Antioxidant activities and phenolic contents of three red seaweeds (Division: Rhodophyta) harvested from the Gulf of Mannar of Peninsular India

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Abstract The antioxidant activities of methanol extract and its solvent fractions (*n*-hexane, dichloromethane and ethyl acetate) of three red seaweeds (*Hypnea musciformis*, *H. valentiae*, and *Jania rubens*) collected from the Gulf of Mannar of South eastern coast of India were evaluated, using different in vitro systems, viz., DPPH, ABTS, HO radical scavenging activities, H₂O₂ scavenging ability, Fe²⁺ ion chelating ability and reducing potential. Folin–Ciocalteu method was used to determine the total phenolic content of the extracts/fractions, and the results were expressed as mg of gallic acid equivalent (GAE)/g of the seaweed extracts/fractions. Thiobarbituric acid-reactive substances (TBARS) inhibition assay was employed to assess the ability of the seaweed extracts/fractions to inhibit lipid oxidation. Ethyl acetate (EtOAc) fractions of *H. musciformis* exhibited significantly higher total phenolic content (205.5 mg GAE/g), DPPH· scavenging activity (IC₅₀ 0.6 mg/mL), ABTS⁺ scavenging activity (IC₅₀ 0.51 µg/mL), Fe²⁺ chelating ability (IC₅₀ 0.70 mg/mL), H₂O₂ scavenging activity (IC₅₀ 0.39 mg/mL), reducing ability (Abs_{700 nm} 1.46) and lipid peroxidation inhibitory ability (2.71 MDAEC/kg) (*P*<0.05) compared to its *n*-hexane, DCM fractions, crude MeOH extract and MeOH extracts/fractions of *H. valentiae* and *J. rubens*. DCM fraction of *J. rubens* showed significantly higher hydroxyl radical scavenging activity (IC₅₀ 0.55 mg/mL) compared with *H. musciformis* and *H. valentiae* (*P*<0.05). This study indicated the potential use of red seaweeds, in particular, *H. musciformis* as candidate species to be used as food supplement for increasing the shelf-life of food industry, and candidates in combating carcinogenesis and inflammatory diseases.

Keywords Antioxidant activity · Red seaweeds · *Hypnea* sp · *Jania rubens* · Phenolics · Lipid peroxidation

Introduction

Seaweeds are the excellent source of bioactive compounds such as carotenoids, dietary fiber, protein, essential fatty acids, vitamins and minerals (Ganesan et al. 2008). Red seaweeds (Rhodophyta) are macroscopic, multicellular, benthic marine red algae (Zubia et al. 2009), commonly traded as food items in East Asia (e.g. sushi wrappings, seasonings, condiments, noodles, and vegetables), and employed in the phycocolloid (alginate, agar and carrageenan) industry as food additives due to gelling, water-retention, emulsifying and other physical properties. The reactive oxygen species (ROS) viz., hydroxyl radical (HO·), hydrogen peroxide (H₂O₂) etc. are physiological metabolites formed during aerobic life as a result of the metabolism of oxygen. DNA, cell membranes, proteins and other cellular constituents are target site of the degradation processes, and consequently induce different kinds of serious human diseases including viz., chronic inflammation, atherosclerosis, cancer, cardiovascular disorders, and ageing (Ruberto et al. 2001). Antioxidant compounds play an important role against these diseases, which explains their considerable commercial potential in medicine, food production and the cosmetic industry (Chakraborty et al. 2013). Therefore, consumption, and addition of antioxidant in food materials protects the body as well as foods against these degradation processes. Since there is an increased interest in the antioxidants of natural origin in recent times in place of synthetic ones, like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) etc., it is rational to explore these sources for their application in food and pharmaceutical applications.

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Seaweeds are photosynthetic organisms, and are highly exposed to a combination of stressful factors, viz., light and oxygen at the origin of the formation of free radicals and other oxidative reagents. But the absence of oxidative damage in the structural components of seaweeds suggests their cells have protective antioxidative defense systems (Chakraborty and Paulraj 2010; Escrig et al. 2001). Therefore these marine floras may be considered as a potential resource of natural antioxidant molecules. Many researchers have found seaweeds to be a rich source of antioxidant compounds (Wang et al. 2009; Heo et al. 2006; Duan et al. 2006; Zubia et al. 2009; Kumar et al. 2008) which can act against lipid oxidation in foods and oxidative stress in target tissues. Some active antioxidant compounds from marine algae were identified as phylophoeophyllin in *Eisenia bicyclis* (Cahyana et al. 1992), phlorotannins in *Sargassum kjellmanianum* (Yan et al. 1996) and fucoxanthin in *Hijikia fusiformis* (Yan et al. 1999).

Among various red seaweeds found in the Gulf of Mannar regions in the southeastern coast of the Indian subcontinent, *Hypnea musciformis*, *Hypnea valentiae* and *Jania rubens* are abundantly available throughout the different seasons. Although antioxidant properties of seaweeds were proved by numerous studies from the past two decades, there is scanty information regarding the antioxidant potential of these species from this very important delta region. Based on this background, the objectives of the present study were to evaluate the antioxidant activities and total phenolic contents of the crude methanol (MeOH) extracts and solvent fractions [*n*-hexane, dichloromethane (DCM) and ethyl acetate (EtOAc)] of these three experimental red seaweeds, to understand their beneficial value as human food or as additives. The correlations between total phenolic contents (TPC) and antioxidant capacities of these seaweeds were also evaluated. The results from the present study will be helpful to develop a new generation of antioxidants for increasing the shelf-life of food products, as nutraceuticals and/or functional foods, and in combating carcinogenesis and inflammatory diseases.

Materials and methods

Seaweed material and description of study area

Red seaweeds used in this study were *Hypnea musciformis* (Wulfen) J.V. Lamouroux (Family: Hypnaceae, Order: Gigartinales), *Hypnea valentiae* (Turner) Montagne (Family: Hypnaceae, Order: Gigartinales), and *Jania rubens* (Linnaeus) J.V. Lamouroux (Family: Corallinaceae; Order: Corallinales) (Fig. 1). They were freshly collected from the Gulf of Mannar in Mandapam region located between 8°48' N, 78°9' E and 9°14' N, 79°14' E on the

south east coast of India (Fig. 1). Samples collected were washed in running water for 10 min, transported to the laboratory and shade dried (35±3 °C) for 36 h. The shade dried seaweeds were powdered and used for further experiments.

Preparation of seaweed extracts and fractions

The powdered seaweed samples (100 g) were extracted three times with methanol (50–60 °C, 3 h), filtered through Whatman No. 1 filter paper and the pooled filtrate was concentrated (50 °C) in rotary vacuum evaporator (Heidolf, Germany) to one-third volume, and then partitioned successively with *n*-hexane (150 mL×3), DCM (150 mL×3) and EtOAc (150 mL×3), concentrated *in vacuo* to furnish *n*-hexane, DCM, and EtOAc fractions, respectively.

Chemicals and reagents

All solvents used were of analytical grade (E-Merck, Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH·), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4'-disulfonic acid sodium salt (ferrozine), Folin-Ciocalteu reagent, ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt), gallic acid, acetyl acetone, ammonium acetate, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), ferrous ammonium sulfate, FeCl₃, potassium ferricyanide, and FeCl₂ were purchased from E-Merck or Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO). All other unlabeled chemicals and reagents were of analytical, spectroscopic or chromatographic reagent grade and were obtained from E-Merck (Darmstadt, Germany).

Determination of total phenolic compounds

Total phenolic content in the seaweed extracts/fractions were determined according to the method as described earlier (Lim et al. 2007) with minor modifications. Briefly, 0.5 mL of the extracts/fractions (5 mg/mL in MeOH) was added into a test tube containing 2.25 mL MeOH. After the addition of 0.25 mL of Folin-Ciocalteu reagent, the mixtures were stirred for 1 min and allowed to stand for 8 min. Then, 2.0 mL of sodium carbonate (7.5 %, w/v) was added and the mixtures were incubated for 120 min at 25 °C. The absorbance, relative to that of the blank (MeOH), was measured at 756 nm using a UV-Vis spectrophotometer (Varian Cary, USA). The concentrations of total phenolic compounds in each extract were determined as milligram of gallic acid equivalent (mg GAE)/g of the extracts/fractions by using the regression equation from the calibration curve of the gallic acid standard. All determinations were performed in triplicate.

Fig. 1 Indicative photographs of *Hypnea musciformis*, *H. valentiae* and *Jania rubens* collected from the Gulf of Mannar region in Southeast coast of India (Lat 8°48' N; Long 78°9' E and Lat 9°14' N; Long 79°14'E). The

geographical indicators showing the location of the sampling area of the red seaweeds have been illustrated

Free radical scavenging ability assays

1, 1-Diphenyl-2-picryl- hydrazil (DPPH•) radical scavenging activity

The antioxidant activity of seaweed extracts/fractions was measured using the stable radical, DPPH•, as a standard reagent. This was determined as described earlier (Lim et al. 2007) with suitable modifications. Briefly, stock solutions of the extracts/fractions were prepared in MeOH. Dilutions were made to obtain concentration ranging from 0.1 to 1.0 mg/mL. Diluted solutions (2.0 mL) were mixed with 2.0 mL of 0.16 mM DPPH• in MeOH. The mixtures were shaken vigorously and maintained for 30 min at ambient temperature (30 °C) in the dark. The absorbance of mixtures was measured at 517 nm against a reagent blank by using a UV–Vis spectrophotometer. The absorbance of the control (2 mL DPPH• solution+2 mL MeOH) and the samples were measured spectrophotometrically at different time intervals (0, 0.5, 1.0, 4.0, 8.0, 16, 24, 48, 72, 96 h). The tests were performed in triplicate. The scavenging activity can be calculated by using the following

equation: $\text{DPPH}\cdot \text{ scavenging activity}(\%) = \frac{(A_{517} \text{ of control} - A_{517} \text{ of sample}) \times 100}{A_{517} \text{ of control}}$. The plot of scavenging activity on DPPH• was recorded and the IC₅₀ value (concentration of the sample to scavenge 50 % of the DPPH radicals; mg/mL) was then calculated.

2,2'-Azino-bis-3 ethylbenzothiozoline-6-sulfonic acid diammonium salt (ABTS) radical scavenging activity

The free radical scavenging activity was also determined by ABTS radical cation decolourization assay as described earlier (Vijayabaskar and Shiyamala 2012) with suitable modifications. Briefly, ABTS^{•+} was dissolved in deionized water to 7 mM concentration, and potassium persulfate (K₂S₂O₈) is added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (12–16 h) in the dark before use. The resultant intensely-colored ABTS^{•+} was diluted with MeOH to give an absorbance of ~0.70 at 734 nm. The ABTS^{•+} scavenging activity was assessed by mixing 5 mL of the above ABTS^{•+} solution with 0.1 mL seaweed extracts/fractions (0.1, 0.2, 0.3,

0.4 and 0.6 $\mu\text{g/mL}$). The final absorbance was measured at 743 nm with UV – Vis spectrophotometer. The percentage of scavenging was calculated by the following formula: $\text{ABTS}^{\cdot+}$ scavenging activity(%) = $((A_0 - A_1)/A_0) \times 100$, where A_0 is the absorbance of control and A_1 is the absorbance of the sample. The plot of scavenging activity on $\text{ABTS}^{\cdot+}$ radical was recorded and the IC_{50} value ($\mu\text{g/mL}$) was then calculated.

Hydroxyl (HO) radical scavenging activity

The capacity of seaweed extracts/fractions to scavenge hydroxyl radicals was measured according to a modification method described by Singh et al. (2002). The extracts/fractions in different concentrations (0.1, 0.2, 0.4 and 0.6 mg/mL) in MeOH were evaporated in vials and the reaction mixture contained 1 mL of iron-EDTA solution, 0.5 mL of EDTA (0.018 %), 1 mL of DMSO (0.85 % v/v in 0.1 M phosphate buffer, pH 7.4) and 0.5 mL of ascorbic acid (0.22 %) was added to each tube. The vials were capped tightly and heated in a water bath at 80–90 °C for 15 min. The reaction was terminated by adding 1 mL of ice-cold TCA (17.5 %). Three mL of Nash reagent (75.0 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL of acetyl acetone and adjust the volume up to 1 L) was added to each vial and left at room temperature for 15 min for color development. The intensity of yellow color was measured spectrophotometrically at 412 nm against blank sample. The mixture without sample was treated as control. All tests were performed in triplicate. The scavenging activity was calculated by following equation: % hydroxyl radical scavenging activity = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the samples. The plot of HO $\cdot$ radical scavenging activity by different concentrations was recorded and the IC_{50} value (mg/mL) was then calculated.

Hydrogen peroxide (H_2O_2) scavenging activity

The ability of the seaweeds to scavenge H_2O_2 was determined according to the method of Ruch et al. (1989) with slight modifications. Briefly, 40 mM H_2O_2 was prepared in phosphate buffer (pH 7.4). Extracts/fractions of different concentrations (0.1 to 1.0 mg/mL) in MeOH (3.0 mL) were added to 3.0 mL of 40 mM H_2O_2 solution and the absorbance of H_2O_2 was determined at 230 nm after 10 min incubation against a blank solution containing phosphate buffer without H_2O_2 . The percentage scavenging of H_2O_2 was calculated as: % H_2O_2 scavenging activity = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of control and A_1 is the absorbance of sample. The plot of scavenging activity on H_2O_2 was recorded and the IC_{50} value (mg/mL) was then calculated.

Lipid peroxidation inhibition activity in model system: Thiobarbituric acid-reactive species (TBARS) formation inhibitory activity

The TBARS formation inhibitory assay was performed by earlier described method (Chakraborty et al. 2013) with suitable modifications. The model system used for this assay was lyophilized green mussel (*Perna viridis* L.) as a lipid source. The lyophilized *P. viridis* (10 mg) was incubated with 1 mL of different seaweed extracts/fractions (1 mL; 2 mg/mL). The incubation was stopped by the addition of cold acetic acid (2 mL, 20 % v/v; pH 3.5), and malondialdehyde (MDA) formation was followed by the addition of TBA (2 mL, 0.78 % w/v in acetic acid). The mixtures were incubated at 95 °C for 45 min, cooled to room temperature and centrifuged (8,000 rpm, 10 min, Superspin PlastoCrafts R-V/Fm, Mumbai, India). The absorbance was measured at 532 nm. All the values are means of three determinations. Antioxidant activity was expressed as equivalent mM of malondialdehyde equivalent compounds (MDAEC) formed per kilogram of the sample related to the control (lyophilized green mussel) undergoing maximum lipid peroxidation on the same assay conditions.

Evaluation of reducing ability

Reducing power of the seaweed extracts/fractions was determined based on the ability of antioxidants to form colored complex with potassium ferric cyanide, TCA and ferric chloride. The reducing power of the extracts was determined as described earlier (Lim et al. 2007) with suitable modifications. 1.0 mL of extracts/fractions (1 mg/mL in MeOH) was mixed with 2.5 mL phosphate buffer (pH 6.6) and 2.5 mL of 1 % potassium ferric cyanide. The mixture was incubated at 50 °C for 20 min. After incubation, 2.5 mL of 10 % TCA was added to the mixture and centrifuged at 6,000 g for 10 min. Then, 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1 % ferric chloride. The reducing ability was represented as the absorbance measured at 700 nm ($\text{Abs}_{700 \text{ nm}}$) after 10 min. Increased absorbance of the reaction mixture indicated increased reducing power. All the measurements were measured in triplicates.

Ferrous ion chelating activity

The ferrous ion chelating activity was measured as described earlier (Lim et al. 2007) with suitable modifications. One mL 0.125 mM FeSO_4 , and 1.0 mL 0.3125 mM ferrozine were mixed with 1.0 mL extracts/fractions (0.1, 0.2, 0.4 and 0.6 mg/mL). The mixture was allowed to equilibrate for 10 min before measuring the absorbance. The decrease in the absorbance at 562 nm of the iron (II)-ferrozine complex was measured. The ability of the sample to chelate ferrous ion was calculated

relative to the control (consisting of iron and ferrozine only) using the equation: Chelating ability $\% = (A_0 - A_1) \times 100 / A_0$, where where A_0 is the absorbance of control and A_1 is the absorbance of sample. The IC_{50} value (mg/mL), which is the concentration of the extracts/fractions that chelate 50 % of the ferrous ion, was calculated from the non linear regression curve.

Statistical analysis

One-way analysis of variance (ANOVA) was carried out with the Statistical Program for Social Sciences 13.0 (SPSS, USA, ver. 13.0) to assess for any significant differences between the means. Differences between means at the 5 % ($P < 0.05$) level were considered significant. The mean variance in the data set was detected using principal component analysis (PCA). The selected variables for PCA were the different antioxidant activities, as exhibited by EtOAc, DCM, *n*-hexane fractions and MeOH crude extracts of the three red seaweeds.

Results and discussion

Yield of MeOH extracts and fractions

The yields of the MeOH extracts, *n*-hexane, DCM, and EtOAc fractions of *H. musciformis*, *H. valentiae* and *J. rubens* were shown in Table 1. Among the MeOH extracts of three seaweeds, *H. valentiae* exhibited higher yield (6.5 g/100 g dry sample) followed by *J. rubens* and *H. musciformis* (5.3 & 4.8 g/100 g dry sample, respectively). The yield of methanolic extract in the present study was higher as compared to the earlier study by Nguyen and Kim (2012) who obtained 4.6 g/100 g dry sample of total MeOH extract in the red seaweed, *Grateloupia lancifolia*. Among the different solvent fractions, *n*-hexane fraction had the highest yield in all the seaweeds (26.2 g/100 g MeOH extract). The higher yield of *n*-hexane fractions compared to the DCM and EtOAc fractions of the same species showed that most of the compounds in these seaweeds were low in polarity and fat-soluble.

Table 1 Yield of methanolic extracts (g/100 g dry seaweed) and fractions (g/100 g methanolic extract) of three red seaweeds ($n=3$)

Seaweeds	MeOH extracts	Fractions		
		<i>n</i> -Hexane	DCM	EtOAc
<i>H. musciformis</i>	4.83 ^a ±0.35	27.02 ^a ±1.05	20.56 ^a ±1.65	22.25 ^a ±0.65
<i>H. valentiae</i>	6.54 ^b ±0.29	30.56 ^a ±1.65	22.32 ^a ±2.05	18.98 ^b ±1.25
<i>J. rubens</i>	5.32 ^b ±0.98	26.21 ^b ±2.03	19.75 ^a ±2.05	22.0 ^a ±1.28

All the values are mean $\pm$ SD ($n=3$); SD standard deviation. a,b Column wise values with different superscripts are significantly different ($P < 0.05$). MeOH Methanol, DCM Dichloromethane, EtOAc Ethyl acetate

Total phenolic content in red seaweed extracts and fractions

The total phenolic content observed for extracts/fractions is shown in Table 2. MeOH extract of *H. musciformis* and *H. valentiae* exhibited significantly higher (6.9 & 9.8 mg GAE/g, respectively) phenolic content ($P < 0.05$). EtOAc fraction of all the seaweeds exhibited high phenolic content (37–205 mg GAE/g) followed by DCM (32–87 mg GAE/g) and *n*-hexane (8–56 mg GAE/g) fractions. These results corroborate well with those studies by Duan et al. (2006), who observed high phenolic content in the EtOAc soluble fraction of red seaweed, *P. urceolata* (73.7 GE/g). In the present study, it was noticed that for all the three seaweeds, higher contents of the phenolic compounds were observed in the solvent fractions than the crude MeOH extract. Similar finding was also reported by Ganesan et al. (2008). This could be due to more interfering substances present in the crude extract as compared to those fractions. Moreover, total phenolic content increased in the fractions with increasing solvent polarity (eg. EtOAc and DCM). It is evident from the present observations that a significantly higher ($P < 0.05$) phenolic content was observed in the polar solvent fractions (EtOAc and DCM) of *Hypnea* sp (54–205 mg GAE/g) indicating their high antioxidant potential (Table 2). Zubia et al. (2007) reported that another *Hypnea* species, *Hypnea spinella* collected from the Gulf of Mexico and Caribbean coast of Yucatan and Quintana Roo showed a phenolic content of 0.67 % (dw). The phenolic content in the EtOAc fraction of *H. musciformis* was significantly higher (205.5 mg GAE/g) compared to *H. valentiae* and *J. rubens* (72.9 & 37.2 mg GAE/g, respectively) ($P < 0.05$) (Table 2). There are earlier reports that substantiate our present observation that solvent extracts of *Hypnea* sp and other red seaweeds are good resources of polyphenolic compounds (Pavia and Aberg 1996; Yoshie et al. 2000).

Free radical scavenging activity of red seaweed extracts and fractions

1, 1-Diphenyl-2-picryl-hydrazil (DPPH•) scavenging activity

DPPH radical scavenging activities (%) of the extracts/fractions were found to be maximum at 48th h. The scavenging activity (48th h) observed at 1 mg/mL are shown in Table 2. MeOH extract of *H. musciformis* and *J. rubens* showed significantly higher ($P < 0.05$) DPPH• scavenging activities (15.4 & 17.7 %, respectively) than *H. valentiae* (7.7 %). However, the EtOAc fraction of *H. musciformis* registered significantly higher DPPH• scavenging activity (82.9 %) than *H. valentiae* and *J. rubens* (20.5 & 29.9 %, respectively). Earlier studies showed high DPPH radical scavenging activities in the EtOAc fractions of red seaweeds, *R. confervoides*, *Polysiphonia urceolata* and *Ecklonia cava* (Duan et al. 2006; Wang et al. 2009;

Table 2 Total phenolic content (mg GAE/g sample), ABTS, DPPH, $\cdot\text{OH}$, H_2O_2 radical scavenging activities (%), lipid peroxidation inhibitory activity (MDAEC/kg), total reduction capability (Absorbance at 700 nm) and Fe^{2+} ion chelating activity (%) of the crude methanolic extracts and solvent fractions (MeOH, *n*-hexane, dichloromethane and ethylacetate) of the three red seaweeds ($n=3$)

Seaweeds	MeOH extracts	Solvent fractions		
		<i>n</i> -Hexane	DCM	EtOAc
Total phenolic content (5 mg/mL)				
<i>H. musciformis</i>	9.84 ^{ap} ±0.03	56.81 ^{aq} ±2.40	87.82 ^{ar} ±3.90	205.48 ^{as} ±2.40
<i>H. valentiae</i>	6.91 ^{bp} ±0.06	8.46 ^{bq} ±0.18	54.68 ^{br} ±0.19	72.95 ^{bs} ±1.25
<i>J. rubens</i>	4.95 ^{cp} ±0.06	2.77 ^{cq} ±0.02	32.48 ^{cr} ±1.04	37.27 ^{cr} ±0.72
DPPH· radical scavenging activity (1 mg/mL)				
<i>H. musciformis</i>	15.40 ^{ap} ±1.05	24.93 ^{aq} ±0.66	69.42 ^{ar} ±1.04	82.98 ^{as} ±0.18
<i>H. valentiae</i>	7.73 ^{bp} ±0.07	2.94 ^{bp} ±0.58	66.36 ^{aq} ±2.81	20.53 ^{br} ±0.86
<i>J. rubens</i>	17.69 ^{ap} ±2.44	14.97 ^{cp} ±0.33	40.38 ^{bq} ±1.46	29.93 ^{cr} ±0.27
ABTS ⁺ radical scavenging activity (0.6 µg/mL)				
<i>H. musciformis</i>	19.60 ^{ap} ±1.44	9.98 ^{aq} ±0.73	25.39 ^{ap} ±0.55	63.30 ^{ar} ±1.73
<i>H. valentiae</i>	14.96 ^{bp} ±0.42	12.90 ^{bp} ±0.64	10.89 ^{bp} ±1.02	27.90 ^{bq} ±0.25
<i>J. rubens</i>	8.78 ^{cp} ±0.30	8.87 ^{ap} ±0.31	28.86 ^{cq} ±0.30	11.09 ^{cp} ±1.16
HO· radical scavenging activity (0.6 mg/mL)				
<i>H. musciformis</i>	15.77 ^{ap} ±0.32	20.87 ^{ap} ±0.11	35.39 ^{aq} ±0.10	21.8 ^{ap} ±1.11
<i>H. valentiae</i>	37.03 ^{bp} ±1.47	26.14 ^{bq} ±1.16	37.42 ^{ap} ±1.42	32.27 ^{bp} ±1.44
<i>J. rubens</i>	42.99 ^{cp} ±1.26	22.31 ^{aq} ±0.61	51.20 ^{br} ±1.45	45.29 ^{cp} ±1.89
H ₂ O ₂ scavenging capacity (1 mg/mL)				
<i>H. musciformis</i>	43.01 ^{ap} ±0.81	32.93 ^{aq} ±0.56	75.54 ^{ar} ±0.72	80.77 ^{ar} ±0.60
<i>H. valentiae</i>	32.75 ^{bp} ±1.03	27.28 ^{bq} ±1.26	37.16 ^{bp} ±0.83	50.5 ^{br} ±0.72
<i>J. rubens</i>	27.63 ^{cp} ±1.36	17.74 ^{cq} ±1.06	37.78 ^{br} ±0.58	53.07 ^{cs} ±1.06
Lipid peroxidation inhibitory activity (2 mg/mL)				
<i>H. musciformis</i>	9.88 ^{ap} ±0.24	13.05 ^{ap} ±0.08	3.61 ^{ap} ±0.37	2.71 ^{aq} ±0.10
<i>H. valentiae</i>	12.68 ^{ap} ±0.48	12.28 ^{ap} ±0.74	4.12 ^{aq} ±0.16	3.93 ^{bq} ±0.15
<i>J. rubens</i>	18.60 ^{bp} ±2.40	17.99 ^{bp} ±1.77	9.82 ^{bq} ±0.26	3.36 ^{cr} ±0.10
Total reduction capability (1 mg/mL)				
<i>H. musciformis</i>	0.74 ^{ap} ±0.01	0.78 ^{ap} ±0.01	0.97 ^{ap} ±0.02	1.46 ^{ap} ±0.02
<i>H. valentiae</i>	0.33 ^{bp} ±0.03	0.38 ^{bp} ±0.01	0.40 ^{bp} ±0.01	0.48 ^{bp} ±0.01
<i>J. rubens</i>	0.43 ^{cp} ±0.01	0.37 ^{bp} ±0.01	0.43 ^{bp} ±0.01	0.45 ^{bp} ±0.01
Fe ²⁺ ion chelating activity (0.6 mg/mL)				
<i>H. musciformis</i>	31.02 ^{ap} ±0.98	13.04 ^{aq} ±0.32	21.42 ^{aq} ±0.95	38.29 ^{as} ±1.71
<i>H. valentiae</i>	12.26 ^{bp} ±0.27	5.26 ^{bq} ±0.20	10.76 ^{bp} ±0.51	18.84 ^{br} ±0.31
<i>J. rubens</i>	23.87 ^{cp} ±1.41	13.06 ^{aq} ±0.72	17.33 ^{cq} ±0.64	37.94 ^{ar} ±1.60

Data are the mean values of triplicate and expressed as mean ± standard deviation. a–c Column wise values with different superscripts of this type indicate significant difference ($P<0.05$). p–s Row wise values with different superscripts of this type indicate significant difference ($P<0.05$). DCM Dichloromethane, EtOAc Ethyl acetate. Concentration of samples for each analysis is given in parentheses

Senevirathne et al. 2006), methanolic and aqueous extracts from red seaweeds, *Gracilaria verrucosa*, *G. textorii*, *Grateloupia filicina*, *Polysiphonia japonica*, *Euchema Kappaphycus* and *Gracilaria edulis* (Heo et al. 2006; Ganesan et al. 2008). Apparently, in *H. valentiae* and *J. rubens*, DCM fractions showed significantly higher ($P<0.05$) DPPH· scavenging activity (40.3–66.3 %) than *n*-hexane and EtOAc fractions. According to the IC_{50} values showed in Fig. 2a, the DPPH· scavenging ability of the three seaweeds exhibited the following order: *H. musciformis* EtOAc fraction (0.6)>*H. valentiae* DCM fraction (0.75)>*J. rubens* DCM fraction (1.24). The present study suggests that the solvent fractions, especially, EtOAc and DCM fractions of *Hypnea* spp may contain compounds having polyphenolic group/s with

multiple -OH groups and/or center of unsaturation in their structural moieties to enable them to donate a proton to DPPH radical thereby neutralizing the latter (Ruberto et al. 2001).

2,2'-Azino-bis-3ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) scavenging activity

ABTS assay has been widely used to investigate free radical scavenging activity of different extracts and fractions and/or its pure compounds. ABTS⁺ scavenging activities of the MeOH extracts/fractions of the red seaweeds at 0.6 µg/mL are recorded in Table 2. Among MeOH extracts, *H. musciformis* recorded significantly higher ABTS⁺ free radical scavenging activity (19.6 %) followed by *H. valentiae*

Fig. 2 IC₅₀ (Inhibition concentration) values for (a) DPPH scavenging activities (mg/mL), (b) ABTS^{•+} scavenging activities (μg/mL), (c) Hydroxyl (HO[•]) radical scavenging activities (mg/mL), (d) H₂O₂ scavenging capacity (mg/mL) and (e) Fe²⁺ ion chelating activity (mg/mL). IC₅₀ values were determined from the linear regression curve of scavenging/

chelating activities against the different concentrations of seaweed extracts/fractions. IC₅₀ value is defined as the amount of antioxidant necessary to decrease the initial DPPH/ABTS/HO[•] radicals, H₂O₂ concentration by 50 % and amount of antioxidant necessary to chelate the Fe²⁺ ion by 50 %

(14.9 %) and *J. rubens* (8.7 %). The present observation correlates well with earlier studies, which reported similar antioxidant activities of the MeOH extract of red seaweed, *Padina* sp (Matanjun et al. 2008). EtOAc fraction of *H.*

musciformis registered significantly higher ABTS^{•+} scavenging activity (63.3 %) followed by *H. valentiae* (27.9 %) and *J. rubens* (11.0 %). Interestingly, DCM fraction of *J. rubens* showed significantly higher ABTS^{•+} scavenging activity

(28.8 %) than its MeOH extract and other solvent fractions ($P < 0.05$). The high $\text{ABTS}^{\cdot+}$ scavenging activity realized by EtOAc and DCM fractions of *Hypnea* sp and *J. rubens* could be due to the presence of carotenes/other pigments with long hydrocarbon chain and aminated compounds (Chew et al. 2008). Apparently, the mechanism of antioxidant action of this EtOAc and DCM fractions can explain as its H-donating property, thereby terminating the oxidation process by converting free radicals to the stable forms. According to the IC_{50} showed in Fig. 2b, the $\text{ABTS}^{\cdot+}$ scavenging ability of the extracts/fractions of three seaweeds exhibited the following order: *H. musciformis* EtOAc fraction (0.51) > *J. rubens* DCM fraction (1.08) > *H. valentiae* EtOAc fraction (1.1). Similar to the DPPH $\cdot$ scavenging activity, the $\text{ABTS}^{\cdot+}$ scavenging capacities of the EtOAc fraction of *H. musciformis* was stronger than other two red seaweed MeOH extracts and fractions.

Hydroxyl radical scavenging activity of red seaweed extracts and fractions

Hydroxyl radical scavenging activity was employed to understand the potential of different seaweed extracts/fractions against short-lived radicals, viz., $\text{HO}^{\cdot}$ radical that abstract H-atoms which causes peroxide reaction of lipids. $\text{HO}^{\cdot}$ radical scavenging activities of the MeOH extracts/fractions of the red seaweeds at 0.6 mg/mL are recorded in Table 2. $\text{HO}^{\cdot}$ scavenging activity of MeOH extracts was in the order: *J. rubens* > *H. valentiae* > *H. musciformis*. DCM fractions of all the three red seaweeds registered significantly higher ($P < 0.05$) $\text{HO}^{\cdot}$ radical scavenging activity (35.4–51.2 %) than other solvent fractions as evident from Table 2. The IC_{50} values (Fig. 2c) established the maximum $\text{HO}^{\cdot}$ scavenging effect of the DCM fractions of all three red seaweeds ($\text{IC}_{50} \leq 0.96$). The present study correlates well with earlier studies that about >90 % $\text{HO}^{\cdot}$ scavenging activity was reported from DCM and BuOH fractions (1 mg/mL) of red seaweeds *Acanthophora spicifera* and *Gracilaria edulis* (Ganesan et al. 2008). The extracts of red seaweeds, *Gracilaria verrucosa*, *G. textorii*, *Grateloupia filicina* and *Polysiphonia japonica* also reported potentially high $\text{HO}^{\cdot}$ scavenging activity (Heo et al. 2006).

Scavenging of hydrogen peroxide by red seaweed extracts and fractions

H_2O_2 , a reactive non radical compound, is of potential biological significance because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it may convert into more reactive species (singlet oxygen and $\text{HO}^{\cdot}$ radicals) (Ruch et al. 1989). H_2O_2 scavenging activity (%) at 1 mg/mL of the MeOH extracts/fractions of the red seaweeds are recorded in Table 2. EtOAc and DCM fractions of *H. musciformis* registered significantly higher ($P < 0.05$) H_2O_2

scavenging activity (80.7 & 75.5 %, respectively) than its MeOH extract (43 %), *n*-hexane fraction (32.9 %) and extracts/fractions of *H. valentiae* and *J. rubens* (< 54 %). According to the IC_{50} showed in Fig. 2d, the H_2O_2 scavenging activity of the EtOAc fraction of three seaweeds exhibited the order: *H. musciformis* (0.39) > *J. rubens* (0.57) > *H. valentiae* (0.64). The strongest H_2O_2 scavenging effect of *H. musciformis* EtOAc fraction can be explained due to the presence of hydrophilic phenolics (Senevirathne et al. 2006). EtOAc fraction of the red seaweed *Ecklonia cava* (Heo et al. 2006) reported high H_2O_2 scavenging activity supporting our observations that red seaweeds are rich source of natural antioxidant compounds, capable to scavenge H_2O_2 .

Lipid peroxidation inhibition ability of red seaweed extracts and fractions: Thiobarbituric acid-reactive substances (TBARS) inhibitory activity

TBARS assay, which reflect the production of low molecular-weight end products, like malondialdehyde is used to indicate free-radical generation. TBARS formation inhibitory activity at 2 mg/mL of the MeOH extracts/fractions of the three red seaweeds was recorded in Table 2. EtOAc (2.7–3.9 MDAEC/kg) and DCM (3.6–9.8 MDAEC/kg) fractions of all three seaweeds exhibited significantly higher TBARS formation inhibitory ability than its corresponding MeOH extract (9.8–18.6 MDAEC/kg) and *n*-hexane fractions (12.3–18.0 MDAEC/kg) ($P < 0.05$). The present study correlates well with earlier studies (Zubia et al. 2009) reporting that EtOAc and DCM fractions are the major seaweed fractions harboring the principle antioxidative components that inhibits lipid peroxidation. Apparently in the present study, EtOAc fraction of *H. musciformis* registered significantly higher TBARS inhibition ability (2.71 MDAEC/kg) than all other extracts/fractions. The inhibition of lipid peroxidation may be due to the presence of polyphenolic antioxidants that were reported to disrupt free-radical chain reaction by donating a proton to fatty acid radicals to terminate chain reactions (Karawita et al. 2005).

Total reduction capability of red seaweed extracts and fractions

Fe^{3+} reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties (Dorman et al. 2003). Total reduction capability at highest concentration (1 mg/mL) of the MeOH extracts/fractions of the three red seaweeds are recorded in Table 1. Significantly higher total reduction capability was observed for MeOH extracts/fractions of *H. musciformis* ($\text{Abs}_{700 \text{ nm}}$ 0.74–1.6) compared with *H. valentiae* and *J. rubens* ($\text{Abs}_{700 \text{ nm}} < 0.5$). EtOAc fraction of

Fig. 3 Correlation between antioxidant activity assays by scatter plot analyses. Scatter plot diagrams showing the correlation of total phenolic content (TPC) *vis-à-vis* (a) DPPH· (n=12; R²=0.657), (b) ABTS·⁺ (n=12; R²=0.765), (c) H₂O₂ scavenging activity (n=12; R²=0.704), (d)

reducing ability (n=12; R²=0.757), (e) lipid peroxidation (TBARS) inhibitory activity (n=12; R²=0.444), (f) Scatter plot showing correlation between DPPH and ABTS radical scavenging activity (n=12; R²=0.403)

H. musciformis ($\text{Abs}_{700\text{ nm}} 1.46$) showed maximum reducing power than *H. valentiae* and *J. rubens* ($\text{Abs}_{700\text{ nm}} 0.48$ & 0.45 , respectively). Our study is in accordance with the earlier reports which reported that the reducing power of MeOH ($\text{Abs}_{700\text{ nm}} 0.07$ – 0.74) and EtOAc extracts ($\text{Abs}_{700\text{ nm}} 0.013$ – 0.467) of red seaweed *Kappaphycus alvarezii* extracts were reported to be higher than *n*-hexanic extract ($\text{Abs}_{700\text{ nm}} 0.017$ – 0.16 at 0.5 – 5 mg/mL) (Kumar et al. 2008). Furthermore, EtOAc fractions of red seaweed *Rhodomela confervoides* exhibited potentially high reducing power (426 mg/g ascorbic acid equivalents) (Wang et al. 2009). Generally, the reducing properties are associated with the presence of compounds, which exert their action by breaking the free radical chain by donating a hydrogen atom or a single electron (Wong et al. 2006).

Fe^{2+} ion chelating activity of red seaweed extracts and fractions

Metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation (Hseu et al. 2008). Fe^{2+} ion chelating activity (%) at 0.6 mg/mL of the MeOH extracts/fractions of the three red seaweeds (Table 2) were in the order: *H. musciformis* > *J. rubens* > *H. valentiae*. EtOAc extracts of *H. musciformis* and *J. rubens* were better chelators of ferrous ion (38.2 & 37.9 %, respectively) compared with *H. valentiae* (18.8 %). Our studies are in accordance with earlier studies which demonstrated that polyphenols derived from seaweeds are potent Fe^{2+} chelators (Senevirathne et al. 2006), and metal chelating potency of phenolic compounds are dependent upon their unique phenolic structure, and the number and location of $-\text{OH}$

groups (Lindsay 1996). According to the IC_{50} values (Fig. 2e), the EtOAc fractions of three seaweeds exhibited strong chelating activity (*H. musciformis* > *J. rubens* > *H. valentiae*) followed by MeOH extracts. Generally, compounds with structures containing two or more of the following functional groups: $-\text{OH}$, $-\text{SH}$, $-\text{COOH}$, $-\text{PO}_3\text{H}_2$, $>\text{C}=\text{O}$, $-\text{NR}_2$, $-\text{S}-$ and $-\text{O}-$ in a favorable structure-function configuration will have chelation activity (Lindsay 1996).

Correlations between phenolic contents and different antioxidant activity assays

A significant correlation between total phenolic contents (TPC) and different radical scavenging assays of seaweed extracts was realized by Pearson correlation analysis [TPC-DPPH $\cdot$ scavenging activity: $R^2=0.657$ (Fig. 3a); TPC-ABTS $^{\cdot+}$ scavenging activity: $R^2=0.765$ (Fig. 3b); TPC- H_2O_2 scavenging activity: $R^2=0.704$ (Fig. 3c); and TPC-reducing ability: $R^2=0.757$ (Fig. 3d)]. DPPH/ $\text{HO}\cdot$ scavenging activity and reducing ability were found to be positively correlated with each other as well as TPC thereby realizes the role of phenolic compounds responsible for the antioxidant properties of seaweed extracts. Other researchers also observed a positive correlation between TPC and antioxidant activities of seaweed extracts (Escrig et al. 2001; Wang et al. 2009; Karawita et al. 2005). The results obtained from the present study suggested that the antioxidant activity registered by extracts/fractions, mainly EtOAc fraction may primarily be due to the presence of polar phenolic compounds. The EtOAc fractions showed no correlation with *n*-hexanic fraction, which further corroborate the above observation. However, a negative correlation was realized between TPC and lipid

Fig. 4 Loading plot diagram (various components viz., principal components, PC-1 and PC-2 in rotated space) of antioxidant activities of extracts/fractions from *H. musciformis*, *H. valentiae*, and *J. rubens*. T – Total phenolic content, D – DPPH $\cdot$ scavenging activity, A – ABTS $^{\cdot+}$ scavenging activity, H – $\text{HO}\cdot$ radical scavenging activity, P – H_2O_2 scavenging activity, L – lipid peroxidation inhibitory activity, R – reducing ability, F – Fe^{2+} ion chelating activity. 1, 2, 3 and 4 represents methanol extract, *n*-hexane, dichloromethane and ethyl acetate fractions, respectively

peroxidation inhibition activity ($R^2=0.444$) (Fig. 3e), apparently indicate that antioxidant activity did not depend only on TPC, but also on other factors or there may be some active metabolites other than phenolics such as polysaccharides capable of inhibiting the TBA-MDA adduct formation (Muzzarelli 1997). TBARS and $\text{HO}^\bullet$ scavenging assay of different extracts/fractions registered positive correlation with each other ($R^2=0.014$; not shown). The TPC and Fe^{2+} ion chelating activity exhibited a positive correlation ($R^2=0.183$; not shown) thus suggesting that phenolics capable of chelating transition metals are present in these red seaweeds. A significant positive correlation registered between DPPH and ABTS radical scavenging assay ($R^2=0.403$) (Fig. 3f) guided us to conclude the identical mechanism of antioxidant activities. A positive correlation has been documented between TPC and antioxidant activity of different seaweed extracts by earlier studies (Escrig et al. 2001; Karawita et al. 2005).

The similarities and differences among different MeOH extracts/organic fractions of the two *Hypnea* spp and *Jania rubens*, and the relationships among different antioxidant activity assays were statistically analyzed using principle component analyses (PCA). Principal component analysis (PCA) was performed to understand how the different antioxidant activities, lipid peroxidation inhibition activity and TPC contribute to total antioxidant activity of the seaweeds. The loading of first and second principal components (PC1 & PC2) accounted for 65.4 % and 34.5 % of the variance, respectively (Fig. 4). PC1 was mainly influenced by TPC of all extracts/fractions (T1 – T4), DPPH $^\bullet$ scavenging activity of DCM and EtOAc fractions (D3 and D4), ABTS $^{+}$ scavenging activity of MeOH extracts and EtOAc fractions, H_2O_2 scavenging activity and reducing ability of all extracts/fractions (P1 – P4 & R1 – R4, respectively). The similarity in the high loadings of TPC, reducing ability, H_2O_2 scavenging activity, ABTS $^{+}$ scavenging activity (MeOH extracts and EtOAc fractions), DPPH $^\bullet$ (DCM fraction) scavenging activity indicated that these are closely related. The high loadings of TPC on PC1 also implicate that phenolic compounds present in red seaweeds are good antioxidants. The DPPH activity of MeOH extracts (D1), ABTS scavenging activity of DCM fraction (A3), lipid peroxidation inhibition activity of *n*-hexane fraction (L2) and Fe^{2+} ion chelating activity of all extracts/fractions (F1 – F4) contributes mainly to PC2. The higher loading of Fe^{2+} ion chelating activity on PC2 indicates that secondary antioxidant properties are not directly related to primary antioxidant property.

Conclusions

The present study reveals the potent antioxidant properties of three red seaweeds viz., *Hypnea musciformis*, *Hypnea valentiae* and *Jania rubens* abundantly available along with

the south east coast of peninsular India. The in vitro antioxidant activities of methanol extracts and different organic fractions of these seaweeds exhibited dose dependency; and increased with increasing concentration. In general, EtOAc fractions of the red seaweeds were found to be more effective than *n*-hexane, DCM fractions and crude MeOH extract. The significant correlation of total phenolic content with different antioxidant activities indicates that phenolic principles present in the red seaweed extracts are endowed with potential antiradical properties. This study provides us with the information regarding the potential of red seaweeds to develop natural sources for antioxidants as food supplements, as nutraceuticals and/or functional foods, and candidates in combating carcinogenesis and inflammatory diseases.

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Conflict of interest declaration The authors declare that they have no conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, the present work.

Submission declaration The authors vouch that the work has not been published elsewhere, either completely, in part, or in any other form and that the manuscript has not been submitted to another journal, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere without the written consent of the copyright-holder. The submitting author certifies that all coauthors have seen a draft copy of the manuscript and agree with its publication.

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