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In vitro antioxidant activities of three selected brown seaweeds of India

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Abstract

In vitro antioxidant activities of three selected Indian brown seaweeds – viz., Sargassum marginatum, Padina tetrastomatica and Turbinaria conoides – were investigated. Total phenolic content and reducing power of crude methanolic extract were also investigated. The activity of total methanolic extract and five different fractions (viz., petroleum ether (PE), ethyl acetate (EA), dichloromethane (DCM), butanol (BuOH) and aqueous) were studied using total antioxidant activity, DPPH radical scavenging and deoxyribose assays. EA fraction of S. marginatum exhibited higher total antioxidant activity of 39.62 mg ascorbic acid equivalent/g extract (or 0.31 mg ascorbic acid equivalent/g seaweed on dry weight basis) among the all the fractions. Among the fractions obtained from different seaweeds, EA fraction of S. marginatum showed higher DPPH scavenging activity of 23.16%; while PE fraction of T. conoides exhibited lower deoxyribose activity of 47.81%. Higher phenolic content (49.16 mg gallic acid equivalent/g extract or 0.86 mg GAE/g of seaweed on dry weight basis) was noticed in aqueous fraction of T. conoides. Reducing power of crude methanolic extract increased with increasing concentration. Reducing power of T. conoides and P. tetrastomatica were higher compared to standard antioxidant (α -tocopherol). Among the seaweeds, total methanolic extract of T. conoides had significantly higher phenol content ($P < 0.05$) compared to the other two species. In vitro antioxidant activity of methanolic extracts from all the three seaweeds showed an increase with increasing concentration indicating the dose dependency of these properties.

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1. Introduction

Seaweeds or marine macroalgae are potential renewable resource in the marine environment. About 6000 species of seaweeds have been identified and are grouped into different classes viz., green (Chlorophytes), brown (Pheophytes) and red (Rhodophytes) algae. The total global seaweed production of the world in the year 2004 was >15 million metric tonnes of which nearly 15–20% is contributed by Indian Ocean region ([FAO, 2006\)](#page-6-0). Seaweed harvest across Indian coast is about 100,000 metric tonnes (wet weight) ([Dhargalkar & Pereira, 2005](#page-5-0)). Seaweeds provide for an excellent source of bioactive compounds such as carotenoids, dietary fibre, protein, essential fatty acids, vitamins

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and minerals [\(Bhaskar & Miyashita, 2005; Fleurence,](#page-5-0) [1999; Mabeau & Fleurence, 1993; Nisizawa, 1988](#page-5-0)). In Asian countries, Japanese are the main consumers of seaweed with an average of 1.6 kg (dry weight) per year per capita ([Fujiwara-Arasaki, Mino, & Kuroda, 1984](#page-6-0)). However, in India, seaweeds are exploited mainly for the industrial production of phycocolloids such as agar-agar, alginate and carrageenan and not as culinary item or for recovering beneficial biomolecules.

Reactive oxygen species such as hydroxyl, superoxide and peroxyl radicals are formed in human cells by endogenous factors and exogenously result in extensive oxidative damage that in turn leads to geriatric degenerative conditions, cancer and a wide range of other human diseases ([Aruoma, 1999; Borek, 1993; Reaven & Witzum, 1996\)](#page-5-0). Carotenoids, the natural pigments from plant origin react rapidly with these free radicals and retard or alleviate the extent of oxidative deterioration ([Akoh & Min, 1997\)](#page-5-0).

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Furthermore, antioxidants from natural sources increase the shelf-life of foods [\(Schwarz et al., 2001](#page-6-0)). Therefore, consumption of antioxidant and/or addition of antioxidant in food materials protect the body as well as foods against these events.

Many researchers have reported various types of antioxidants in different kinds of higher plants [\(Larson, 1988;](#page-6-0) [Shon, Kim, & Sung, 2003](#page-6-0)). More recent reports revealed seaweeds to be a rich source of antioxidant compounds [\(Duan, Zhang, Li, & Wang, 2006; Kuda, Tsunekawa, Goto,](#page-5-0) [& Araki, 2005; Lim, Cheung, Ooi, & Ang, 2002; Park, Shah](#page-5-0)[idi, & Jeon, 2004\)](#page-5-0). Further, reports on the antioxidant properties of seaweed extracts from India are very limited. Hence, the present study was aimed to investigate the antioxidant properties of three different brown seaweed extracts and their different fractions using various in vitro free radical scavenging procedures. In addition, relationship between antioxidant activity and total phenolic content and total antioxidant activity were also considered in this study.

2. Materials and methods

2.1. Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-deoxy-D-ribose and a-tocopherol were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethylenediaminetetraacetic acid (EDTA), Folin-Ciocatteau's phenol reagent and hydrogen peroxide were purchased from Merck (Mumbai, India). Thiobarbituric acid (TBA) was purchased from Hi-Media (Mumbai, India). All other solvents and chemicals were of analytical grade.

2.2. Seaweed material

Brown seaweeds used in this study were Sargassum marginatum, Padina tetrastomatica and Turbinaria conoides. They were collected freshly from East and West coast of India. Samples collected were washed thoroughly with freshwater, transported to the laboratory immediately in an iced condition and shade dried $(38 \pm 2 \degree C)$ in a drier (Kilburns-024 E, Mumbai, India) for about 20 h. The shade dried seaweeds were powdered and used for further experiments.

2.3. Preparation of seaweed extracts and fractions

First extraction of each seaweed was prepared by pouring methanol into the bottle containing 50 g of seaweed powder at the ratio of 10:1 (v/w) , the mixture was flushed with nitrogen and kept under orbital shaking incubator (M/s Technico, India) at room temperature (29 \pm 2 °C) for 24 h under dark condition. Likewise, second extraction with chloroform and methanol mixture $(1:1 \, (v/v))$ and third extraction with chloroform were prepared from the same powder. Three extracts (representing both lower polar, polar and non-polar components) of each sample

were pooled together and evaporated under reduced pressure using rotary flash evaporator (Superfit, India). The crude extract of each sample was weighed and then dissolved in 90% aqueous methanol for fractionation based on polarity with PE extracting the non-polar components while the aqueous fraction representing the more polar substances ([Duan et al., 2006\)](#page-5-0). First fractionation was carried out with 3×100 ml petroleum ether. Petroleum ether (PE) fraction was collected and aqueous methanol phase was evaporated under reduced pressure to give a semisolid. Then semisolid portion was dissolved in 200 ml distilled water and further fractioned with ethyl acetate (EA), dichloromethane (DCM) and n-butanol (BuOH). Resulting fractions including aqueous were evaporated to dryness. Dried fractions were dissolved in methanol and stored in colored vials for further analysis. Extracts used for all experiments were at the concentration of $1000 \mu g$. Further, a-tocopherol was used as the positive control in case of reducing power, DPPH radical scavenging and hydroxyl radical scavenging (by deoxyribose assay) activity estimations.

2.4. Total phenolic content

Phenolic contents of crude methanolic extract and factions were estimated by the method of [Taga, Miller, and](#page-6-0) [Pratt \(1984\).](#page-6-0) Briefly, 100 μ l aliquot of sample were mixed with 2.0 ml of 2% Na₂CO₃ and allowed to stand for 2 min at room temperature. After incubation, 100 µl of 50% Folin-Ciocalteau's phenol reagent were 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 (Shimadzu, Kyoto, Japan). Phenolic contents are expressed as gallic acid equivalents per gram (GAE/g) of extract.

2.5. Total antioxidant activity

Total antioxidant activities of crude methanolic extract and fractions were determined according to the method of [Prieto, Pineda, and Aguilar \(1999\).](#page-6-0) Briefly, 0.3 ml of sample was mixed with 3.0 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95 °C for 90 min under water bath. Absorbance of all the sample mixtures was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid in milligram per gram of extract.

2.6. Reducing power

Reducing power of crude methanolic extract obtained brown seaweeds was determined by the method explained by [Oyaizu \(1986\).](#page-6-0) Briefly, 1.0 ml of methanol containing different concentration of sample was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium

ferricyanide (1%). Reaction mixture was incubated at 50 $^{\circ}$ C for 20 min. After incubation, 2.5 ml of trichloroacetic acid (10%) was added and centrifuged $(650g)$ for 10 min. From the upper layer, 2.5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml FeCl₃ (0.1%) . Absorbance of all the sample solutions was measured at 700 nm. Increased absorbance indicates increased reducing power.

2.7. DPPH radical scavenging activity

The scavenging effects of crude methanolic extract and fractions were determined by the method of [Yan and Chen](#page-6-0) [\(1995\)](#page-6-0). Briefly, 2.0 ml of 0.16 mM DPPH solution (in methanol) were added to the test tube containing 2.0 ml aliquot of sample. The mixture was vortexed for 1 min and kept 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. \(2006\)](#page-5-0). Sample blank and control samples were performed according to the method.

2.8. Deoxyribose radical scavenging activity

Deoxyribose non-site specific hydroxyl radical scavenging activity of methanolic extract and fractions were determined according to the method of [Chung, Osawa, and](#page-5-0) [Kawakishi \(1997\).](#page-5-0) Briefly, 2.0 ml aliquots of sample were added to the test tube containing reaction mixture of 2.0 ml $FeSO_4 \cdot 7 H_2O$ (10 mM), 0.2 ml EDTA (10 mM) and 0.2 ml deoxyribose (10 mM). The volume was made up to 1.8 ml with phosphate buffer (0.1 M, pH 7.4) and to that 0.2 ml $H₂O₂$ (10 mM) was added. The mixture was incubated at 37 °C under dark for 4 h. After incubation, 1 ml of TCA (2.8%) and TBA (1%) were added to the mixture, and then left to stand under boiling waterbath for 10 min. After treatment absorbance was measured at 532 nm. If the mixture was turbid, the absorbance was measured after filtration. Scavenging activity (%) was calculated using the equation given by [Heo, Park, Lee, and](#page-6-0) [Jeon \(2005\)](#page-6-0). All the other treatments were performed according to the method.

2.9. Statistical analysis

The experiments were carried out in four different batches of seaweeds. The means of all the parameters were examined for significance by analysis of variance (ANOVA) and in case of significance, mean separation was accomplished by Duncan's multiple range test using STATISTICA software (Statsoft, 1999).

3. Results and discussion

3.1. Extract and fractions yield

Yield of total methanolic extract and fractions of three brown seaweeds are given in [Table 1](#page-3-0). Among the total methanolic extracts of three seaweeds, P. tetrastomatica had a yield of 12.31% followed by T. conoides (5.76%) and S. marginatum (5.45%). Among the fractions, higher yield was observed in petroleum ether (28.40%), ethyl acetate (27.54%) and aqueous fraction (30.27%) of T. conoides. P. tetrastomatica and S. marginatum, respectively. The total extract yield of 12.1% and aqueous fraction yield of 36.36% was observed by [Duan et al. \(2006\)](#page-5-0) in red alga, Polysiphonia urceolata.

3.2. Total phenolic content

Phenolic compounds are commonly found in plants and have been reported to have several biological activities including antioxidant activity. Aqueous fraction of S. marginatum and T. conoides exhibited higher phenolic content of 24.61 and 49.16 mg GAE/g of seaweed extract (or 0.29 and 0.86 mg GAE/g of seaweed on dry weight basis), respectively ([Table 2](#page-3-0)), as compared to other fractions and total methanolic extract. Aqueous fraction of P. tetrasromatica also showed higher content (20.04 mg GAE/g of extract or 0.61 mg GAE/g seaweed on dry weight basis) as compared to phenolic content of other fractions from the same species ($P \le 0.05$). Earlier report revealed that phenolic compounds are one of the most effective antioxidants in brown algae ([Nagai & Yukimoto, 2003\)](#page-6-0). Phenolic content of total metholic extract of S. marginatum and P. *tetrastomatica* were not significantly different ($P > 0.05$); while, total methanolic extract of T. conoides had significantly higher phenol content as compared to that of other two seaweeds ($P \le 0.05$). [Kuda et al. \(2005\)](#page-6-0) observed the phenolic content of 0.18 mg catechin equivalents/g in ethanolic extract of brown seaweed, Papenfussiella kuromo.

3.3. Total antioxidant activity

Total antioxidant activity of total methanolic extract and fractions of three brown seaweeds are presented on [Table 3](#page-3-0). In this phosphomolybdenum method, Mo (VI) is reduced to form a green phosphate/Mo (V) complex. Higher activity of 39.62, 9.79 and 9.65 mg ascorbic acid equivalent/g extract (or 0.31, 0.08 and 0.17 mg ascorbic acid equivalent/g of seaweed on dry weight basis) was observed in EA fraction of S. marginatum, DCM fraction of P. tetrastomatica and aqueous fraction of T. conoides, respectively. The total antioxidant activity of total methanolic extracts was significantly different between the three seaweeds ($P < 0.05$). [Kumaran and Karunakaran \(2007\)](#page-6-0) have reported total antioxidant activity in the range of 245 to 376 mg ascorbic acid equivalents/g in the case higher plant extracts. Higher activity was observed in fractions as compared to total methanolic extract. Higher activity in fractions may be due to the interferences of other compounds present in crude (methanolic) extract; and, it has also been reported that solvents used for extraction have dramatic effect on the chemical species [\(Yuan, Bone, &](#page-6-0) [Carrington, 2005\)](#page-6-0). In addition, the fact that phenolic

Yield of total extract (as % w/w of seaweed on dry weight basis) and fractions (as % of total methanolic extract) of three brown seaweeds $(n=4)$

All the values are mean \pm SD; SD: Standard Deviation.

ME: Methanol; PE: petroleum ether; EA: ethyl acetate; DCM: dichloromethane; BuOH: butanol.

a,b: Column wise values with same superscripts of this type indicate no significant difference $(P > 0.05)$.

Table 2

All the values are mean \pm SD; SD: Standard Deviation.

ME: Methanol; PE: petroleum ether; EA: ethyl acetate; DCM: dichloromethane; BuOH: butanol.

a, b, c: Column wise values with same superscripts of this type indicate no significant difference $(P > 0.05)$.

p, q, r, s, t: Row wise values with different superscripts of this type indicate significant difference ($P < 0.05$).

Table 3

Total antioxidant activity (mg ascorbic acid equivalents/g extract) of total extract and fractions obtained from of three brown seaweeds (Concentration of extracts used $= 1000 \text{ µg}$) ($n = 4$)

All the values are mean \pm SD; SD: Standard Deviation.

ME: Methanol; PE: petroleum ether; EA: ethyl acetate; DCM: dichloromethane; BuOH: butanol.

a, b, c: Column wise values with same superscripts of this type indicate no significant difference $(P > 0.05)$.

p, q, r, s, t: Row wise values with different superscripts of this type indicate significant difference ($P \le 0.05$).

content of seaweeds was not different significantly $(P > 0.05)$ barring T. conoides indicates the role of other compounds in antioxidant activity of seaweed extracts. It has been earlier reported that some major active compounds from brown seaweed that have antioxidative properties are phlorotannins and fucoxanthin [\(Yan, Li, Zhou,](#page-6-0) [& Fan, 1996; Yan, Chuda, Suzuki, & Nagata, 1999](#page-6-0)). Also, antioxidative activities of brown seaweeds can not be attributed to their characteristic pigment (fucoxanthin) or any other carotenoids alone ([Yan, Nagata, & Fan, 1998\)](#page-6-0).

3.4. DPPH radical scavenging activity

DPPH has been used extensively as a free radical to evaluate reducing substances ([Cotelle et al., 1996](#page-5-0)) and is a useful reagent for investigating the free radical scavenging activities of compounds ([Duan et al., 2006\)](#page-5-0). DPPH radical scavenging activity (%) of total methanolic extract and fractions of three brown seaweeds are presented on [Table](#page-4-0) [4.](#page-4-0) Methanolic extract of T. conoides showed significantly higher activity ($P \le 0.05$) of 17.35% followed by P. tetrastomatica (14.78%) and *S. marginatum* (11.00%). Scavenging activity of 23.16% was observed in EA fraction of S. marginatum, 19.55 and 17.79% in PE fraction of T. conoides and P. tetrastomatica, respectively. The DPPH radical scavenging activity of enzymatic extract has been attributed to the ability of these extracts in pairing with the odd electron of DPPH radical ([Park et al., 2004](#page-6-0)). [Duan](#page-5-0) [et al. \(2006\)](#page-5-0) also observed higher radical scavenging activity in fractions when compared to crude extract. No activity was observed in DCM and BuOH fraction of S. marginatum, and BuOH fraction of P. tetrastomatica.

3.5. Deoxyribose scavenging activity

Deoxyribose scavenging activity (% inhibition) of total methanolic extract and fractions of three brown seaweeds are presented on [Table 5](#page-4-0). The striking aspect of deoxyribose assay is that it involves the hydroxyl radical which is the most active reactive oxygen species [\(Yan et al.,](#page-6-0) [1998\)](#page-6-0). The effect of extracts and fraction in scavenging OH radicals to prevent oxidative degradation of deoxyri-

Table 1

All the values are mean \pm SD; SD: Standard Deviation; ND: no activity detected; NA: Not analysed.

ME: Methanol; PE: petroleum ether; EA: ethyl acetate; DCM: dichloromethane; BuOH: butanol.

a, b, c: Column wise values with same superscripts of this type indicate no significant difference $(P > 0.05)$.

p, q, r, s, t: Row wise values with different superscripts of this type indicate significant difference ($P < 0.05$).

Table 5

Deoxyribose scavenging activity $(\%)$ of total extract and fractions obtained from of three brown seaweeds (Concentration of extracts used = 1000 µg) $(n = 4)$

All the values are mean \pm SD; SD: Standard Deviation; NA: not analysed.

ME: Methanol; PE: petroleum ether; EA: ethyl acetate; DCM: dichloromethane; BuOH: butanol.

a, b, c, d: Column wise values with same superscripts of this type indicate no significant difference ($P > 0.05$).

Row wise values with different superscripts of this type indicate significant difference ($P < 0.05$).

bose substrate was determined. The percent of inhibition was more than 90% in DCM, BuOH and aqueous fraction of all the three seaweeds. Lower inhibition rate of 47.81% was observed in PE fraction of T. conoides. However, deoxyribose scavenging acticity of total methanolic extracts from the three different seaweeds were statistically not significant ($P > 0.05$). Similarly, [Heo et al. \(2005\)](#page-6-0) found 47% inhibition in enzymatic extract of S. fulvellum and concluded that enzymatic extracts of seaweed possessed little effect on scavenging the hydroxyl radical. The ability of the seaweed extracts to quench reactive hydroxyl radical species has potential application to extend shelf life of products ([Yuan et al., 2005](#page-6-0)).

3.6. Dose dependency of antioxidative activities

Concentration dependency of antioxidant activity was investigated as a function of reducing power (Fig. 1) as this gives a general view of reductones present in the sample. Reducing power increased with increasing concentration in all the samples. Same trend has also been reported by [Kumaran and Karunakaran \(2007\)](#page-6-0) in methanolic extracts of higher plants. All concentrations exhibited the OD value <1.0. Similar findings were also reported by [Kuda et al.](#page-6-0) [\(2005\)](#page-6-0). This property is associated with the presence of reductones that are reported to be terminators of free radical chain reaction ([Duh, 1998\)](#page-6-0). Also, it was observed that at any given concentration (between 100 and $1000 \mu g$)

Fig. 1. Reducing power of total methanolic extract obtained from three brown seaweeds.

methanolic extracts of T. conoides and P. tetrastomatica had higher reducing power than S. marginatum. The total methanolic extract of T. conoides showed higher reducing power (from 100 to 1000 µg concentrations) as compared to α -tocopherol (Fig. 1). Similarly, *P. tetrastomatica* exhibited higher reducing activity up to a concentration of 500 lg as compared to the positive control.

Effect of concentration of antioxidant compounds on the DPPH radical scavenging and deoxyribose scavenging activity are shown in Figs. 2 and 3. The results indicate that the scavenging activity of seaweed extracts were concentration dependent. However, the positive control $(\alpha$ -tocopherol) did not show dose dependency and had a DPPH and hydroxyl radical scavenging activities of >95% and 78%, respectively [\(Tables 4 and 5\)](#page-4-0). The scavenging activity $(\%)$ was found to increase with increasing concentration of extract in both the DPPH and deoxyribose assay. However, the methanolic extracts showed less DPPH scavenging activity (Fig. 2) as compared to standard antioxidant (a-tocopherol). This present finding corroborates well with earlier reports in other higher plants including brown/red seaweeds [\(Kuda et al., 2005; Kumaran & Karunakaran,](#page-6-0) [2007\)](#page-6-0) and their enzymatic extracts ([Park et al., 2004\)](#page-6-0).

Fig. 2. DPPH radical scavenging activity (%) of total methanolic extract from three brown seaweeds.

Fig. 3. Hydroxyl radical scavenging activity (%) of total methanolic extract from three brown seaweeds as assessed by deoxyribose assay.

Methanolic extracts of all the three brown seaweeds showed higher hydroxyl radical scavenging activity, in dose dependent manner, compared to standard antioxidant (Fig. 3). The seaweed extracts were effective in scavenging hydroxyl radicals to prevent degradation of deoxyribose substrate in a dose dependent manner elaborates the fact that these seaweed extracts are able to prevent deoxyribose damage associated with the direct binding of iron to deoxyribose and the subsequent attack by hydroxyl radicals generated via the Fenton reaction [\(Yuan et al., 2005\)](#page-6-0).

4. Conclusions

It can be concluded that seaweeds or marine macroalgae can be utilized as a source of natural antioxidant compounds as their crude extracts and fractions exhibit antioxidant activity. The results indicate that different solvent fractions obtained from total (methanolic) extract exhibit higher antioxidant activities as compared to the total extract. The findings of this work are useful to further research to identify, isolate and characterize the specific compound which is responsible for higher antioxidant activity. Bioactive compounds found in seaweeds await a major breakthrough for a variety of food/medical applications as they have the potential for application of such compounds as natural antioxidants in different food/ pharamaceutical products.

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