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Antioxidant properties and total phenolic content of a marine diatom, *Navicula clavata* and green microalgae, *Chlorella marina* and *Dunaliella salina*

A. Hemalatha, K. Girija, C. Parthiban, C. Saranya and P. Anantharaman*

CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, India

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ABSTRACT

The study focuses on determining the antioxidant properties of the different solvent extracts, namely methanol, acetone and hexane, from the microalgae. The evaluation of total phenolic content and antioxidant properties was determined by total antioxidant activity, Ferric reducing antioxidant power, DPPH radical scavenging activity, Hydrogen peroxide radical scavenging activity and Nitric oxide radical scavenging assay. Among the tested microalgae, the maximum antioxidant activity was recorded in methanolic extract of C. marina. Whereas methanolic extract of green microalgae and diatom showed good antioxidant potential. This study suggests that different solvent extracts contain different potential antioxidant compounds capable to scavenge different types of free radicals.

Keywords: *Navicula clavata*, *Chlorella marina*, *Dunaliella salina*, Antioxidant activity, Total phenolic content

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INTRODUCTION

Microalgae are photoautotrophic organisms that are exposed to high oxygen and radical stresses and consequently have developed several efficient protective systems against reactive oxygen species and free radicals (Pulz and Gross, 2004). Microalgae represent an almost untapped resource of natural antioxidants, due to their enormous biodiversity and much more diverse than higher plants. The value of microalgae as a source of natural antioxidants is further enhanced by the relative ease of purification of target compounds (Li *et al.*, 2001). However, not all groups of microalgae can be used as natural sources of antioxidants, due to their widely varied contents of target products, growth rate or yields, ease of cultivation, and/or other factors. Reports on the antioxidant activities of microalgae are limited, especially concerning the relationship between their phenolic content and antioxidant capacity (Li *et al.*, 2007). Therefore, the present investigation was attempted to study the antioxidant properties of methanolic, acetone and hexane extracts of marine diatoms *Navicula clavata* and a Green Microalgae, *Chlorella marina* and *Dunaliella salina* by using three different solvents (methanol, acetone and hexane) extracts.

MATERIALS AND METHODS

2.1. Sample collection and extraction

The marine diatom *Navicula clavata* was isolated from the Vellar estuary, Parangipettai, south east coast of India. This strain was isolated in serial dilution methods. The green microalgae of *Chlorella marina and Dunaliella salina* strain was obtained from CMFRI (Central Marine Fisheries Research Institute), Tuticorin, Tamilnadu, India. The unialgal cultures was developed and maintained with F/2 media. The three selected microalgae were cultured in MoES-HABs "Harmful Algal Blooms" project, algal culture laboratory, CAS in Marine Biology, Annamalai

University and the cells were harvested by centrifuged at 4000 rpm for 10 min. The harvested cells were lyophilized under reduced pressure. A precisely weighed (2 g) amount of ground freeze dried microalgae 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.

2.2. Antioxidant activities of microalgae

2.2.1. Total phenolic content

Phenolic contents of crude extracts were estimated by the method of Taga *et al*., 1984. 100 µl of aliquot sample was mixed with 2.0 ml of 2% Na_2CO_3 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).

2.2.2. Total antioxidant activity

Total antioxidant activity was measured by the method of Prieto *et al*., 1999. Total Antioxidant Capacity (TAC) reagent consist of 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) were mixed together 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 695 nm. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid.

2.2.3. DPPH radical scavenging assay

The scavenging effects of samples for DPPH radical were determined by the method of Yen and Chen, 1995. 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 then left to stand 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*., 2006.

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

2.2.4. Hydrogen peroxide radical scavenging assay

The ability of microalgae crude extract to scavenge hydrogen peroxide was determined by the standard procedure of Gulcin *et al*., 2004. 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 37ºC.

2.2.5. Nitric oxide radical scavenging assay

The nitric oxide radical were measured by following the method of Gulcin, 2006. 2 ml of sodium nitroprusside (10 mm) was mixed with 1 ml of the test extracts in phosphate buffer (pH 7.4). The 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% napthyl 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.

2.2.6. Ferric reducing antioxidant power assay

Reducing power of different crude extract was determined by the method of Oyaizu, 1986. 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 50ºC 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 $FeCl_3$ (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.

2.3. Statistical analysis

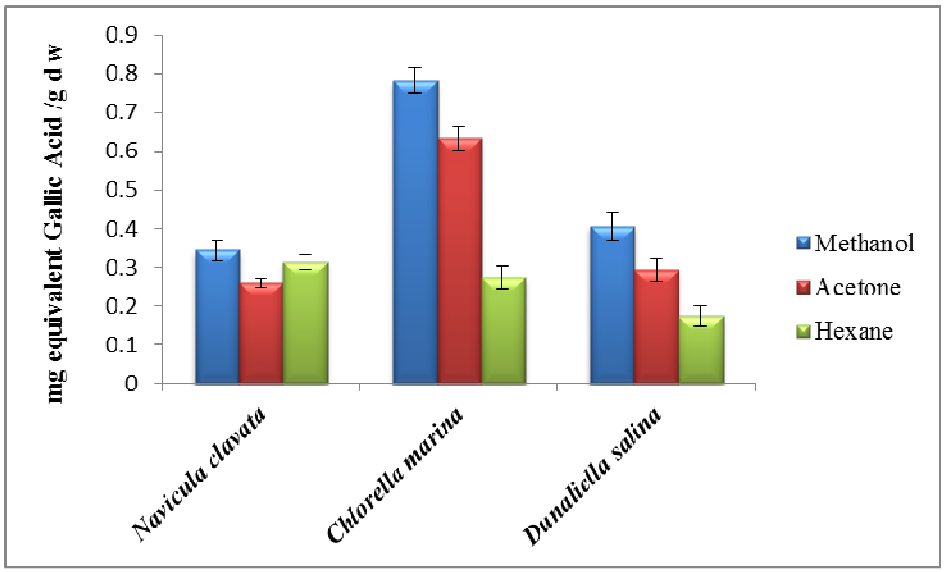
The data were subjected to two way and one way ANOVA using statistics software package (SPSS, ver.16) to analyze the statistical significance.

RESULTS

3.1. Total phenolic content

The total phenolic content of three different solvent extracts (methanol, acetone and hexane) of microalgae *Navicula clavata*, *Chlorella marina* and *Dunaliella salina* were determined and the results are presented in Fig.1. The highest phenolic content was found to be higher in methanol and acetone extract of 0.78 ± 0.032 and 0.63 ± 0.031 mg/g gallic acid equivalent were observed in *C. marina*, followed by the hexane extract of *N. clavata* (0.34 ± 0.028 mg/g gallic acid equivalent). The lowest phenolic content was recorded in hexane extracts of *D. salina* (0.17 ± 0.024mg /g gallic acid equivalent). Variance in total phenolic content between the tested species and solvent extract (P>0.05) was statistically differed.

Fig. 1: Total phenolic content of the microalgae



3.2. Total antioxidant activity

Total antioxidant activity of different solvent extracts of three marine diatoms was showed in Fig.2. The highest activity of 1.03 ± 0.02 and 0.96 ± 0.031 mg/g ascorbic acid equivalent were observed in methanol extract of *C. marina* and *D. salina*, followed by the acetone and hexane extract of (0.88 ± 0.02 and 0.72 ± 0.037 mg/g ascorbic acid equivalent) were observed in *D. salina.* The lowest activity was noticed from the hexane extract of *N. clavata* (0.50 ± 0.03 mg/g ascorbic acid equivalent). Changes in total antioxidant activity between the tested species and solvent extract (P<0.05) was statistically differed.

3.3. DPPH radical scavenging assay

DPPH radical scavenging activities (%) of different extracts of three microalgae are presented in Fig.3. All these microalgae extracts possessed the ability to scavenging DPPH at various degrees, with the methanol extract of *C. marina* (23.08%) and *D. salina* (17.66%) was found to be the most potent scavenger followed by the acetone extract of *C. marina* (20.54%) and *D. salina* (12.62%). The acetone extract of *N. clavata* extract showed the minimum DPPH radical scavenging activity at 9.1%. The scavenging effect of standard on the DPPH radical decreased in the order of BHT > gallic acid, which was 15.25% and 12.51%. Variance in DPPH activity between the tested species (P<0.05) and solvent extract (P<0.01) was statistically differed.

Fig. 2: Total antioxidant activity of the microalgae

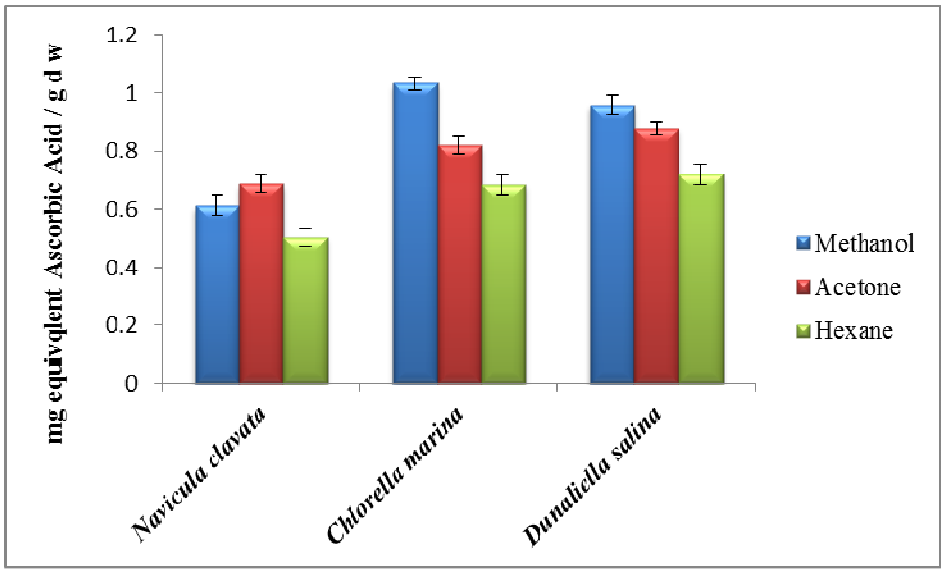
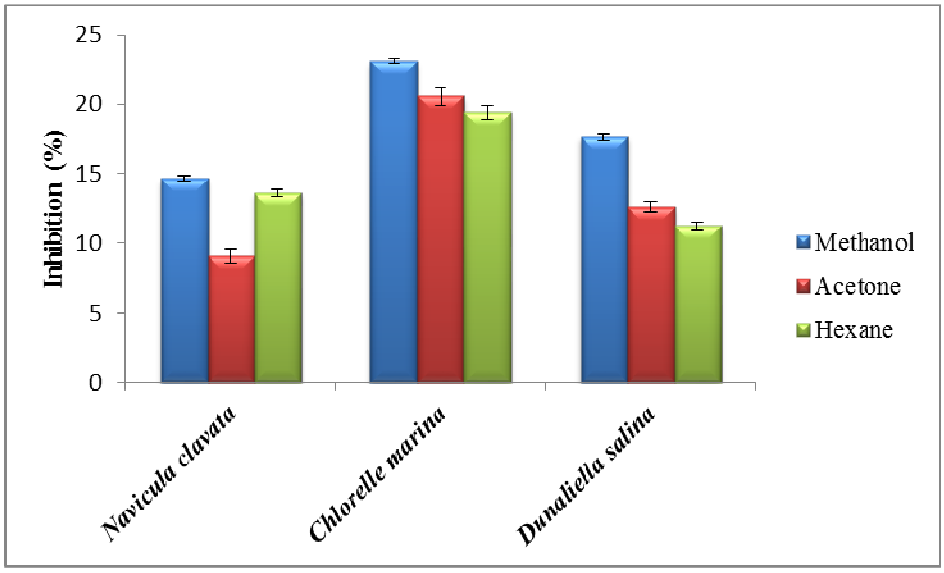


Fig. 3: DPPH radical scavenging assay of the microalgae



3.4. Hydrogen peroxide radical scavenging assay

Hydrogen peroxide radical scavenging activities of different solvent extracts of three marine diatoms were shown in Fig. 4. Hydrogen peroxide Radical Scavenging Activity (%) was found to be maximum in methanol extract (61.33%) and hexane extract (55.3%) of *C. marina*, whereas minimum in acetone extract (15.54%) of *N. clavata*. Variance in hydrogen peroxide activity between the tested species ($P>0.05$) and solvent extract ($P<0.001$) was statistically differed.

3.5. Nitric oxide radical scavenging assay

Nitric oxide radical scavenging assay of different solvent extracts of three marine diatoms were shown in Fig. 5. Results indicated that the highest scavenging activity was observed in methanol and hexane extract of *C. marina* (25.76% and 21.73%) respectively whereas minimum in acetone extract of *N. clavate* (6.4%). Variance in nitricoxide scavenging assay between the tested species ($P<0.01$) and solvent extract ($P<0.001$) was statistically differed.

Fig. 4: Hydrogen peroxide scavenging assay of the microalgae

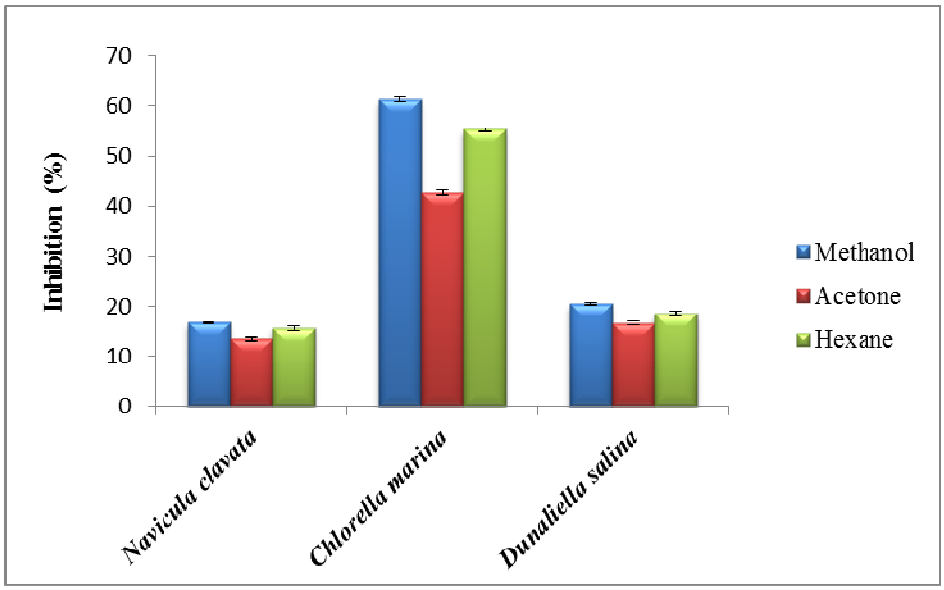
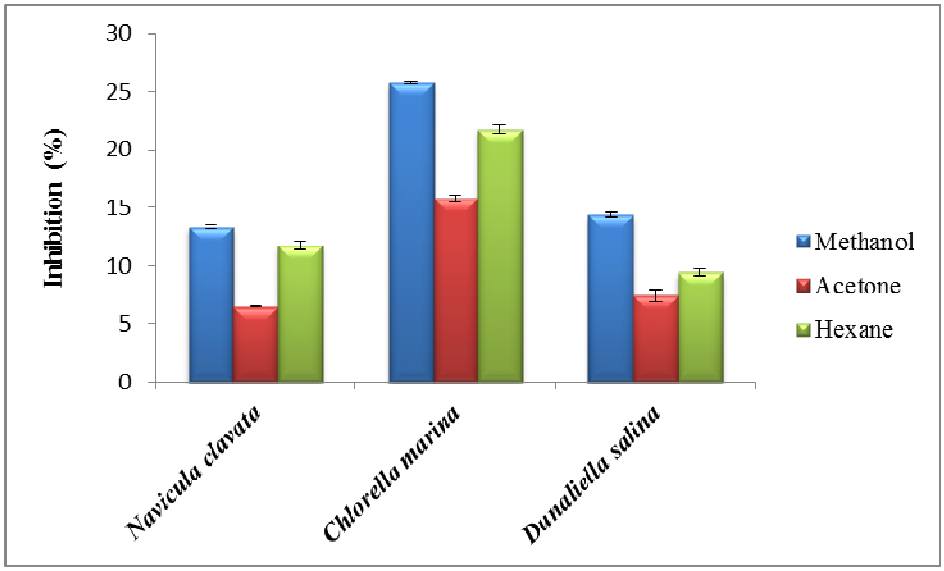


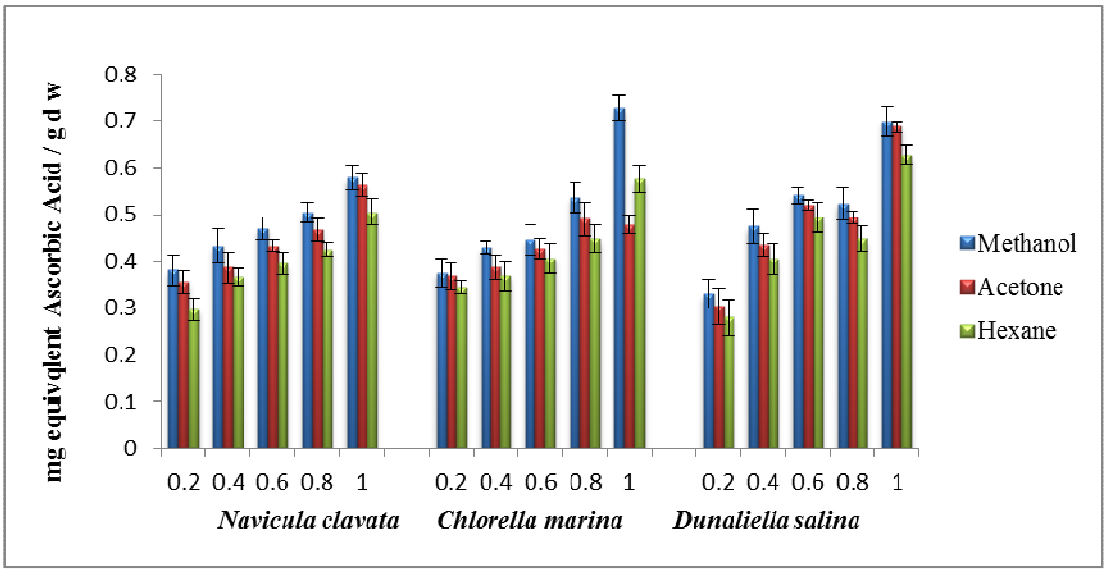
Fig. 5: Nitric oxide radical scavenging assay of the microalgae



3.6. Ferric reducing antioxidant power

The reducing activity of the microalgae extracts as determined by reducing power assay varied as seen in Fig.6. The antioxidant activity of the methanol, acetone and hexane microalgae extract determined by reducing power assay was as follows: The reducing power were found to be higher in methanolic extract of *C. marina* (0.73 ± 0.026 mg/g ascorbic acid equivalent) at 1 ml concentration and *D. salina* (0.70 ± 0.031 mg/g ascorbic acid equivalent) at1 ml followed by acetone extract of *D. salina* (0.69 ± 0.012 mg /g ascorbic acid equivalent) at 1ml. The lowest reducing power was recorded in lowest concentration of hexane extracts of *D. salina* (0.27 ± 0.03 mg/g ascorbic acid equivalent) at 0.2 ml concentration of micro algal extracts. Differences in ferric reducing antioxidant power between the tested species was statistically non-significant (P>0.05).

Fig. 6: Ferric reducing antioxidant power assay of the microalgae



DISCUSSION

The highest phenolic content was observed in methanolic extract of *C. marina* (0.78 ± 0.032 mg/g gallic acid equivalent). This result is more or less similar with the earlier investigation Uma *et al*., 2011 and it clearly explain that the methanolic extract found to be having higher phenolic content in *D. olivaceous* and flavonoid content was high in acetone extract of *C. humicola*. According to Manivannan *et al*., 2012 methanol extract of *Chlorella marina* exhibited higher activity which as followed by diethyl ether and hexane extract. This may be due to the differences in the polarity of the solvents used.

The highest antioxidant activity of *C. marina* methanol extract is (1.03 ± 0.02 mg/g). Similarly, Sivakumar and Rajagopal, 2011 reported that the highest antioxidant activity was observed in methanol extract from eight green algal species. Uma *et al*., 2011 observed that the methanolic extracts displayed greater potential in all antioxidant assays when compared to ethanolic and acetone extract of green microalgae *Desmococcus olivaceous* and *Chlorococcum humicola*.

In this study, methanol extract of *C. marina* (23.08%) and *D. salina* (17.66%) and acetone extract of *C. marina* (20.54%) was found to be the most potent scavenger. The hexane extract of *N. clavata* showed the minimum DPPH radical scavenging activity at 9.1% respectively. Similarly, both methanolic and acetone extracts of *D. olivaceous* and *C. humicola* showed a significant dose dependent reduction of DPPH radicals, Uma *et al*., 2011. Lee *et al*., 2010 reported that the 80% methanol extract and organic solvent fractions of both algae showed notable activities indicating the higher efficacy for scavenging of free radicals. The implications are important as radical scavengers may protect cell tissues from free radicals, thereby preventing diseases such as cancer.

Hydrogen peroxide Radical Scavenging Activity (%) was found to be maximum in methanol (61.33%) and hexane extract (55.3%) of *C. marina*, whereas minimum in acetone extract (15.54%) of *N. clavata*. According to Uma *et al*., 2011 methanolic extracts of *D. olivaceous* exhibited 39% scavenging activity and the acetone extracts of *C. humicola* exhibited 15% scavenging activity. In this study, highest nitric oxide scavenging activity was observed in methanol and hexane extract of *C. marina* (25.76% and 21.73%) respectively. This is lined with the finding of Lee *et al*., 2010, 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.

The reducing power was found to be higher in methanolic extract of *C. marina* (0.73 ± 0.026 mg/g). Similarly, Kuda *et al*., 2005 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*., 2005 explained that these polar compounds can be extracted to a higher extent, in this way increasing the yield of extract.

In the present study, the methanolic extracts showed antioxidant activity in the order of *C. marina* >*D. salina* > *N. clavata.* The antioxidant mechanisms of microalgae 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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