolic content and phytochemical properties. Furthermore, the methanolic extract was evaluated for its in vitro antioxidant activity by using different assays such as DPPH radical scavenging activity, hydrogen peroxide scavenging activity and reducing power assay. Measurement of total phenolic content of the methanolic extract of mixed algal sample was achieved by using Folin-Ciocalteau reagent containing 134 ± 0.91 mg GAE g⁻¹ of phenolic content, which was found significantly potent when compared to reference standard gallic acid. The flavonoid content of methanolic extract of mixed algal sample was found to be 20.11 ± 2.13 mg QE g⁻¹. The DPPH activity of methanolic extract of mixed algal sample (20-100 µg mL⁻¹) was increased in a dose-dependent manner, which was found in the range of 45.68–67.68% as compared to ascorbic acid 64.26–82.58%. The results obtained in this study clearly indicate that fresh water algal sample has a significant potential to be used as a supplement of antioxidant agent.

[Keywords: Total phenolic content; Total flavonoid content; Phytochemicals; Antioxidant activity]

Introduction
Algae are a large and diverse group of simple, typically autotrophic organisms, ranging from unicellular to multicellular forms. Cyanobacteria, also known as blue-green algae, blue-green bacteria or cyanophyta, are common members of the plankton of marine, brackish and freshwaters throughout the world¹. Marine algae are one of the largest producers of biomass in the marine environments. They produce a wide variety of chemically active metabolites in their surroundings, potentially as an aid to protect themselves against the other settling organisms¹. These active metabolites such as halogenated compounds, alcohols, aldehydes and terpenoids are produced by several species of marine macro- and micro-algae and have antibacterial, anti-algal, and antifungal properties which are effective in the prevention of biofouling as well as show several other benefits such as their antibiotic activity². Marine organisms are rich sources of structurally new and biologically active metabolites³. About 30,000 species of algae are found the world over which occur at all places where there is light and moisture and are found in abundance in ocean and fresh water. Algae are also used as a medicine and fertilizer. In recent years, there have been many reports of macro-algae derived compounds that have a broad range of biological activities. Terpenoids (isoterpenoids), a subclass of the prenyllipids (terpenes, prenylquinones and sterols) represent the oldest group of small molecular product synthesized by plants and probably the most widespread group of natural product⁴.

Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidences suggest that antioxidants reduce the risk for chronic diseases including cancer and heart disease⁵. Free radicals are highly reactive molecules or chemical species capable of independent existence. Generation of highly reactive oxygen species (ROS) is an integral feature of normal cellular function like mitochondrial respiratory chain, phagocytosis, arachidonic acid
metabolism, ovulation, and fertilization, and their production however, multiplies several folds during pathological conditions. The release of oxygen free radicals has also been reported during the recovery phases from many pathological noxious stimuli to the cerebral tissues. Antioxidants have been reported to prevent oxidative damage caused by free radical, they can interfere with the oxidation process by reacting with free radicals, and also by acting as oxygen scavengers.

Although significant amount of research on the biological and therapeutic efficacy of a marine alga has been conducted by various worldwide researchers, very little research has been carried out so far on organic extract of mixed algal material sample as a source of potent antioxidant representative with respect to phenolic and flavonoid contents. Therefore, present study was undertaken to evaluate the quantitative profile of phenolic and flavonoid contents present in the methanolic extract of fresh water mixed algal sample along with its antioxidant activities.

Materials and Methods

Preprocessing and cleaning of sample:
Fresh water, unicellular, non-motile green algae water samples were collected from the lower lake of Bhopal city, in Madhya Pradesh state of India. Algal culturing was carried out with 100 mL Bold’s basal medium supplemented with sterile compressed air and kept under fluorescent light with 16 h light period and at 25°C temperature. Alga samples were cleaned and epiphytes and necrotic parts were removed and stored at 4°C for further extraction procedure.

Extraction:
The stored algal samples were centrifuged at 2500 rpm for 10 minutes and supernatant was discarded (to obtain water free algal material) and algal material was dried under shade. A 25 g of dried fresh water algae sample was extracted for 15 min with 50 mL of 70% methanol. Further, the extracts were dried using a rotary evaporator and pooled. Dried methanolic extract sample of fresh water algal material was stored at -20°C for further use.

Qualitative phytochemical analysis:
Phytochemical screening of the methanol extract of collected fresh water algal sample was carried out according to the standard procedures. The methanol extract was subjected to preliminary phytochemical screening to identify the various bioactive constituents i.e., alkaloids, terpinoids, glycosides, steroids, triterpenoids, flavonoids, carbohydrates, saponins and tannins.

Quantitative determination of total phenolic content:
The total phenolic content in the methanolic extract of fresh water algal sample was determined spectrophotometrically by Folin-Ciocalteau colorimetric method. Briefly, 20 µL of diluted methanolic extract sample was mixed with 100 µL of Folin-Ciocalteau reagent and kept for 3 min at room temperature. Further, 80 µL (10% aqueous sodium carbonate solution) was added to the reaction mixture and the reaction solution was allowed to stand for 1 h at room temperature. The absorbance of the resulting blue colored mixture was measured at 765 nm against a blank sample containing only the solvent (200 µL). The amount of total phenolics was calculated as gallic acid equivalents (GAE) from the calibration curve plotted from gallic acid standard solution and was expressed as mg GAE g⁻¹ dry mass.

Quantitative determination of total flavonoid content:
Total flavonoid content of methanol extract of fresh water algal sample was determined by the colorimetric method. Briefly, 100 µL of methanol extract of fresh water algal sample or standard reagent and 400 µL of ethanol were mixed with the 500 µL of 2% solution of AlCl₃ diluted in distilled water. After 1 h incubation at room temperature, the absorbance was measured at 430 nm. Quercetin was used to plot the standard curve, and the results were expressed as mg of quercetin equivalents (QE) g⁻¹ dry mass.

Determination of DPPH (1,1-diphenyl 2-picrylhydrazyl) radical scavenging activity:
The modified method of Shukla et al. (2012) was used for the determination of scavenging activity of DPPH free radical of the methanol extract of fresh algal material. A solution of 1 mM DPPH in methanol was prepared as a control and 50 µL of this solution was added to 2.95 mL of methanol extract prepared in different concentrations (20, 40, 60, 80 and 100 µg mL⁻¹). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical-
scavenging activity. The ability of methanol extract to scavenge DPPH radical was calculated by the equation:

\[
\text{DPPH radical scavenging activity (\%) = \left[\frac{(\text{AC} - \text{AS})}{\text{AC}}\right] \times 100}
\]

Where AC is the absorbance of the control and AS is the absorbance in the presence of the sample of methanolic extract or standard. Each experiment was carried out in triplicates.

**Hydrogen peroxide based antioxidant activity:**

The ability of the methanol extract of collected fresh water algal material to scavenge hydrogen peroxide was determined according to the method of Ruch et al (1989)\(^\text{11}\). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Methanolic extract (100 µg mL\(^{-1}\)) of various concentrations (20, 40, 60, 80 and 100 µg mL\(^{-1}\)) was added to a hydrogen peroxide solution (0.6 mL, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by both methanol extract and standard compound was calculated by the following equation:

\[
\text{Hydrogen peroxide radical scavenging activity (\%) = \left[\frac{(\text{AC} - \text{AS})}{\text{AC}}\right] \times 100}
\]

Where AC is the absorbance of the control and AS is the absorbance in the presence of the sample of methanolic extract or standards.

**Determination of reducing power ability:**

Reducing power ability of the methanolic extract of fresh water algal sample was determined according to the previously described method\(^\text{12}\). Briefly, 250 µL of each methanol extract or standard compound (ascorbic acid as a positive control) at various concentrations (10, 20, 40, 60, 80 and 100 µg mL\(^{-1}\)) was mixed with 250 µL of 0.2 M sodium phosphate buffer (pH 6.8) and 250 µL of 1% (w/v) potassium ferricyanide solution. Then, the mixture was incubated at 50°C in a water bath for 20 min, mixed with 250 µL of 10% (w/v) trichloroacetic acid (TCA), and centrifuged at 3,000 x g for 10 min. Then, 500 µL of supernatant was mixed with 500 µL of distilled water, after which 100 µL of 0.1% (w/v) FeCl\(_3\) was added to the mixture. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

**Results and Discussion**

Although collection of fresh algal sample varies from species to species in a region, the highest percentage occurrence of fresh algal material was of cyrophyceae (46.17%), chlorophyceae (39.27%) and bavillarior phyceae (26.65%), respectively (Table 1). Fresh water and plant kingdom have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them\(^\text{13}\).

<table>
<thead>
<tr>
<th>Class</th>
<th>Percentage occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillarion phyceae</td>
<td>26.65</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>39.27</td>
</tr>
<tr>
<td>Cyrophyceae</td>
<td>46.17</td>
</tr>
</tbody>
</table>

Selection of proper extraction method and solvent is very important in order to obtain extract with acceptable yield and consumer acceptability, since separation of secondary metabolites usually occurs through the selective use of solvents which may affect the quantity and quality of extract\(^\text{14}\). Since different phyto- and or algal-chemicals including polyphenols, proteins, polysaccharides and flavonoids, have significantly higher solubility in polar solvents, hence, in the present study, we used methanol to extract the majority of polar and non-polar compounds from the collected fresh water alga sample.

The percentage yield values of methanol extract of fresh algal sample was found to be 4.12% (Table 2). The preliminary phytochemical screening of methanol extract of fresh water algal sample showed the presence of number of phyto-constituents such as alkaloids, terpenoids, carbohydrates, phenolic compounds, flavonoids, and glycosides. The phytochemical constituents of methanolic extract of fresh water algal sample has been shown in Table 3. The results are in justification with the studies on antioxidant activities of methanolic leaf extract of *Jasminum humile* carried out by Cheikh-Rouhou et al (2007)\(^\text{15}\).

Phenolic compounds are considered to be most significant and biologically active compounds with various health beneficial properties\(^\text{16}\). In this study, the amount of total phenolic content in the methanolic
extract of fresh water algal sample was expressed as GAE g⁻¹ dry mass obtained from the standard calibration curve of gallic acid as reference drug. The content of total phenolics in methanolic extract of fresh water algal sample was found to be 134 ± 0.91 mg GAE g⁻¹ (Table 2). Phenolic compounds have received considerable attention because of their potential antioxidant activity.\(^1\)

### Table 2-Chemical composition profile of methanolic extract of fresh water mixed algal sample.

<table>
<thead>
<tr>
<th>Chemical components</th>
<th>Methanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Percent Yield (%)</td>
<td>4.12 %</td>
</tr>
<tr>
<td>Total phenolic content (mg GAE/g)</td>
<td>134 ± 0.91 mg GAE g⁻¹</td>
</tr>
<tr>
<td>Total flavonoid content</td>
<td>20.11 ± 2.13 mg QE g⁻¹</td>
</tr>
</tbody>
</table>

### Table 3-Preliminary phytochemical screening of fresh water mixed algal sample.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Tests</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Mayer’s Test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Wagner’s Test</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hager’s Test</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Salkowski test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Shinoda Test</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Zinc hydrochloride test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Alkaline reagent test</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Molish test</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>FeH₃O⁺ test</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>FeCl₃ test</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>Froth test</td>
<td>-</td>
</tr>
<tr>
<td>Steroids &amp; terpinoids</td>
<td>Salkowski test</td>
<td>-</td>
</tr>
<tr>
<td>Amino acids</td>
<td>Ninhydrin test</td>
<td>-</td>
</tr>
</tbody>
</table>

+= Presence, -= Absence.

Flavonoids are the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants and blue-green algae. The content of flavonoid compounds in methanolic extract of fresh water algal sample was determined from the standard calibration curve of quercetin and expressed in QE g⁻¹ dry mass. The flavonoid content of methanol extract of fresh water algal sample was found to be 20.11 ± 2.13 mg QE g⁻¹.

Antioxidants have a wide range of biological and pharmacological activities and are considered to be of great benefits in nutrition and health, since oxidative stress is an important factor in cell damage and it has been implicated in the development of various chronic diseases such as diabetes, certain cancers and neurodegenerative diseases.\(^1\) There are restrictions on the use of synthetic antioxidants such as ascorbic acid as they are suspected to be carcinogenic.\(^1\)

The methanolic extract of fresh water algal sample was investigated for its antioxidant activity using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), hydrogen peroxide radical scavenging assay and reducing power assay methods. The reduction capability of DPPH was determined by the decrease in its absorbance, which is induced by antioxidant compounds. Positive DPPH test suggests that the sample was free radical scavengers. The scavenging effect of methanol extract of fresh water algal sample and ascorbic acid on DPPH radical was compared. On the DPPH radical, methanol extract had significant scavenging effects with increasing concentration in the range of 20–100 µg mL⁻¹. However, when compared, the scavenging effect of methanol extract of algal sample was lower than that of ascorbic acid. The different concentrations of methanol extract of algal sample (20, 40, 60, 80 and 100 µg mL⁻¹) showed antioxidant activities in a dose-dependent manner (45.68%, 51.26%, 57.86%, 65.11% and 67.68% inhibition), respectively in DPPH radical scavenging assay (Fig. 1). Similar dose-dependent results were observed in methanol extract of *Camellia sinensis*, *Ficus bengalensis* and *Ficus racemosa* as they contained relatively higher levels of total phenolics than acetone extract.\(^2\)

The hydrogen peroxide radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells as described previously.\(^2\) Also as reported earlier, hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage.\(^2\) This radical has the capacity to join nucleotides in DNA and can cause strand breakage which contributes to carcinogenesis, mutagenesis and cytotoxicity.\(^2\) Hydrogen peroxide scavenging capacity of an extract is directly related to its antioxidant activity.\(^2\)

The percent inhibition of methanol extract of fresh water algal sample on hydrogen peroxide scavenging at the concentrations of 20, 40, 60, 80 and 100 µg mL⁻¹ was found to be 26.31%, 28.61%, 31.59%, 34.50% and 37.33%, respectively (Fig. 2).
The results showed antioxidant activity in a dose-dependent manner. The ability of the above mentioned methanolic extract to quench hydrogen peroxide radical seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen species, thus reducing the rate of the chain reaction. Hagerman et al. (1998) explained that high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical-scavenging activity by phenolics than their specific functional groups. It is reported that the reducing properties are generally associated with the presence of reductions possessing the hydrogen donating ability.

Biologically active compounds or extracts show antioxidant action by breaking the free radical chain via this property. In our study, based on the reducing power results, the methanol extract of the fresh water algal sample may contain higher amounts of reduction compounds thus reflecting higher antioxidant potential. In reducing power assay, the higher the absorbance of the reaction mixture, the higher would be the reducing power. In the present study, during reducing power assay, the methanol extract of fresh water algal sample at the concentrations of 10, 20, 40, 60, 80 and 100 µg/ml showed absorbance as 0.046, 0.088, 0.157, 0.191, 0.278 and 0.294, respectively (Fig. 3). Reducing power of sample is directly associated with its antioxidant activity as also reported and evaluated by Oktay et al (2003) and Yildirim et al (2000). Several biological active compounds or extracts are known to possess potent antioxidant activity.
Fig. 3-Reducing power ability of methanolic extract of fresh water algal sample compared with standard drug ascorbic acid.

Hence, the observed antioxidant activity may be due to the presence of any of these constituents. The presence of high contents of terpinoids in the methanolic extract of fresh water algal sample could also be responsible for its antioxidant potential.

Conclusion
In conclusion, this study provides evidences that methanolic extract of fresh water algae sample possessed potent health-protecting antioxidant effects. The methanolic extract of fresh water algal sample was found to contain various important phytochemicals such as phenolic, alkaloid, carbohydrate, and terpinoids which showed remarkable synergistic effects on antioxidant activity. Here, we have demonstrated that the methanolic extract of fresh water algal sample contained significantly high level of total phenolic as well as flavonoid compounds and was capable of inhibiting and quenching free radicals to terminate the radical chain reactions, thus acting as a reducing agent. Significant antioxidant activity of methanolic extract of collected fresh water algae provides a scientific validation for the important use of such algal-based fresh water samples as an accessible source of natural antioxidants with consequent health benefits.

References
23 Babu BH, Shylesh BS, Padikkala J, Antioxidant and


