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RESEARCH PAPER

In Vitro Antioxidant and Free Radical Scavenging Activity and Chemometric Approach to Reveal Their Variability in Green Macroalgae from South Andaman Coast of India

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Abstract

Total phenol, flavonoid, carotenoid and antioxidant activity of four green macroalgae (*Halimeda tuna, Halimeda macroloba*, *Enteromorpha* sp. and *Acetabularia acetabulum*) collected from South Andaman coast were analysed in this study. The results revealed that methanol extract of *Enteromorpha* sp. showed maximum phenolic, flavonoid and carotenoid content of 5.72±0.13 mg GAE/g, 21.15±1.05 mg RE/g and 47.78±0.46 µg/g respectively. All the analysed macroalgae species exhibited antioxidant activities in dose dependent manner among which *Enteromorpha* sp. demonstrated greater antioxidant potential. *A. acetabulum* exhibited greater ABTS (2, 2-azinobiz-3-ethylbenthiazoline-6-sulfonic acid) radical scavenging ability (92%) as evident by its low IC_{50} (6.30 mg/ml) in comparison to other species. Based on factor analysis, up to two principal components were chosen to explain variability in 10 independent phytochemicals. The antioxidant potential reveals their potential for future applications in medicine, dietary supplements as well as the natural source of immunostimulants.

Keywords: Marine macroalgae, total phenol, flavonoids, carotenoids, antioxidant activity.

Introduction

Andaman and Nicobar Islands are very rich in diversity and distribution of marine macroalgae or seaweed flora (Karthick, Mohanraju, Ramesh, & Murthy, 2013). Out of 105 species of algae recorded, dominant macroalgae such as Rhodophyceae (39.38%), Chlorophyceae (35.71%) and Phaeophyceae (25%) (Mohanraju & Tanushree, 2012) were widely distributed in the Islands. Macroalgae are considered to be beneficial to human health as they contain various organic and inorganic compounds (Kuda, Taniguchi, Makoto, & Arakiz, 2002) and also an excellent source of bioactive compounds such as carotenoids, dietary fibres, proteins, essential fatty acids, vitamins and minerals. Macroalgae extracts are considered as an important source of plant nutrition for sustainable agricultural production. The fact that even under harsh environmental conditions, macroalgae proliferate without suffering any phytodynamic damage suggesting their antioxidant potential to quench free radicals generated in unfavorable climate (Matsukawa *et al*., 1997). Macroalgae, like all other plants possess phenolic compounds that act as antioxidant by chelating metal ions preventing the formation of free radicals and improving the antioxidant endogenous systems. It has been reported that algae generally exhibit higher antioxidant activity due to the presence of non-enzymatic antioxidant compounds (Cahyana, Shuto, & Kinoshita, 1992). Various reports consider macroalgae to be a rich source of antioxidants and their extracts have been studied to produce a variety of compounds and some of them have even reported to possess bioactivity of potential medicinal value (Cahyana *et al*., 1992; Konig, Wright, Sticher, Anghofer, & Pezutto, 1994; Tutour *et al*., 1998). This ability of natural antioxidants to react rapidly with the free radicals and retard or alleviate the extent of oxidative damage has drawn attention of various researchers towards the marine resources for development of new drugs and healthy food.

Very recently, the pharmaceutical and agri-food industries have been at the origin of a great expansion in the demand for macroalgae due to their significant applications as ingredients in functional foods and richness in antioxidant ingredients. It was reported that macroalgae are rich source of bioactive compounds, such as terpenoids, phlorotanins, fucoidans, sterols and glycolipids, and that the extracts or isolated pure components from macroalgae posses a wide range of pharmacological properties such as anticancer, antibacterial, antifungal, anti-viral, anti-inflammatory, anticoagulant, antioxidant,

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hypoglycaemic, hypolipidemic, antimelanogenic, anti-bone loss, hepatoprotective and neuroprotective activities (Chakraborty & Paulraj, 2010).

The importance of macroalgae as a source of novel bioactive substances is growing rapidly and researchers revealed that marine algal originated compounds exhibit various biological activities (Wijesekara & Kim, 2010). The antioxidant activities of various macroalgae have been reported by various research groups (Ganesan, Chandini, & Bhaskaran, 2008; Ismail & Hong, 2002; Matsukawa *et al*., 1997; Megha & Sabale, 2013; Boonchum *et al*., 2011; Foon, Ai, Kuppusamy, Yusoff, & Govindan, 2013; Seenivasan, Rekha, Indu, & Geetha, 2013; Farasat, Nejad, Seyed, & Namjooyan, 2014). Therefore, various natural pigments isolated from marine algae have attracted much attention in the field of food, cosmetic and pharmacology. Free radicals are often generated as by-products of biological reactions or from exogenous factors. Reactive oxygen species, generated in organisms as an integral part of metabolism are highly reactive and can cause cellular dysfunction and cytotoxicity. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented. However, to the best of our knowledge there are no reports on the antioxidant activity of green macroalgae from Andaman Islands. Thus, the aim of this study is to estimate the total phenol, flavonoid, carotenoid content and to assess the antioxidant activity of the four selected green macroalgae namely *Halimeda tuna* (J. Ellis & Solander) J. V. Lamouroux., *Halimeda macroloba* Decaisne, *Enteromorpha* sp. *and Acetabularia acetabulum* (Linnaeus) P. C. Silva.

Chemometrics as the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods. Principal Component Analysis (PCA) is a class of chemometrics, mostly used as a tool in exploratory data analysis and for making predictive models. The central idea of PCA is to reduce the dimensionality of a data set consisting of a large number of interrelated variables, while keeping maximum variation in the data set. It can be done by eigen value decomposition of a data covariance (or correlation) matrix or singular value decomposition of a data matrix, usually after mean centering (and normalizing or using Z-scores) the data matrix for each attribute (Abdi & Williams, 2010). Factor analysis typically incorporates more domain specific assumptions about the underlying structure and solves eigenvectors of a slightly different matrix. In the language of factor analysis, the proportion of variance of a particular item that is due to common factors (shared with other items) is called communality. Therefore, an additional task has to be faced when applying this model to estimate the communalities for each variable, that is, the proportion of variance that each item has in common with other items.

Materials and Methods

Collection and Preparation of Macroalgae Extract

Four species of green macroalgae such as *H. tuna, H. macroloba, Enteromorpha* sp. and *A. acetabulum* were collected along the South Andaman coast at three different locations during low tide and GPS coordinates for each sampling station was recorded (Table 1). The samples were thoroughly washed with seawater to remove epiphytes, sand and pebbles and immediately transported to the laboratory in ice box, where they were washed with freshwater to remove salt. The samples were shade dried at room temperature for 3-4 days. The dried samples were then homogenised with pestle and mortar and stored at 4ºC for further analysis. One gram of dried sample was extracted with 50 ml of 80% methanol twice at room temperature followed by centrifugation at 4500 g for 10 minutes. The supernatant was pooled and filtered through Whattman No. 1 filter paper into fresh tubes and stored at 4ºC for further analysis. For carotenoid estimation, 80% of acetone was used.

Total Phenolic Content

Estimation of total phenolic content (TPC) of the sample was done by Folin-Ciocalteau (FC) assay described by Singleton and Rossi (1985) with minor modification. 200 µl of extracted sample was mixed with 200 µl FC reagent (0.5 N) followed by 1.6 ml 7.5% Na_2 CO_3 and incubated for 2 hours in dark at room temperature. The absorbance was measured at 765 nm using UV-visible spectrophotometer. Gallic acid was used as standard and the phenolic contents were expressed as Gallic Acid Equivalents (mg GAE/g) of macroalgae extract.

Table 1. Sample collection details

Sl No.	Name of the macroalgae sample	Location	GPS Coordinates
1.	*Halimeda tuna* (J. Ellis & Solander) J. V. Lamouroux	Old Wandoor Beach	N 11˚34’01.2”; E 92˚37’05.1”
2.	*Halimeda macroloba* Decaisne	Old Wandoor Beach	N 11˚34’01.2”; E 92˚37’05.1”
3.	*Enteromorpha sp.*	Carbyn’s Cove Beach & Marina Park	N 11˚38’35”; E 92˚44’54” N 11˚40’15.6”; E 92˚44’15.7”
4.	*Acetabularia acetabulum* (Linnaeus) P. C. Silva	Marina Park	N 11˚40’15.6”; E 92˚44’15.7”

Total Flavonoid Content

Flavonoid content of the macroalgae extract was determined by spectrophotometric method of Zishen, Mengcheng, and Jianming (1999) with slight modification. 0.5 ml of sample was mixed with 0.3 ml 15% sodium nitrite, 0.6 ml 10% ammonium chloride hexahydrate and 3 ml of 1N sodium hydroxide after an interval of 5 minutes. Absorbance was immediately measured at 510 nm. Flavonoid content was expressed as Rutin equivalents of extract (mg RE/g).

Total Carotenoid Content

Carotenoid content of the acetone extracted samples were determined by spectrophotometric method of Ranganna (1997).

DPPH (1, 1-Diphenyl-2-Picrylhydrazyl) Radical Scavenging Activity

The scavenging effects of the macroalgae extracts were determined according to the method of Yen and Chen (1995) with minor modifications. 200 µl aliquot of each extracts were mixed with 100 µl of 0.16 mM DPPH methanolic solution. The mixture was incubated in dark for 30 minutes and then the absorbance was recorded at 517 nm in spectrophotometer. The half-maximal inhibitory concentration (IC50) was calculated by the linear regression analysis and expressed as mean of determinations. Ascorbic acid was used as control.

% inhibition of DPPH radical (scavenging activity) = $(A_{control} - A_{sample}/ A_{control})$ x 100

where $A_{control}$ is the absorbance of the control (without extract) and A_{sample} is the absorbance in the presence of the extract/standard.

The IC50 (concentration for 50% inhibition) values of all the methods were calculated by using linear regression analysis. All the experiments were repeated thrice. Higher the IC50 value, lower is the scavenging activity.

ABTS (2, 2-Azinobiz-3-Ethylbenthiazoline-6-Sulfonic Acid) Radical Cation Scavenging Activity

ABTS radical cation scavenging activity of the extract was determined by Re *et al*. (1999) with minor modification. ABTS was prepared by mixing 20 mM ABTS solution with 70 mM potassium peroxodisulphate, 24 hours prior to use. 100 µl aliquot of the extracts were made up to 10 ml with distilled water and mixed with 3.9 ml of ABTS reagent and absorbance of these solutions was measured at 734 nm after 5 minutes. Trolox was used as standard.

% inhibition of ABTS radical=$(A_{control}-A_{sample}/A_{control})$ x100

where $A_{control}$ is the absorbance of the control (without extract) and A_{sample} is the absorbance in the presence of the extract/ standard.

FRAP (Ferric Reducing Antioxidant Power)

The ferric reducing activity of the samples was determined by the method of Benzie and Strain (1999) with minor modification. Sample in different aliquot, were made up to 1 ml with distilled water and mixed with 900 µl reagent solution [10 mM 2,4,6-trispyridyl trizine, 20 mM ferric chloride hexahydrate and acetate buffer (0.2 M acetic acid and 0.02 M of sodium acetate at pH- 3.6)]. Absorbance was measured at 595 nm and ferric sulphate was used as standard. The radical scavenging activity was measured using similar equations as DPPH and ABTS methods.

Total Antioxidant Activity (TAA)

Total antioxidant activity of methanolic extracts was quantified by Prieto, Pineda, and Aguilar (1999) method with slight modification. 0.2 mg/ml of samples in different aliquots were mixed with 1 ml each of the three reagent solutions (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The samples were incubated at 95° C for 90 minutes in water bath. Absorbance was measured at 695 nm. Total antioxidant activity was expressed as mg of ascorbic acid per gram of extract.

Hydrogen Peroxide (H_2O_2) Radical Scavenging Activity

The H_2O_2 scavenging activity was determined by Ruch, Cheng, and Klaunig (1989) method with minor modification. 0.6 ml of 40 mM H_2O_2 solution prepared in phosphate buffer (pH-7.4) was added to the extracts and ascorbic acid (control) and the absorbance of H_2O_2 were determined at 230 nm.

% inhibition of H_2O_2 radical=$(A_{control}-A_{sample}/A_{control})$ x100

where $A_{control}$ is the absorbance of the control (without extract) and A_{sample} is the absorbance in the presence of the extract.

Metal Chelating Agent (MCA)

Metal chelating activity of macroalgae samples were determined by Decker and Welch (1990) method. 0.1 ml of samples was mixed with reagent (0.1 ml of 2 mM ferric chloride and 0.2 ml of 5 mM of ferrozine). The mixture was stirred fastly and kept for incubation at room temperature for 10 minutes. Absorbance was measured at 562 nm. EDTA was used as standard.

$$\% \text{ Metal chelating activity} = (A_{control} - A_{sample} / A_{control}) \times 100$$

where, $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the extract/standard.

Reducing Power

Reducing power of the methanol extract was determined by Oyaizu (1986) method with minor modification. Different concentration of sample was mixed with 2.5 ml of phosphate buffer (0.2 M, 6.6 pH) and 2.5 ml of potassium ferric cyanide (1%) and incubated for 30 minutes at 50°C. 2.5 ml of Trichloroacetic acid (10%) was added after incubation and centrifuged at 3000 rpm for 10 minutes. 2.5 ml solution from the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml ferric chloride (0.1%). Absorbance was measured at 700 nm. Butylated hydroxytoluene (BHT) methanolic solution was used as standard.

Statistical Analysis

Data were expressed as mean ± standard error in three replicates. All the statistical analysis was carried out using SPSS 17.0. To determine whether there were any difference among the means, one way analysis (ANOVA) and the Duncan's multiple range test were applied to the result and P values<0.05 regarded to be significant. The correlation and factor analysis was performed using SAS (Version 9.3, SAS Institute Inc., Carry, NC). For a visualisation of the data discrimination, PCA plots mapped variables (10 phytochemicals) and samples (n=4) through loadings and scores in dimensional spaces determined by PCs with eigen values >1.0 based on a Kaiser's rule (Kaiser, 1960).

Results and Discussion

Total Phenol, Flavonoid and Carotenoid Content

The total phenol content of the selected four green macroalgae is shown in Table 2. Total phenolic content varied between 2.506±0.16 (*H. tuna*) and 5.72±0.13 mg GAE/g (*Enteromorpha* sp*)* with the coefficient of variation (C.V) of 37.97%. Significant difference (P<0.05) was observed in all species. In this study, the highest phenolic content is 10 times lower (49.75 mg GAE/g) as per report by Ghannam, Cox, and Gupta (2010). The reason might be the difference in growth period, geographic location, storage type, genetic diversity, etc., solvent used (methanol) may not be efficient to extract the phenolic compounds or due to the loss of some phenolic compounds during sample preparation from the tissue matrix causing low phenolic content. Similar trend was found for flavonoid content which varied from 6.3±0.1 to 21.15±1.05 mg RE/g with the C.V of 64.01%. The highest content of flavonoids may be due to high content of total phenolics in *Enteromorpha* sp. Flavonoid content ranging from 7.66 to 42.5 mg QE/g was recorded in different species of brown algae by Ghannam *et al.* (2010). The flavonoid content of the two *Halimeda* sp. collected from the same location was significantly different. Similar results was recorded by Farasat *et al.* (2014) in *Ulva* sp. Carotenoid content was also found to be highest in *Enteromorpha* sp. (47.78±0.46 µg /g) and lowest in *H. macroloba* (36.00±1.00). On contrary, Vimala and Poonghuzhali (2015) reported only 1.38 µg/g of carotenoid content in *Ulva reticulata* Forsskal. Similar results were observed in *Chaetomorpha* sp., (6.743±0.1 to 18.17±2.2) by Farasat, Nejad, Seyed, and Namjooyan (2013) and *Codium adharens* C. Agardh (38.5 µg/g) by Seenivasan *et al.* (2013). The high content of these phytochemicals could explain its high radical scavenging activity.

In-Vitro Free Radical-Scavenging Activity

The basic structure of the phenols and other structural factors play a fundamental role in the mechanism by which these compounds are able to scavenge free radicals. As per the earlier report of Lata, Trampczynska, and Paczesna (2009), it is difficult to compare the phenolic content of macroalgae, as many variations can be principally caused by different growth period, geographic location, storage type, genetic diversity and many other factors. Scavenging activity is attributed to the presence of phenolic hydroxyl groups, particularly in

Table 2. Phytochemical and antioxidant activity of green macroalgae extracts

Macroalgae sample	*H. tuna*	*H. macroloba*	*Enteromorpha sp*	*A. acetabulum*	C.V
Phenol (mg GAE g^{-1})	2.506±0.16^{d}	3.445±0.155^{b}	5.72±0.13^{a}	3.116±0.422^{c}	37.976
Flavonoid (mg RE g^{-1})	6.3±0.1^{c}	8.8±0.1^{b}	21.15±1.05^{a}	7.15±0.25^{bc}	64.01
Carotenoid (µg g^{-1})	41.02±0.21^{b}	36.00±1.00^{c}	47.78±0.46^{a}	36.02±0.42^{c}	13.868
DPPH (mg g^{-1})	20.78±0.32^{c}	19.72±0.27^{c}	27.1±0.35^{a}	25.34±0.20^{b}	15.267
ABTS (mg g^{-1})	4±0.011^{c}	5.875±0.16^{c}	10.43±23^{a}	12.375±0.18^{a}	47.639
FRAP (µg g^{-1})	152.89±2.53^{b}	154.74±1.28^{b}	164.3±1.04^{a}	156.21±0.78^{b}	3.203
TAA (mg g^{-1})	5.23±0.03^{a}	4.11±0.004^{a}	5.39±0.04^{a}	2.06±0.73^{b}	36.559
H_2O_2 (mg g^{-1})	132.73±2.38^{b}	108.28±2.94^{c}	153.84±3.49^{a}	84.16±1.28^{d}	25.185
MCA (mg g^{-1})	12.68±0.12^{c}	10.62±0.49^{d}	18.25±0.30^{a}	14.86±0.32^{b}	23.132
RP (mg g^{-1})	6.405±0.01^{d}	8.745±0.17^{c}	12.87±0.27^{b}	13.74±0.05^{a}	33.15

the 3′OH and 4′OH of the three-carbon chain.

DPPH Radical Scavenging Activity

Antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colorless/ bleached product (i.e., 2, 2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in decrease in absorbance at 517 nm. The DPPH activity of methanolic extract varied from 19.72±0.27 to 27.1±0.35 mg/g with the C.V of 15.26% (Table 2). The DPPH activity was maximum for *Enteromorpha* sp. which is also significantly different ($P<0.05$) from the other species. There was a steady increase in inhibition of radicals with concomitant increase in concentration of the extract establishing dose dependence of the extract in scavenging DPPH radicals. The inhibition effects of extract on DPPH radicals increased with increasing concentration (0.2-2 mg/ml), being highest in *H. macroloba* (70.1%) and lowest in *H. tuna* (68.8 %) at 2 mg/ml (Figure 1 (a)). The level of inhibition observed in this present study is higher than the inhibition reported by Seenivasan *et al*. (2013) for *Sargassum wightii* Greville ex J. Agardh which is having high scavenging activity to curb against free radical. Foon *et al*. (2013) reported 46.07 % and 56.03% of DPPH activity in *Padina* sp. and *Eucheuma cottonii* Weber-van Bosse at 2 mg/ml. The variation in DPPH radical scavenging activity may be due to different extraction methods and solvents used in various studies which may influence antioxidant ranges. Our data is in agreement with the report for green macroalgae namely *Chaetomorpha antennina* (Bory) Kutzing and *Enteromorpha intestinalis* (Linnaeus) Nees by Megha and Sabale (2013) and red macroalgae namely *Gracilaria corticata* (J. Agardh) J. Agardh and *Gelidiella acerosa* (Forsskal) Feldmann & Hamel by Ismail and Hong (2002). The interaction between antioxidant and DPPH is shown to be dependent on the structural conformation of antioxidant. This structural feature of the DPPH test may enable the assay to differentiate phenolic compounds among several compounds

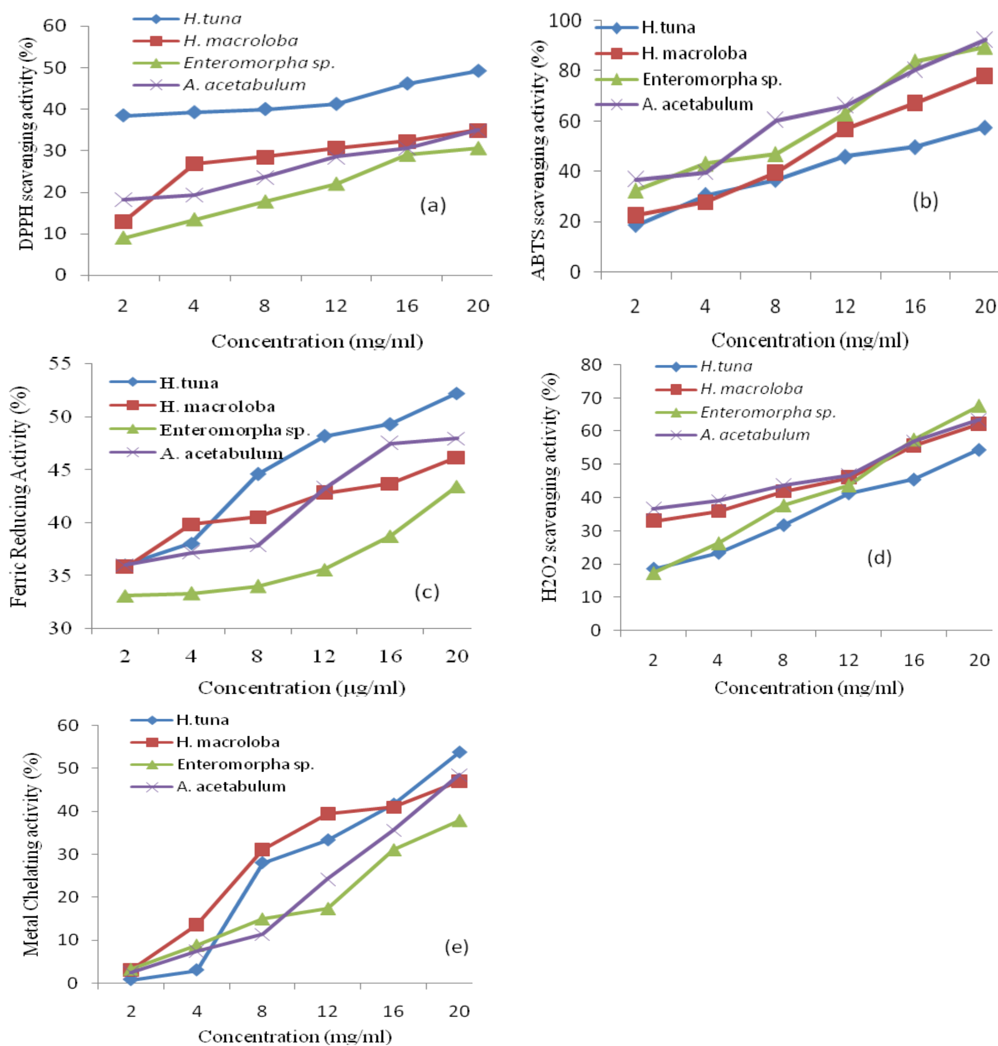

Figure 1. Radical scavenging activities of different green macroalgae.

usually present in natural system capable of expressing antioxidant activity. These natural compounds have shown higher scavenging ability indicating their potential free radical inhibition. The IC50 value was found to be highest in *H. tuna* indicating lowest potential to scavenge DPPH radical. However, there was non-significant difference between other three species namely *Halimeda macroloba*, *Enteromorpha* sp. and *Acetabularia acetabulum* suggesting equal potential in quenching DPPH radical.

ABTS Assay

ABTS activity was lowest in *H. tuna* (4 mg/g) and highest in *A. acetabulum* (12.37 mg/g) with the C.V of 47.64%. The lowest activity of *H. tuna* may be due to low level of phenol as supported by Farasat *et al*. (2014). But, the highest activity did not corroborate with the highest phenolic content. This may be due to steric obstruction and absence of free 3-OH in the flavonoid structure (mainly flavones) where the absence of hydroxylation at the 3-position on the C-ring rendering electron delocalization difficult so that the end products of the chain reaction was unstable non-resonating structure (Heim, Tagliaferro, & Bobilya, 2002). Like the result obtained in DPPH activity, the scavenging activity also increased with the increase in concentration of extract (2-20 mg/ml), being highest in *A. acetabulum* (92.61%) and lowest in *H. tuna* (57.59%) at 20 mg/ml as shown in figure 1(b) The scavenging activity increased from 33.1 to 53.5% (Figure 1 (c)) with the increase in concentration (2-20µg/ml) but it was lower as compared to DPPH and ABTS scavenging activity. Seenivasan *et al*. (2013) and Boonchum *et al*. (2011) reported only 15.2% and 17.55% for *Chaetomorpha linum* (O. F. Muller) Kutzing and *H. macroloba* respectively. IC50 value indicated that *A. acetabulum* (lowest IC50=6.3 mg/ml) is the highest scavenger of ABTS radical while *H. tuna* (IC50=16.1 mg/ml) is the weakest scavenger. When compared with DPPH scavenging activity, the IC50 value is high for all the macroalgae. The reason may be due to the fact that ABTS assay is based on electron transfer only while DPPH assay is based on both hydrogen and electron transfer. So the lower concentration of extract was sufficient to nullify free radical mediated chain reactions in this assay.

FRAP Assay

FRAP method is based on the comparison of total amount of antioxidant with the reducing capacity of the sample. The highest activity was observed in *Enteromorpha* sp. (164.3 µg/g) and the lowest activity was recorded in *H. tuna* (152.89±2.53) as shown in Table 3 with the C.V of 3.2%. The FRAP value of *Enteromorpha* sp. was not significantly different ($P<0.05$) from other macroalgae. Fe^{2+} ion chelating ability of the methanolic extracts of brown macroalgae *Turbinaria conoides* (J. Agardh) Kutzing and *T. ornata* (Turner) J. Agardh are 58.29±3.66 and 27.63±1.34 mg/g respectively. High FRAP values for brown macroalgae *Sargassum polycystum* C. Agardh and *Padina* sp. have been shown by Matanjun, Muhamed, Mustapha, and Muhammad (2008). The FRAP scavenging effect of the tested extracts decreased in the order of *A. acetabulum*>*Enteromorpha* sp.>*H. macroloba*>*H. tuna* shown in figure 1(c). Budhiyanti, Raharjo, Marseno, and Iwan (2012) have reported a higher ferrous ion chelating ability in *Sargassum hystrix* J. Agardh. An extract with higher binding ability would prevent or inhibit reaction such as fenton type reaction which generates reactive hydroxyl radicals.

TAA

A significant difference ($P<0.05$) of total antioxidant activity (TAA) between four species of green macroalgae was observed. The maximum antioxidant activity was estimated in *Enteromorpha* sp. (5.39 mg/g) and the minimum activity was recorded in *A. acetabulum* (2.06 mg/g) with the C.V of 36.56%. Even though *H. tuna* possessed lowest phenolic content, it had the second lowest value of TAA. The result may be due to some other non-phenolic compounds having antioxidant activities as reported by Souza *et al*. (2012). Similar result was

Table 3. Pearson's correlation coefficient between phytochemicals in green macroalgae

	Phenol	Flavonoids	Carotenoids	DPPH	ABTS	FRAP	MCA	H_2O_2	RP
Phenol	1.000								
Flavonoids	0.975**	1.000							
Carotenoids	0.672	0.798	1.000						
DPPH	0.965**	0.942**	0.556	1.000					
ABTS	0.952**	0.891	0.439	0.985**	1.000				
FRAP	0.921**	0.972**	0.907**	0.838	0.770	1.000			
MCA	0.917**	0.884	0.726	0.783	0.783	0.905**	1.000		
H_2O_2	0.975**	0.968**	0.632	0.995***	0.967**	0.884	0.809	1.000	
RP	0.959**	0.933**	0.536	0.999***	0.986**	0.824	0.769	0.992***	1.000

, * Significant at 5% and 1% respectively.

also reported by Ganesan *et al*. (2008), Seenivasan *et al*. (2013) and Megha and Sabale (2013).

Hydrogen Peroxide (H_2O_2) Radical Scavenging Assay

Many species of macroalgae possess scavenging ability of hydrogen. H_2O_2 activity was ranged between 153.84 mg/g (*Enteromorpha* sp) and 84.16 mg/g EDTA (*A. acetabulum*). The dose dependent scavenging activity was found highest in *Enteromorpha* sp (67.66%) and lowest in *H. tuna* (54.49%) shown in figure 1(d). Here, the highest phenolic content did not correspond to the highest H_2O_2 activity. The reason may not only be due to phenolic compounds, but also other hydrophilic compounds, for example peptides, fucoidan and Maillard reaction products (Kuda & Ikemori, 2009). The IC50 value was observed to be highest in *A. acetabulum* (1.19 mg/ml) and lowest in *H. tuna* (1.189 mg/ml) reflecting the highest and lowest value of phenolic content respectively.

Metal Chelating Activity (MCA)

In the presence of chelating agents, the complex formation (Ferrozine-Fe^{2+}) is disrupted with the result that the red colour of the complex is decreased. These results indicated that the significant ($P<0.05$) variation of chelating activity, being lowest for *H. macroloba* (10.62 mg/g) and highest for *Enteromorpha* sp. (18.25 mg/g) with C.V of 23.12%. Boonchum *et al*. (2011) reported that hydrophobic phenolic compounds did not contain antioxidant potential. In this present study, the lowest MCA activity observed might be due to the presence of more hydrophobic phenolic compounds in *H. macrobola* and also due to the extraction effect of solvent (80% methanol) which may not be sufficient for the flavonoids responsible for the chelating effect. With the increase in concentration (2-20 mg/ml), the chelating effect was increased, being maximum in *H. tuna* (53.78%) and minimum in *Enteromorpha* sp. (37.87%) shown in figure 1(e). The value of IC50 was highest in *Enteromorpha* sp. (20.0 mg/ml) and lowest in *A. acetabulum* (17.92 mg/ml). In contrary, the lowest IC50 value of *Enteromorpha* sp. did not corroborate with the highest chelating activity indicating slow rate of inhibition. It was also reported that the chelating agents that form S-bonds with metal, are effective as secondary antioxidants. This may be explained for the low value of *Enteromorpha* sp. even it has high phenolic and flavonoid content. In this case, *A. acetabulum* might have bound with metal through S-bond by reducing the redox potential of the reactions, thereby stabilizing the oxidized form of the metal ion. Again, metal binding capacities of dietary fibres are well known, e.g. the inhibitory effects on ferrous absorption of algal dietary fibres such as carrageenan might have caused the decrease of ferrous ion in the assay system (Chew, Lim, Omar, & Khoo, 2008).

Reducing Power (RP)

A. acetabulum exhibited highest reducing power of 13.74 mg/g while *H. tuna* followed the lowest value (6.405 mg/ml). The result is in line with the report by Boonchum *et al*. (2011) who reported the reducing capacity of 14.323 mg/ml in *H. macroloba*, which is higher than our study. The result may be due to different growth period, geographic location, storage type and genetic diversity as discussed earlier. With the increase in concentration (2-20 mg/ml), the reducing power increased in all the four macroalgae suggesting that an increase in concentration leads to higher electron donating ability. Thus, the reducing property indicated that the antioxidant compounds are electron donors and can reduce the oxidized intermediates of the lipid peroxidation process. The result obtained in the present study is in accordance with the earlier reports in brown macroalgae collected from different regions were found to be endowed with potential reducing abilities (Cho, Lee, Kang, Won, & You, 2011).

Correlation Analysis

The correlation coefficients among phenolics, flavonoid, carotenoids and total antioxidant capacity are listed in table 3. Phenolic content had a strong positive correlation with the flavonoid content and antioxidant capacity (r^2=0.917-0.975; $P<0.05$), which was in agreement with many of previous studies of Luo, Wang, Yu, Qu and Su (2010) and Zakaria, Ibrahim, Sulaiman and Supardy (2011) for marine algae. However, non-significant correlation was observed between phenolic and flavonoid content as well as other antioxidant values except FRAP. This shows the carotenoid had antioxidant activities in reducing Fe^{3+} radical. ABTS radical is rather stable, but it reacts energetically with an H-atom donor such as phenolics, being converted into a non-colored form of ABTS. Here, non-significant correlation between flavonoids with ABTS radicals may be due to the kind of flavonoids present in the macroalgae species. This phenomenon might be due to the strong intramolecular hydrogen bond between the 5-hydroxyl and the 4-one in the C ring in flavonoids present in all macroalgae which reduces the contribution of the carbonyl group to the electron delocation. The significant correlation between H_2O_2 and RP indicated that both are reducing in nature where the former is helpful in producing OH^- ion and the later gains electron in quenching free radicals. Similarly, the lower correlation between carotenoids with DPPH, ABTS, H_2O_2 and RP indicated that only the phenolic compounds are not involved in the antioxidant activity through this pathway but there might be some effects involving other active compounds.

Factor and Principal Component Analysis

Phytochemical data from all macroalgae were submitted to factor analysis to identify associations between variables and their ability to discriminate parameters among them. To know how much of the variance in the variables has been accounted for by the variables is based on communalities values. From Table 4, it is observed that over 99.9 % (maximum) of variability is accounted for ABTS value while 88.9 % (minimum) variability was accounted for phenolic content. Out of ten variables used, only two factor was selected, according to the criteria of an eigen value of>1.0. The two factors accounted for 95.97% of total variance (Table 4). After computation of a varimax rotation, it was found that factor 1 and 2, with an eigen value of 6.323 and 3.274, represents 63.23 % and 32.74 % of overall variance. Varimax rotation assumes that each factor has a small number of large loadings and a large number of zero (or small) loadings (Kim and Mueller, 1978). The loadings and factor scores describes the patterning of the data in the analysis. The higher the absolute value of the loading, the more the factor contributes to the variable.

The information present in the loadings can be displayed in two-dimensional loading plots, for instance, by plotting the loading of each variable on PC1 against its loading on PC2. It is often visualized using arrows or lines as in Figure 2 because the direction in which the variables lie on this plot as seen from the origin is used in the interpretation. Variables, for which the angle between the lines connecting them with the origin is small, are strongly correlated. This is because variables that have the same weights have the same effect on the scores: they give the same information about the samples and are, therefore, strongly positively correlated. When the direction from the origin is the same but the points representing the variables are not close to each other, the ratio between the weights on the two PCs is still the same for those variables, which means that they are also strongly correlated. Variables that are close to the origin have small weights and are not important. In our study, ABTS and RP are very strongly correlated because they fall on nearly the same line from the origin. TAA and H_2O_2 are also strongly correlated with each other but not with ABTS and RP. Based on the guidelines provided by Stevens (1992), an attribute was correlated to load heavily on a given component if the factor loading was greater than 0.72. PC1 describes 63.23% of the variance in the data set, and its loadings (Table 4) indicate that it has high contributions from RP (0.985), ABTS(0.971), DPPH (0.942), MCA (0.825) and FRAP (0.776). PC2 showed a high positive loading for H_2O_2 (0.994), TAA (0.929), carotenoids (0.926) and flavonoids (0.769). The factor axes act as a reference frame to determine where the data-variable vectors can be placed by giving factor loadings or coordinates – that is, the numerical labels on the axes represent factor loadings (Comrey & Lee, 1992). The correlation between a vector and one of the factors or with another variable (vector) can be determined as a function of the cosine angle between them. In Fig. 2, the PC1–PC2 plot is explanatory to 63.23% of the total variation in the data set, and is composed of all positive loadings except ABTS and RP as per by their relationship to one another and the x-axis. H_2O_2 scavenging activity had the highest loading (0.994) at the PC2 axis thereby denoting its significant contribution to the variance explained in PC2. From scree plot, the curve flattened after two components. Hence, up to two principal components were chosen to explain variability in 10 investigated phytochemicals.

Conclusion

The present study showed that four green macroalgae such *as H. tuna, H. macroloba, Enteromorpha* sp. and *A. acetabulum* possessed total phenol, total flavonoids and varying degrees of antioxidant activity in methanolic extracts. Among all

Table 4. Eigen analysis of the correlation matrix loadings of the significant principal components (PCs)

Variables	PC1	PC2
Total phenol	0.658	0.676
Flavonoids	0.594	0.769
Carotenoids	0.312	0.926
DPPH	0.942	0.232
ABTS	0.971	-0.238
FRAP	0.776	0.608
TAA	-0.371	0.929
H_2O_2	-	0.994
MCA	0.825	0.479
RP	0.985	-0.158
Eigen value	6.323	3.274
Variance explained (%)	63.232	32.745

Extraction method: Principal component analysis.
Rotation method: varimax with Kaiser normalisation (Eigen value >1).
*The most significant loadings are highlighted in boldface.

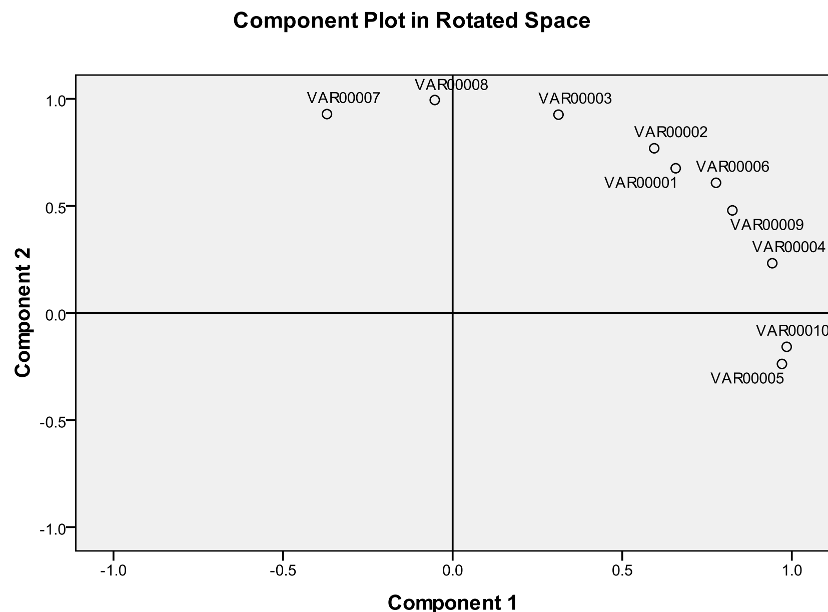

Figure 2. Loading plot of PC1–PC2 for four macroalgae [VAR001-Phenol, 002-Flavonoids, 003- Carotenoids, 004-DPPH, 005-ABTS, 006-FRAP, 007-TAA, 008-H2O2, 009-MCA, 010-RP].

the species, *Enteromorpha* sp. showed maximum phenol content, flavonoid content and highest antioxidant potential. Acetone extract of *Enteromorpha* sp. exhibited high carotenoid content when compared to other green macroalgae. Therefore, the green macroalgae extracts could provide valuable and promising source of natural antioxidants, which could decrease or retard the risk of cancer, inflammation, obesity, etc. and can replace synthetic antioxidants. The high content of these phytochemicals explain its high radical scavenging activity. The results of the present study confirmed that macroalgae are rich source of phytoconstituents which can be isolated and further screened for various biological activities like disease treatment for mankind. As there is a growing trend of disease and an increased requirement for medicine or drugs, this study recommends that macroalgae being an underutilized bioresource could be exploited in a sustainable manner for the welfare of mankind.

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