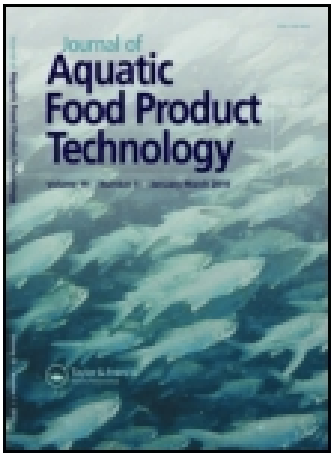

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Biological Activities of Freshwater Algae, *Spirogyra singularis* Nordstedt

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Spirogyra is commonly found as accessible algae in freshwater areas all over the world. Some medical uses have been reported from this genus. Biological activities of *Spirogyra singularis* were investigated employing eight *in vitro* assays. The extract showed different antioxidant activity. IC_{50} for DPPH radical-scavenging was 4.71 ± 0.11 μg mL^{-1}. The extract showed very strong nitric oxide-scavenging activity with $IC_{50} = 77.3 \pm 2.07$ μg mL^{-1}, but its Fe^{2+} chelating ability was weak. The extract also exhibited high antioxidant activity in hemoglobin-induced linoleic acid peroxidation tests and scavenging of hydrogen peroxide. The extract also showed good antihemolytic activity. The total amount of phenolic content in the extract was determined as gallic acid equivalents, and total flavonoid content was calculated as quercetin equivalents. Biological activities may be attributed, at least in part, to the presence of phenol and flavonoid contents in the extract.

Keywords: antioxidant activity, antihemolytic activity, green algae; radical scavenging, *Spirogyra singularis*

INTRODUCTION

Free radicals are implicated in the initiation of many chronic disorders—such as cancer, arteriosclerosis, nephritis, diabetes mellitus, rheumatism, cardiovascular diseases, neurodegenerative disease, gastrointestinal tract disorders, and inflammatory injury (Halliwell, 1997). The red blood cell membrane is the main target of free radical attacks. Hemolysis caused by free radical attack is implicated in different disorders. For example, in glucose-6-phosphate dehydrogenase deficiency,

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oxidative stress induced by oxidant substance has a cellular toxic role (Vanella et al., 1991). Antioxidants are of vital importance in protection of tissue damage caused by free radicals (Valko et al., 2007).

A previous study has demonstrated the important role of nitric oxide (NO) in initiation or progression of numerous diseases, such as cancer and inflammation (Sreejayan and Rao, 1997). The antioxidants may have the ability to counteract the effect of NO formation and, in turn, may be of considerable interest in preventing the ill effects of excessive NO generation in the human body.

The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease. Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in humans and thereby improves quality of life and overall survival from some diseases, such as Thalassemia major (Hebbel et al., 1990). Previous studies have also shown that iron chelators and hydroxyl-radical scavengers protect against acute renal failure, especially aminoglycoside antibiotic-mediated nephrotoxicity (Moghaddam et al., 2010). In recent years, the search for natural iron chelators with lower side effects has increased (Grazul and Budzisz, 2009).

Synthetic antioxidants are widely used, but their side effects such as carcinogenic effect have been reported (Madhavi & Salunkhe, 1996). Polyphenolic compounds, such as flavonoids and phenols, are widely found in food products derived from plant sources, and they have been shown to possess pharmacological and biological activities that relate to their antioxidant ability (van Acker et al., 1996). Therefore, the search for natural antioxidants has increased.

Spirogyra is a genus of filamentous green algae of the order Zygnematales. It is commonly found in freshwater areas, and there are more than 400 species of *Spirogyra* in the world (John et al., 2002). Previous studies reported antimicrobial, antioxidant, and anticancer activities from *Spirogyra varians* (Moon et al., 2009). In addition, the bacteriostatic effect (Stangenberg, 1968), anti-HSV-1, and anti-HIV activities of *Spirogyra* species have been reported previously (Hayashi et al., 1996). Numerous biological activities including antioxidant and anticholinesterase activities have been reported from *Spirogyra gratiana* Transeau (Kartal et al., 2009). Phytochemical studies show that *Spirogyra gratiana* Transeau has a high content of chlorogenic, gentisic, gallic acid, coumaric acid, ferulic acid, benzoic acid, gallic acid, ethyl ester, vitamin C, asthaxanthin, beta-carotene, and canthaxanthin (Kartal et al., 2009). To the best of our knowledge, there are no scientific reports on the antihemolytic and antioxidant activities of *Spirogyra singularis.*

MATERIALS AND METHODS

Sample Collection

Spirogyra singularis was collected from the Sari, Mazandaran, Iran; identified by Dr. Naser Jafari, Assistant Professor of Algology and Ecology; and deposited in the Herbarium of the Faculty of Biology, Department of Biology, College of Sciences, University of Mazandaran, Babolsar.

Chemicals

Ferrozine, linoleic acid, trichloroacetic acid (TCA), 1, 1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide, cumene hydroperoxide (CuOOH) and hydrogen peroxide were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Gallic acid, quercetin, butylated hydroxyanisole (BHA), vitamin C, sulfanilamide, n-(1-naphthyl) ethylenediamine dihydrochloride, EDTA, and

ferric chloride were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade or purer.

Extraction Procedure

The extraction was executed in triplicate. In brief, 10 g of *S. singularis* powder sample was extracted at room temperature by the percolation method using aqueous ethanol (70%). The resulting extract was concentrated over a rotary vacuum until a crude extract was obtained, which was then freeze-dried for complete solvent removal. The yield of crude extract was 2.4 g.

Determination of Total Phenolic and Flavonoid Contents

Total phenolic content was determined by the Folin-Ciocalteau method (Hertog et al., 1993). Briefly, the sample (0.5 mL) was mixed with 2.5 mL of 0.2 N Folin-Ciocalteau reagent for 5 min, and 2.0 mL of 75 g L^{-1} sodium carbonate were then added. The absorbance of reaction was measured at 760 nm with a double beam spectrophotometer (Perkin Elmer, Waltham, MA, USA) after 2 h of incubation at room temperature. The results were expressed as gallic acid equivalents. Total flavonoid content was determined by colorimetric method (Hertog et al., 1993). Briefly, a 0.5-mL solution of extract was separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm. The results were expressed as quercetin equivalents.

Antioxidant Activity

DPPH Radical-Scavenging Activity

The stable 1, 1-diphenyl-2-picryl hydrazyl radical was used for determination of free radical-scavenging activity of the sample (Lee et al., 2003). Different concentrations of sample were added, at an equal volume, to ethanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated three times. Vitamin C, BHA, and quercetin were used as controls. The half maximal inhibitory concentration (IC_{50}) values denote the concentration of sample which is required to scavenge 50% of DPPH free radicals.

Reducing Power Determination

Briefly, 2.5 mL of sample (25–800 μg mL^{-1}) in water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$], (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm (Yen and Chen, 1995). Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Assay of Nitric Oxide-Scavenging Activity

For the experiment, 1 mL sodium nitroprusside (10 mM) in phosphate-buffered saline was mixed with 3 mL of different concentrations of sample dissolved in water and incubated at room temperature for 150 min (Sreejayan and Rao, 1997). The same reaction mixture, without sample but with an equivalent amount of water, served as control. After the incubation period, 0.5 mL of Griess reagent

was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control.

Metal Chelating Activity

The chelation of ferrous ions by the sample was determined according to Dinis et al. (1994). Briefly, the sample (0.2–3.2 mg mL^{-1}) was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL); then the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0-A_s)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of the tested sample/standard. Na_2EDTA was used as positive control.

Scavenging of Hydrogen Peroxide

Briefly, a solution of hydrogen peroxide (40 mM, 0.6 mL) was prepared in phosphate buffer (pH 7.4). Then, 1.4 mL of sample (0.1–1 mg mL^{-1}) in distilled water was added to a hydrogen peroxide solution. The absorbance of the sample at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the sample and standard was calculated as follows: $\%\text{Scavenged}\,[H_2O_2] = [(A_o-A_1)/A_o] \times 100$, where A_o was the absorbance of the control, and A_1 was the absorbance in the presence of the extract and standard (Ebrahimzadeh et al., 2010).

Antioxidant Activity in a Hemoglobin-Induced Linoleic Acid Peroxidation Test

The antioxidant activity of the sample was determined by a modified photometry assay (Nabavi et al., 2010). Reaction mixture (200 mL) containing 10 mL of sample (10–400 mg), 1 mmol of linoleic acid emulsion, 40 mmol of phosphate buffer (pH 6.5), and 0.0016% hemoglobin was incubated at 37°C for 45 min. After the incubation, 2.5 mL of 0.6% HCl in ethanol was added to stop the lipid peroxidation. The amount of peroxide value was measured in triplicate using the thiocyanate method by reading the absorbance at 480 nm after coloring with 100 mL of 0.02 mol L^{-1} of $FeCl_2$ and 50 mL of ammonium thiocyanate (0.3 g mL^{-1}). Vitamin C was used as positive control.

Antihemolytic Activity

Preparation of Rat Erythrocytes

All the animal experiments were carried out with the approval of the Institutional Animal Ethical Committee. Eight-week-old male rats in the body weight range of 180–220 g were housed in individual polypropylene cages (1 week before experiment) and had free access to food and water. The animals were fed with standard diet. The animals were killed under anesthesia and blood was collected by heart puncture in heparinized tubes. Erythrocytes were isolated and stored according to our recently published articles (Nabavi et al., 2011). Washed erythrocytes were stored at 4°C and used within 6 h for further studies.

Antihemolytic Activity of Sample Against H_2O_2 Induced Hemolysis

Antihemolytic activity of the sample was assessed as described by Nabavi et al. (2010). Erythrocytes from male rat blood were separated by centrifugation and washed with phosphate buffer saline (PBS; pH 7.4). Erythrocytes were then diluted with PBS to give 4% suspension. Two mL of 1 g mL^{-1} of sample of saline buffer was added to 2 mL of erythrocyte suspension, and the volume was made up to 5 mL with PBS. The mixture was incubated for 5 min at room temperature,

and then 0.5 mL of H_2O_2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H_2O_2 in the reaction mixture was adjusted to bring about 90% of hemolysis of blood cells after 240 min. After incubation, the reaction mixture was centrifuged at 1,500 rpm for 10 min, and then extent of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation (mean values of three determinations were used for the calculation).

Antihemolytic Activity of Sample Against CuOOH-Induced Hemolysis

The antioxidant activity of the sample was determined in rat erythrocytes (RBC) exposed to CuOOH by measuring the erythrocyte membrane resistance to free radical-induced hemolysis (Aldini et al., 2006). Aliquots (3.5 mL) were incubated at 37°C for 210 min in the presence of 50 μM CuOOH (dissolved in ethanol), and the cellular integrity determined turbidimetrically at 710 nm at 30-min intervals. The sample (dissolved in EtOH; final concentrations 0.5 μg mL^{-1}) was preincubated for 30 min with RBC (1% suspension in PBS) before the addition of CuOOH [blanks were RBC added with ethanol, at a final concentration always less than 0.1% (v/v)]. Percentages of hemolysis were determined by setting as a 100% hemolysis the absorbance value determined in RBC suspensions sonicated for 5 s at 50% power (mean values of three determinations were used for the calculation).

Statistical Analysis

Experimental results are expressed as means ± *SD*. All measurements were replicated three times. The data were compared against the blank tube without extract or standards using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Differences were considered statistically significant at $p < 0.05$. The IC_{50} values were calculated from linear regression analysis.

RESULTS AND DISCUSSION

Total phenolic compounds, as determined by Folin Ciocalteu method, are reported as gallic acid equivalents. The total phenolic content of *S. singularis* was 47.51 ± 1.37 mg gallic acid equivalent/g of extract. Also, the total flavonoid content of the extract was 35.52 ± 1.10 mg quercetin equivalent/g of extract powder. *S. singularis* showed potent DPPH radical-scavenging activity with $IC_{50} = 4.71 \pm 0.11$ μg mL^{-1}. The IC_{50} values for ascorbic acid, quercetin, and BHA were 5.05 ± 0.1, 5.28 ± 0.2, and 53.96 ± 3.1 μg mL^{-1}, respectively. The potent DPPH radical scavenging activity may be the result of the high phenol and flavonoid content of the extract. Phenol and flavonoids can scavenge the DPPH radical via electron or hydrogen donation mechanism and so changes the color from violet to yellow. Substances which are able to perform this reaction can be considered radical scavengers.

In reducing power assay, phenols or other electron donor compounds in the sample can reduce Fe^{3+} to Fe^{2+}. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Shon et al., 2003). Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1 shows the dose-response curves for the reducing power of the extract. It was found that the reducing power of the sample increased with increasing concentration. Vitamin C showed higher activity than extract ($p > 0.05$).

The nitric oxide assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. *S. singularis* demonstrates good nitric oxide

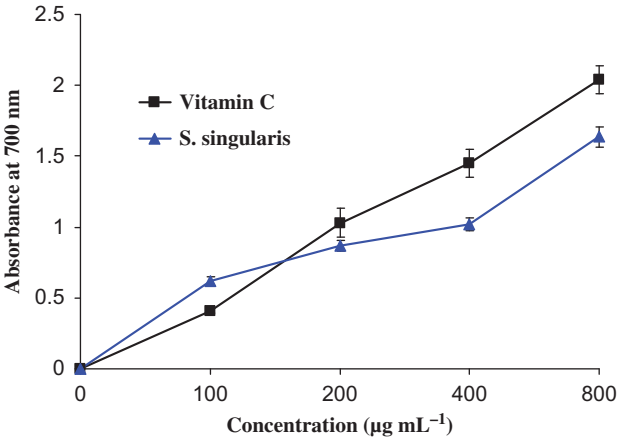

FIGURE 1 Reducing power of *Spirogyra singularis* extract. Results are expressed as the mean ± *SD* of three experiments (color figure available online).

radical-scavenging activity (IC_{50} for *S. singularis* was 77.3 ± 2.07 μg mL^{-1} and 17.01 ± 0.03 μg mL^{-1} for quercetin). Quercetin showed very potent nitric oxide radical scavenging, but its carcinogenic activity has been reported (Dunnik and Hailey, 1992). In addition to reactive oxygen species, NO is also implicated in inflammation, cancer, and other pathological conditions (Ebrahimzadeh et al., 2010). The scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health.

S. singularis showed weak iron chelating activity, with IC_{50} equal to 973.06 ± 32.01 μg mL^{-1}. EDTA showed very powerful activity (IC_{50} = 18 ± 0.5 μg mL^{-1}). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. In this assay, extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures the ferrous ion before ferrozine.

S. singularis was capable of scavenging hydrogen peroxide in a concentration dependent manner. IC_{50} was 19.6 ± 0.97 μg mL^{-1}. The IC_{50} values for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6 μg mL^{-1}, respectively. Phenolics and other reductant compounds can donate electrons to H_2O_2, neutralizing it to water (Shon et al., 2003). Hydrogen peroxide itself is not very reactive, but it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell (Shon et al., 2003).

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Erythrocytes are considered as prime targets for free radical attack owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the O_2 transport associated with redox active hemoglobin molecules, which are potent promoters of reactive O_2 species (Yu, 2001). The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (Nabavi et al., 2011). A tested sample showed good activity in hemoglobin-induced linoleic acid peroxidation tests. Its activity was comparable with vitamin C (Figure 2). Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation.

The effect of *S. singularis* was tested and no harmful effects on erythrocytes were found. The IC_{50} of antihemolytic activity of *S. singularis* was 323.01 ± 10.98 μg mL^{-1} and 235 ± 9 μg mL^{-1} for vitamin C. The antihemolytic activity of quercetin and other flavonoids has been reported

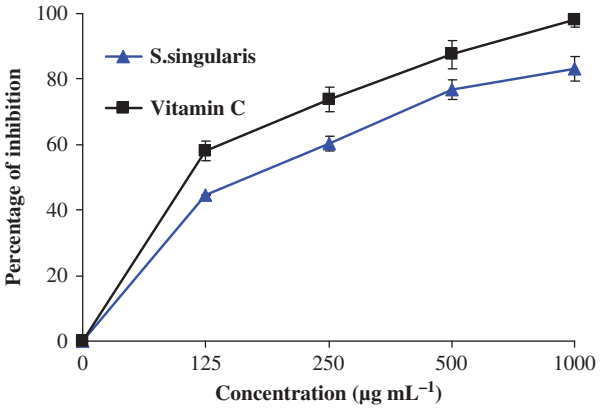

FIGURE 2 Antioxidant activities of *Spirogyra singularis* against hemoglobin-induced linoleic acid peroxidation tests. Results are expressed as the mean ± *SD* of three experiments. Vitamin C used as positive control (color figure available online).

previously. Good antihemolytic activity of extract could be the result of high flavonoid content, especially quercetin (Chaudhuri et al., 2007).

In RBC exposed to CuOOH (Figure 3), hemolysis started after 30-min incubation. *S. singularis* dose dependently delays the onset of the CuOOH-induced hemolysis; at 120 min, hemolysis was inhibited by 16.9% at 4 µg mL^{-1}, by 18.9, 20, and 21.4% at 2, 1, and 0.5 µg mL^{-1}, respectively, and totally inhibited at the highest concentration.

The extract showed very good activity in some models. These results can be useful for further applications of *S. singularis* and its constituents in pharmaceutical preparations after performing clinical in vivo studies.

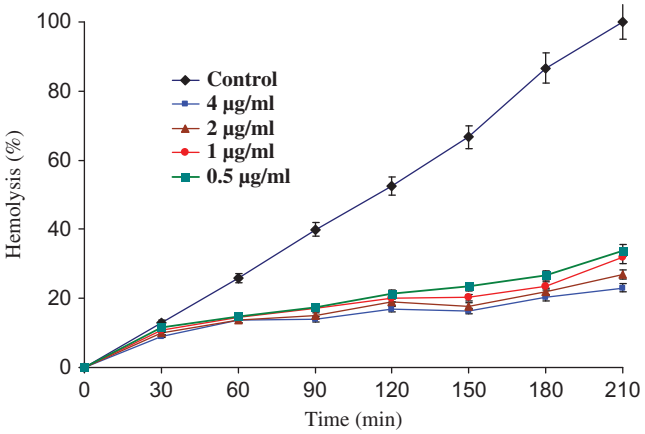

FIGURE 3 Protective effect of *Spirogyra singularis* on red blood cell hemolysis induced by CuOOH (50 µM). Values are the mean ± *SD* of three independent experiments (color figure available online).

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