Evaluation of antioxidant activities and total phenolic contents of different solvent extracts of selected marine diatoms

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Antioxidant properties and total phenolic content were evaluated in three different solvent extract of three selected marine diatoms, Chaetoceros curvisetus, Thalassiosira subtilis and Odontella aurita. Diatoms were extracted using methanol, acetone and hexane by a three-step sequential extraction procedure. Total phenol content, and the antioxidant properties such as total antioxidant activity, DPPH radical scavenging activity, nitric oxide radical scavenging activity, hydrogen peroxide radical scavenging activity and ferric reducing power of diatom extracts (methanol, acetone and hexane) were determined. Results showed that total phenolic content and antioxidant properties were higher in methanolic extract of O. aurita. Total phenol content (0.55 ± 0.031 mg/g gallic acid equivalent), total antioxidant activity (0.97 ± 0.044 mg/g ascorbic acid equivalent), DPPH radical scavenging activity (15.25%), hydrogen peroxide radical scavenging activity (54.73%), Ferric reducing power assay (1.032 ± 0.031 mg/g ascorbic acid equivalent) and nitric oxide radical scavenging activity were maximum in C. curvisetus (32.37%). Thus, phenolic compounds were not the only major contributor to the antioxidant capacities of these marine diatoms. The microalgae could contain different antioxidant compound than the other plants.

[Key words: Antioxidant activity, phenolic contents, diatoms, marine diatoms]

Introduction

In recent years, the use of photosynthetic microorganisms, such as microalgae, in life sciences has received increasing attentions due to their diverse phytometabolic contents with various chemical structures and biological activities. They also have nutritionally potential applications as functional foods which are able to provide additional physiological and pharmacological benefits for human health. Polysaturated fatty acids, sulfated polysaccharides, phycosterols, heat-induced proteins, phenolic compounds, and pigments including carotenoids are the naturally origin functional ingredients which have positive effects on the health of man and animals. A large number of studies on the microalgal bioactive compounds have oriented to the anti-inflammatory, antiviral, antimicrobial, antihelmintic, cytotoxic, immunological, and enzyme inhibition properties.

The oxidative damage is caused by reactive oxygen species on lipids, proteins and nucleic acids may trigger various chronic diseases, such as coronary heart disease, atherosclerosis, cancer and ageing. Epidemiological studies have demonstrated an inverse association between intake of fruits and vegetables and mortality from age related diseases, such as coronary heart disease and cancer, which may be attributed to their antioxidant activity. On the other hand, some synthetic antioxidants, such as BHT and BHA, need to be replaced with natural antioxidants, as they were found to be toxic and carcinogenic in animal models. Thus, it is important to identify new sources of safe and inexpensive antioxidants of natural origin.

Algal biomass and algae-derived compounds have a very wide range of potential applications, from animal feed and aquaculture to human nutrition and health products. Most, if not all, commercially available natural antioxidants are derived from terrestrial plants (e.g. rosemary, tea, grape seeds, pine bark, cocoa). It is however believed that unicellular microalgae are a promising alternative source of antioxidants. There are a number of reports on the evaluation of antioxidant activity in microalgae and cyanobacteria belonging to the genera Botryococcus, Chlorella, Dunaliella, Nostoc, Phaeodactylum, Spirulina, Haematococcus and Chaetoceros. These studies concluded that several microalgal genera contain potent antioxidants, both from lipophilic and hydrophilic nature.

Present investigation was attempted to study the antioxidant activities and total phenolic content of methanolic, acetone and hexane extracts of marine diatoms Odontella aurita, Chaetoceros curvisetus and Thalassiosira subtilis.
Materials and Methods

Marine diatoms such as *Odontella aurita*, *Cheatoceros curvisetus* and *Thalassiosira subtilis* were isolated from the Vellar estuary, Parangipettai, southeast coast of India. These strains were isolated by serial dilution methods. Unialgal cultures were developed and maintained with F/2 media. Three selected marine diatoms were cultured in MoES-HABs “Harmful Algal Blooms” project, algal culture laboratory, CAS in Marine Biology, Annamalai University and the cells were harvested by centrifugation at 4000 rpm for 10 min. The harvested cells were lyophilized under reduced pressure. A precisely weighed (2 g) amount of ground freeze dried marine diatoms were extracted for 24 h in 40 ml of methanol, acetone and hexane at room temperature. The extraction was twice repeated and filtered through glass funnel and Whatmann No. 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure using a rotary flash evaporator. Finally the dry extracts were lyophilized and stored in refrigerator for further analysis.

Phenolic content of crude extracts were estimated by the method of Taga *et al.* 31. 100 µl of aliquot sample was mixed with 2.0 ml of 2% Na₂CO₃ and allowed to stand for 2 min at room temperature. After incubation, 100 µl of 50% Folin Ciocalteau’s phenol reagent was added and the reaction mixture was mixed thoroughly and allowed to stand for 30 min. at room temperature in the dark. Absorbance of all the sample solutions was measured at 720 nm using spectrophotometer (Phenolic content is expressed as Gallic acid equivalent per gram).

Total antioxidant activity was measured by the method of Prieto *et al.* 32. Total Antioxidant Capacity (TAC) reagent was prepared by adding 7.45 ml of sulphuric acid (0.6 mM solution), 0.9942 g of sodium sulphate (28 mM solution) and 1.2359 g of ammonium molybdate (4 mM solution) in 250 ml distilled water. 300 µl of extract was dissolved in 3 ml of TAC reagent. Blank was maintained with distilled water replacing the TAC reagent. Absorbance of all sample mixtures was measured at 720 nm using spectrophotometer (Phenolic content is expressed as Gallic acid equivalent per gram).

Reducing power of different crude extract was determined by the method of Oyaizu 33. Briefly, 1.0 ml of different solvent extract containing different concentration of samples were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferric cyanide (1%). Reaction mixture was kept in a water bath at 50°C for 20 min. After incubation, 2.5 ml of Trichloroacetic acid (10% of TCA) was added and centrifuged at 650 rpm for 10 min. From the upper layer, 2.5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml of FeCl₃ (0.1%). Absorbance of all the solution was measured at 700 nm. Ferric reducing antioxidant power is expressed as the number of equivalents of ascorbic acid.

The scavenging effects of samples for DPPH radical were determined by the method of Yen and Chen, 34. Briefly, 2.0 ml of aliquot of test samples was added to 2.0 ml of 0.16 mM DPPH methanolic solution. The mixture was vortexed for 1 min and incubated at room temperature for 30 min in the dark. The absorbance of all the sample solutions was measured at 517 nm. The scavenging effect (%) was calculated by using the formulae given by Duan *et al.* 35.

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\text{Scavenging effect} (%) = \left[1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}} \right] \times 100
\]

The ability of marine diatoms crude extract to scavenge hydrogen peroxide was determined by the standard procedure of Gulcin *et al.* 36. Hydrogen peroxide 10 mM solution was prepared in the phosphate buffer saline of 0.1 M, pH 7.4, 1 ml (0.25 mg) of the extract was rapidly mixed with 2 ml of hydrogen peroxide solution. The absorbance was measured at 230 nm in the UV spectrophotometer against a blank (without hydrogen peroxide) after 10 min. of incubation at 37°C.

Nitric oxide radical was measured by following the method of Gulcin, (2006) 37, 2 ml of sodium nitroprusside (10 mm) was mixed with 1 ml of the test extracts in phosphate buffer (pH 7.4). Mixture was incubated at 25°C for 150 min. To 0.5 ml of the incubated solution, 1 ml of sulphanilic acid reagent (0.33% sulphanilamide in 20% acetic acid) was added and allowed to stand for 5 min for completing diazotization. 1 ml of 0.1% naphthyl ethylene diamine dihydrochloride was added and incubated at room temperature for 30 min. Absorbance was measured at 540 nm. Ascorbic acid was used as positive control. The nitric oxide scavenging activity of the crude extracts was represented as % of scavenging.
Statistical analysis

The data were subjected to two-way and one-way ANOVA using statistics software package (SPSS, ver.16) to analyze the statistical significance.

Results

Total phenolic content of three different solvent extracts (methanol, acetone and hexane) of diatoms such as *O. aurita*, *C. curvisetus* and *T. subtilis* were determined which the results were presented in Fig.1. Phenolic content was found to be higher in methanolic extract of *O. aurita* (0.55 ± 0.031 mg/g gallic acid equivalent) followed by acetone extract of *C. curvisetus* (0.44 ± 0.027 mg/g gallic acid equivalent) and hexane extract of *O. aurita* (0.29 ± 0.011 mg/g gallic acid equivalent). Lowest phenolic content was recorded in hexane extracts of *T. subtilis* (0.17 ± 0.024 mg/g gallic acid equivalent). Changes in total phenolic content between the tested species (P<0.01) and solvent extracts (P<0.05) were statistically differed.

Total antioxidant activities of different solvent extracts (methanol, acetone and hexane) of selected diatoms were presented in Fig.2. Highest activity of 0.97 ± 0.044 and 0.72 ± 0.052 mg/g ascorbic acid equivalent were observed in methanol and acetone extracts of *O. aurita* and hexane extract of *T. subtilis* (0.64 ± 0.044 mg/g ascorbic acid equivalent). Lowest activity was noticed in the hexane extract of *O. aurita* (0.43 ± 0.037 mg/g ascorbic acid equivalent). Changes in total antioxidant activity between the tested species (P<0.01) and solvent extracts (P>0.05) differed statistically.

Ferric reducing activity of the different solvent extracts (methanol, acetone and hexane) of diatoms such as *O. aurita*, *C. curvisetus* and *T. subtilis* was determined by reducing power assay varied as seen in Fig.3. Reducing power was found to be higher in methanolic extract of *O. aurita* (1.032 ± 0.031 mg/g ascorbic acid equivalent) at 1 ml concentration and *C. curvisetus* (0.68 ± 0.08 mg/g ascorbic acid equivalent) at 1 ml followed by hexane extract of *T. subtilis* (0.89 ± 0.04 mg/g ascorbic acid equivalent) at 1ml. The lowest reducing power was recorded in lowest concentration of hexane extracts of *C. curvisetus* (0.22 ± 0.01 mg/g ascorbic acid equivalent) at 0.2 ml concentration. Differences in ferric reducing antioxidant power between the tested species (P>0.05) and solvent extract concentration were statistically non-significant (P>0.05).

DPPH radical scavenging activities (%) of different solvent extracts (methanol, acetone and hexane) of diatoms *O. aurita*, *C. curvisetus* and *T. subtilis* are presented in Fig. 4. All these marine diatoms extracts possessed the ability to scavenge DPPH at various degrees. Methanol extract of *O. aurita* (15.25%) and *T. subtilis* (12.51%) was
found to be the most potent scavenger followed by the acetone extract of *O. aurita* (13.65%) and *C. curvisetus* (9.4%). Acetone extract of *T. subtilis* and hexane extract of *C. curvisetus* showed the minimum DPPH radical scavenging activity at 7.39% and 4.66% respectively. Scavenging effect of standard on the DPPH radical decreased in the order of BHT > gallic acid, which was 15.25% and 12.51%. Variation in DPPH activity between the tested species (P<0.05) and solvent extracts (P<0.05) differed statistically.

Nitric oxide radical scavenging assay of different solvent extracts (methanol, acetone and hexane) of diatoms *O. aurita, C. curvisetus* and *T. subtilis* were shown in Fig. 6. Results indicated that the highest scavenging activity was observed in methanol and hexane extract of *C. curvisetus* (32.37% and 31.32%) respectively whereas the minimum activity was noticed in acetone extract of *T. subtilis* (14.64%). Changes in nitric oxide radical scavenging activity between the tested species (P>0.05) and solvent extracts (P>0.05) differed statistically.

**Discussion**

Maximum phenolic content was observed in extracts of *C. vulgaris* obtained by pressurised liquid extraction at elevated temperatures using 90% ethanol reported that the phenolic content was high in the water fraction of *C. vulgaris* and *Nostoc ellipsosporum*, *Nitzschia laevis*. Nevertheless, Li et al., HaJimahmoodi et al. and Cha et al. were reported that the phenolic content was very low in the non-polar solvent of hexane fraction of marine diatoms. However in the present study, the highest phenolic content was observed in methanolic extract of *O. aurita* (0.55 ± 0.031) while lowest phenolic content in hexane extract of *T. subtilis* (0.17 ± 0.024). These results are more or less similar with the earlier investigation of Uma et al., and it clearly explains that the methanolic extract found to have higher phenolic content in *D. olivaceous* and flavonoid content was high in acetone extract of *C. humicola*. According to Manivannan et al. methanol extract of *Chlorella marina* exhibited higher activity which was followed by diethyl ether and hexane extracts. This may be due to the differences in the polarity of the solvents used.
In concern with the antioxidant activity, the maximum antioxidant activity of methanol extract (0.97 ± 0.044 mg/g) and acetone extract (0.72 ± 0.052 mg/g) were observed in O. aurita, and hexane extract in T. subtilis (0.64 ± 0.044 mg/g). Lowest activity was noticed in the hexane extract of O. aurita (0.43 ± 0.037 mg/g). Similarly, Sivakumar and Rajagopal \(^4\) reported that highest antioxidant activity was observed in methanol extract from eight green algal species. Uma et al. \(^3\) observed that the methanolic extracts displayed greater potential in all antioxidant assays when compared to ethanolic and acetone extracts of green microalgae *Desmococcus olivaceous* and *Chlorococcum humicola*. Although some other authors have described the presence of high content of sterols in C. vulgaris. High antioxidant activity of C. vulgaris could be due to its chemical composition\(^3\). On the other hand, the high antioxidant activity was observed in C. vulgaris, was due to its high content of polyunsaturated fatty acids. Possibly, the additional use of a less polar extracting solvent such as hexane or ethyl acetate would improve the antioxidant activity of the extract, as this could lead to the extraction of some phenolic compounds and it has been previously reported in several *Chlorella* species\(^5\).

The reducing power was found to be higher in methanolic extract of O. aurita (1.032 ± 0.031 mg/g) at 1ml concentration and the minimum reducing power was present in lowest concentration of hexane extracts of C. curvisetus (0.22 ± 0.01 mg/g) at 0.2ml concentration. Similarly, Kuda et al. \(^4\) reported that the highest amount of reducing power was observed in the highly polar water extract of S. lomentaria and the minimum reducing power was observed in ethanol extract and crude fucoidan, these were dose-dependent. Supportively, Herrero et al. \(^2\) explained that these polar compounds can be extracted to a higher extent, in this way increasing the yield of extract. Gordan \(^4\) found that the antioxidant effect of reductones is based on the destruction of the free radical chain by donating a hydrogen atom. Reduction of ferric ion (Fe\(^{3+}\)) to ferrous ion (Fe\(^{2+}\)) is measured by the strength of the green-blue colour in solution which absorbs at 700 nm. The marked ferric reducing power activity of extract was due to the presence of polyphenols which may act in a similar way as reductones react with free radicals to turn them into more stable products and abort free radical chain reactions\(^4\).

In this study, methanol extract of *O. aurita* (15.25%) and *T. subtilis* (12.51%) was found to be the most potent scavenger. Acetone extract of *T. subtilis* and hexane extract of *C. curvisetus* showed the minimum DPPH radical scavenging activity at 7.39% and 4.66% respectively. Similarly, both methanolic and acetone extracts of *D. olivaceous* and *C. humicola* showed a significant dose dependent reduction of DPPH radicals\(^4\). This assay revealed that the extracts might prevent reactive radical species from damaging biomolecules such as lipoprotein, DNA, aminoacids, sugar, proteins and PUFA in biological and food systems. Lee et al. \(^2\) reported that 80% methanol extract and organic solvent fractions of both algae showed notable activities indicating the higher efficacy for scavenging of free radicals. Nakamura et al. \(^4\) suggested that carbohydrates and proteases have the capability to liberate bioactive compounds for radical scavenging. The implications are important as radical scavengers may protect cell tissues from free radicals, thereby preventing disease such as cancer.

Hydrogen peroxide radical scavenging activity (%) was maximum in methanol (54.73%) and hexane extract (53.54%) of *O. aurita* whereas it was minimum in acetone extract of *T. subtilis* (28.41%). According to Uma et al. \(^4\) methanolic extracts of *D. olivaceous* exhibited 39% scavenging activity and the acetone extract of *C. humicola* exhibited 15% scavenging activity. Gulcin \(^3\) stated that hydrogen peroxide is not very reactive, but sometimes it can be toxic to cells because of rise in the hydroxyl radicals in the cells.

Nitric oxide is a gaseous free radical, which is of concern in cancer, inflammation and other pathological conditions. In nitric oxide scavenging, the enzymatic digests showed higher activities than the solvent fractions and those activities were significantly higher than the standard antioxidants. Therefore, it can be suggested that those bioactive compounds were relatively hydrophilic. In addition, enzymatic hydrolysis was effective in extracting biochemical compounds that were responsible for nitric oxide inhibition. The activities of nitric oxide and O$_2^\cdot$ were found to be relatively low, but their metabolites ONOO$^-$ (peroxynitrite) are extremely reactive and directly induce toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA.
modifications. In this study, highest nitric oxide radical scavenging activity was observed in methanol and hexane extracts of *C. curvisetum* (32.37% and 31.32%) respectively whereas minimum in acetone extract of *T. subtilis* (14.64%). This is lined with the finding of Lee et al. who found that the ethyl acetate of *H. porphyrae* (30.1%) and the 80% of methanol extract of *O. unicellularis* (49.3%) exhibited significantly higher nitric oxide radical scavenging effects than those of the commercial antioxidants.

**Conclusion**

In the present study, the methanolic extracts showed the predominant antioxidant activity in *O. aurita* > *C. curvisetuum* > *T. subtilis*. The antioxidant mechanisms of marine diatoms extracts may be attributed to their free radical-scavenging ability. In addition, phenolic compounds appear to be responsible for the antioxidant activity of microalgae extracts. On the basis of the results obtained, microalgae can be used for a variety of beneficial chemo-preventive effects.

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