ANTI-OXIDANT POTENTIAL OF INDIAN *PORPHYRA*

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Summary

The present study explores the anti-oxidant potential exhibited by the different fractions of red alga, *Porphyra vietnamensis* (Bangiales, Rhodophyta). Antioxidant activity of aqueous and alcoholic extracts as well as an isolated polysaccharide, Porphyran was determined using various *in vitro* models like 1,1-Diphenyl-2-picrylhydrazyl (DPPH), nitric oxide, superoxide, hydroxyl and peroxide radical scavenging activity. The anti-lipid peroxidation potential and total phenolic content were also determined. Preliminary phytochemical screening of drug showed the absence of alkaloid, essential oil, coumarin and steroidal content however it gave the confirmation for presence of carbohydrates, resins, pigments, glycosides, tannins, gums, mucilages, amino acids, saponins and phenols. Among the various fractions, phenol content ranged from 513.0 to 941.0 mg gallic acid equivalent (GAE) /100 g dry sample with maximum content found in polysaccharide fraction, porphyran (941.0 mg GAE/gm DW). Porphyran also showed maximum antioxidant activity with an IC_{50} value of 0.272 mg/mL for DPPH radical, 0.258 mg/mL for H_2O_2 radical, 0.301 mg/mL for superoxide radical and 0.233 mg/mL for nitric oxide radical. Porphyran even showed maximum anti-lipid peroxidation effect (0.512 mg/mL).The results were significant as compared to standard antioxidants such as l-ascorbic acid and α-tocopherol. On the basis of above results it was concluded that the various methanol, aqeous and polysaccharide fractions of *Porphyra* showed significant antioxidant activity.

Key words: *Porphyra vietnamensis*, Free radicals, porphyran, polar pigments.

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Introduction

Oxidative modification of DNA, proteins, lipids and small cellular molecules by reactive oxygen species (ROS) plays a role in a wide range of common diseases and age-related degenerative conditions (1). A vast amount of circumstantial evidence implicates oxygen-derived free radicals (especially superoxide and hydroxyl radical) as mediators of inflammation, shock, and ischemia/reperfusion injury. Furthermore the radicals also play a role in the process of ageing and carcinogenesis (2). The free radical theory of ageing suggests that damage produced by the interaction of such free radicals with cellular macromolecules results in cellular senescence and ageing (3). Furthermore, interest in employing antioxidants from natural sources to increase the shelf life of foods is considerably enhanced by consumer preference for natural ingredients and concerns about the toxic effects of synthetic antioxidants. Seaweeds have recently received a great deal of attention from scientific researchers. A number of investigators have found that these traditional sources of food provide not only nutritional benefits, but also help fight diseases and contribute to the maintenance of good health (4). Therefore, algal species as alternative materials to extract natural anti oxidative compounds have attracted much attention of biomedical scientists. There are some evidences that seaweeds contain compounds with a relatively high antioxidant and antiproliferative activity. Certain fatty-acids, polyphenolic and phyco-billiproteins content exert free radical scavenging activity (5). Additionally sulfated polysaccharide and mycosporines further helps in reducing oxidave stress. *Porphyra* (Bangiales, Rhodophyta) popularly known as 'Nori' in Japan, 'Kim' in Korea and 'Zicai' in China has an annual value of over US $ 1.8 billion. It is also used as a drug in traditional Chinese medicine (6-9). Numerous reports regarding its anti-oxidant (10-11), anti-cancer (12-13), anti-aging (14), anti-fatique (15), anti-coagulants anti-hyperlipidimic (16), sunscreen agent (17) and anti-viral (18) activities has been found. Various speices has been explored so far, however *P.vietnamensis* still needs more attention. The aim of this study is to evaluate the *in vitro* antioxidant activity of *P.vietnamensis* methanol extract, aqeous extract (polar pigments) and isolated polysaccharide compound, porphyran and to characterize the relationship between chemical constituents and antioxidant potential.

Methods

Collection of algal material

Algal sample of *P.vietnamensis* was collected from the Ratnagiri coast, Maharashtra, India. The algal sample was authenticated by the Chief Botanist, Botany department, Pune university. (Ref. No. : Bot/ 571 /09)

Drugs and Chemicals

Chemicals used in this study were of analytical grade. Sodium nitroprusside, sulphanilamide, N-(1-naphthyl) ethylene diamine dihydrochloride, thiobarbituric acid, potassium nitrite, potassium ferricyanide, ascorbic acid, and ferric chloride were supplied by Sigma Chemicals. 1,1-Di-Phenyl-2-picrylHydrazyl, nitro blue tetrazolium, riboflavin and deoxyribose were purchased from Hi Media Chemicals Ltd. Mumbai, India. All reagents used for the experiment were of the analytical grade.

Preparation of extract

Alcoholic fraction

Algal material of *P.vietanmensis* was collected, thoroughly washed and dried at 55ºC in an air dryer for 48 h. Dried materials were powdered separately with a Wiley mill (Model 4276-M, Thomas Scientific, USA) to pass a 20 mesh sieve and stored in sealed plastic bags. About 500 mg of the various powders were taken in 5 mL volumetric flask, mixed with 5 mL of MeOH and vortexed for 2 min followed by sonication (33 MHz, Roop Telesonic, India) at room temperature for 5 min. The process was repeated thrice for complete extraction. After sonication, methanolic extracts were combined and evaporated to dryness *in vacuo*. Dried extract was dissolved in methanol to prepare dilutions in a range of 100-500 µg/mL.

Polysaccharide fraction (Porphyran)

Porphyran was isolated according to the method of Nishide *et al.* (19-20). Dried "Nori" (225g) was soaked in 2250 ml of 7.5% formalin for 12 hr. at room temperature. An equal volume of water was added and the solution refluxed on boiling water for 8hr. The mixture was centrifuged at 10,000 R.P.M for 20 min and supernatant was filtered through diatomite. The resulting filtrate was adjusted to pH 7.0 with NaOH or HCL and then evaporated to 1/4 to 1/5 of its starting volume at 65°C. Four fold volumes of methanol were added to the residual solution to precipitate polysaccharide content. The mixture was centrifuged at 10,000 rpm for 20 min, and the supernatant was discarded. The precipitate was successively washed 2-3 times with 80% aqueous methanol and acetone. The residue was freeze-dried, after evaporating the acetone under a blast of hot air, to yield white powdered polysaccharide. The polysaccharide obtained was treated with $NaBH_4$–NaOH a, 100 mg of polysaccharide was dissolved in 20 mL distilled water with 0.05% (w/v) $NaBH_4$ and maintained overnight under stirring at 20°C. NaOH (3 M) containing 0.3% (w/v) $NaBH_4$ was added up to 0.6 M as the final concentration. After being maintained under agitation for 3 h at 80°C, the solution was neutralized with HCl, dialyzed exhaustively against distilled water, and freeze-dried. Now the sample is again heated with 1M NaOH at 50, 60, 70, 80, 90°C and 100°c. Samples were taken at intervals, the reaction stopped and the solution neutralized with 1 M hydrochloric acid. Finally acid hydrolysed aqueous extract was lyophilized.

Pigments fraction

1. For polar pigments

One gram of dried algae was taken and dipped it in sufficient quantity of water. The material was placed in mortar and triturated for 5 min. After that added one gram of sand and triturated till pink colour of sample did not appear. After that it was centrifuged at 1200rpm. The supernatant was collected and left material was discarded.

2. For non polar pigments

To one gram of dried sample sufficient quantity of acetone was added to make it wet and then placed in to mortar for trituration. After 5 min of trituration add one gram of sand and again triurate it for 10 min. all the material was collected in 50 ml of centrifuge tubes and centrifuged at 1200rpm. The supernatant was collected and the left residual material was discarded.

Preliminary phytochemical screening
Preliminary phytochemical screening was carried out by using standard procedures (21, 22).

Determination of total phenolic content
The total phenolic content (TPC) of the methanolic extract of *P.vietnamensis* was determined by the method of Folin-Ciocalteu reaction using gallic acid as standard (23). To 100 μL of extract (100 μg/mL), add 500 μL of (50%) Folin-Ciocalteu reagent followed by the addition of 1 mL of 20% Na_2CO_3 solution. A mixture was incubated at room temperature for 20 min. and the absorbance was measured at 730 nm. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram samples.

Anti-oxidant studies

Determination of 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity
The antioxidant activity, based on scavenging of stable DPPH free radical, was determined (24). Different concentrations of test sample were added to 3 mL of 0.004% methanol solution of DPPH. Absorbance at 517 nm was measured after 30 min and the percent inhibition activity was calculated as:
DPPH Scavenged (%) = (A cont. - A test) / (A cont.) x 100
Where, A cont. = Absorbance of control reaction
A test = Absorbance of test reaction
The antioxidant activity of the extract was expressed as IC_{50}.

Determination of nitric oxide (NO) radical scavenging activity
Nitric oxide (NO) was generated from sodium nitropruside (SNP) and measured by Greiss reaction. SNP in aqueous solution at physiological pH instinctively generates NO, which intermingles with oxygen to generate nitrite ions that can be anticipated by Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). Scavengers of NO with oxygen leading to reduced production of NO. SNP (5 mM) in phosphate buffer saline (PBS) was mixed with different concentration of (100-500 μ g/mL) drug dissolved in suitable solvent and incubated at 25°C for 150 min. The above samples were reacted with Greiss reagent. The absorbance of chromophore created during diazotization of nitrite with sulphanilamide and following coupling with naphthyl ethylene diamine was read at 546 nm and referred to the absorbance of standard solution of potassium nitrite treated in the same way with Greiss reagent (25).

Superoxide anion (O_2^{-}) radical scavenging activity
Measurement of superoxide anion (O_2^{-}) scavenging activity of extracts was based on the method described with slight modification (26, 27). O_2^{-} radicals are generated non-enzymatically in Phenazine methosulphate–Nicotinamide adenine dinucleotide phosphate (PMS–NADH) systems by the oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide radicals were generated in 1 mL of Tris-HCl buffer (16 mM, pH 8.0) containing NBT (50 μM) solution and NADH (78 μM) solution. The reaction was started by adding PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm in a spectrophotometer was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

(%) I = (A0 – A1) / (A0) x 100
where A0 was the absorbance of the control and A1 was the absorbance of extract and the standard compound.

Determination of hydroxyl radical (OH^-) scavenging activity
Non-site-specific hydroxyl radical (OH^-) scavenging activity assay was evaluated by a previously reported method (29). The mixture containing FeCl3 (100 µM), ascorbic acid (100 µM), ethylene diamine tetra acetic acid (EDTA, 100 µM), H2O2 (10 mM), deoxyribose (2.8 mM), and test sample in 500 µL phosphate buffered saline (PBS, 20 mM, pH 7.4) was incubated for 1 h at 37°C. After adding 250 µL of trichloroacetic acid (10% w/v) and 250 µL of thiobarbituric acid (1% w/v), the reaction mixture was boiled for 15 min in a water bath. The colour development was measured at 532 nm and the scavenging activity of test sample was expressed as the percentage inhibition of the deoxyribose degradation to malondialdehyde. The site specific assay for hydroxyl radical scavenging activity was performed in a similar manner to that of the non site specific assay, except that EDTA was discarded.

Determination of H_2O_2 radical scavenging activity
The ability of extract to scavenge H_2O_2 was determined. A solution of H_2O_2 was prepared in PBS (pH 7.4). H_2O_2 concentration was determined spectrophotometrically, by measuring absorption with extinction coefficient for H_2O_2 of 81 m-1 cm-1. Extracts (100-500 µ g/mL) in distilled water were added to H_2O_2 solution (0.6 mL, 40 mM). Absorbance of H_2O_2 at 230 nm was determined 10 min later against a blank solution containing PBS without H_2O_2 (30).

Lipid peroxidation assay
Thuong et al. (2007) reported that inhibitory activity of kudingcha against mitochondrial lipid peroxidation was measured by the thiobarbituric acid reactive substance (TBARS) method. They also reported the preparation of mitochondria and measurement of lipid peroxidation (LPO) was reported previously (31).

Statistical analysis
Experimental results were mean ±SEM of three parallel measurements. Analysis of variance was performed by ANOVA followed by Newmans-Keul multiple comparison test. $p < 0.05$ was considered as significant.

Results and Discussion

Preliminary phytochemical screening
Phyto-chemical screening proved that *Porphyra* fractions had no alkaloid, essential oil, coumarin and steroidal content however it gave the confirmation for carbohydrates, resins, pigments, glycosides, tannins, gums and mucilages, amino acids, saponins and phenols (Table 1).

Total phenolic content
The antioxidant activity of natural products is due to the antioxidant principles like polyphenols, carotenoids, and vitamins C and E . Polyphenols constitute a large and complex category of antioxidants including flavonoids, the largest and most-studied group of polyphenols (32). The total phenol content of porphyran (941 mg GAE /gm DW) was found

to be highest and the methanol extracts showed lowest phenol content (513 mg GAE /gm DW) (Table 2).

DPPH radical scavenging activity
Different samples of *P.vietnamensis* significantly ($p< 0.05$) scavenged DPPH radical in a concentration dependent manner. The polysaccharide fraction showed more scavenging activity than other isolated fractions with IC_{50} value of 0.272 mg/mL (Table 3). The antioxidant reacts with DPPH radical (purple colour) and converts it into a colourless α-α-diphenyl-β-picrylhydrazyl. It has characteristic absorbance maxima at 517 nm, widely used to evaluate the free radical scavenging effect of natural antioxidants (33). Many researchers have reported positive correlation between free radical scavenging activity and total phenolic compound. DPPH radical scavenging activity increased with the increase of phenolic compound content (34-36).

Nitric oxide radical scavenging activity
The different fractions quenched NO released by Sodium nitroprusside (nitric oxide donor). The fractions significantly and dose dependently decreased the release of NO. ROS like O_2^{-} may react with NO results in reactive nitrogen species (RNS) such as NO_2, N_2O_4. Both ROS and RNS causing cellular damage (37). Again porphyran showed maximum scavenging activity with IC_{50} value 0.233 g/mL. All plant materials showed significant scavenging activity as compared to control (Table 3).

Superoxide radical scavenging activity
Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals (38). From the investigations, it was found that the all fractions inhibit superoxide radicals in a dose dependent manner. In the PMS/NADH-NBT system, superoxide anions derived from dissolved oxygen by PMS/NADH coupling reaction reduce NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anions in the reaction mixture. The porphyran fraction again exhibited maximum superoxide scavenging activity (IC_{50} value 0.301) (Table 3).

H_2O_2 radical scavenging activity
H_2O_2 radical itself not very reactive but it can sometimes be toxic to cell because it generates OH^{-} radical in the cell (39). Porphyran significantly inhibited peroxide radical with IC_{50} value 0.258 mg/mL (Table 3).

Lipid peroxidation assay
Decomposition of lipid membrane in the body leads to the formation of Malondialdehyde (MDA) along with other aldehydes and enals as the end product. These react with thiobarbituric acid to form coloured complexes. Hence these are called as the Thiobarbituric Acid Reactive Substances (TBARS). The complex of TBA-MDA is selectively detected at 532 nm using UV spectrophotometer. Porphyran showed maximum anti-lipid peroxidation effect in liver homogenate with IC_{50} value of 0.512 mg/mL (Table 3). The result showed that inhibition of TBARS formation in rat liver homogenate increased with increasing concentration. (40)

Inhibition of OH^- radical

The highly reactive OH^- radical can cause oxidative damage to DNA, lipid and proteins. The effect of extract on the inhibition of free radical mediated deoxyribose damage was assessed by means of iron (II) dependent DNA damage assay. Among all these porphyran showed maximum inhibition with IC_{50} value of 0.434 mg/mL in comparison with polar pigments and methanol extract with IC_{50} value of 0.312 mg/mL and 0.234 mg/mL (Table 7.5.1).

Table 1: Phytochemical tests of petroleum ether, aqueous and alcohol extract of *Porphyra Sp.*

Chemical test	Pet.ether extract	Alcohol extract	Aqueous extract
Alkaloids	-	-	-
Glycosides	-	-	+
Resins	-	-	+
Tannins	-	+	+
Coumarins	-	-	-
Phenols	-	+	+
Saponin	+	-	+
Essential oil	-	-	-
Fixed oil	+	+	-
Gums and Mucilages	-	-	+
Carbohydrates	-	-	+
Amino acids	-	+	+
Terpenoids	-	+	-
Steroids	-	-	-
Pigments	-	-	+

(+ Present, - Absent)

Table 2: Total phenol content in different samples of *P. vietnamensis*

Samples	Phenol Content (mg GAE/100 g sample)
Porphyra (alcohol extract)	513.0±0.01**
Polar Pigments	712.0±0.05*
Porphyran	941.0±0.001***

All values are expressed as mean ± SEM (n=3);

*P<0.05 considered significant as compare to control

Table 3: Free radical scavenging activity of *P. vietnamensis*

IC_{50} value (mg/ml)

Free radical	Porphyra (alcohol extract)	Polar Pigments	Porphyran
DPPH	0.223±0.05*	0.270±0.01**	0.272±0.01**
H_2O_2	0.223±0.05*	0.230±0.001***	0.258±0.05*
Superoxide	0.235±0.001***	0.259±0.05*	0.301±0.01**
Nitric Oxide	0.236±0.001***	0.279±0.01**	0.233±0.001***
Hydoxyl	0.234±0.001***	0.312±0.01**	0.434±0.05*
LPO (Liver)	0.418±0.01**	0.467±0.05*	0.512±0.05*

All values are expressed as mean ± SEM (n=3);

*P<0.05 considered significant as compared to control.

Conclusion

Various species of *Porphyra* are widely studied by the researchers for their food and non-food applications. Besides of various pharmaceutical applications, various nations utilized this red alga for dietary purposes. However, Indian Porphyra is unexplored yet. This nutritious alga contains wide amount of anti-oxidative agents. At present several reports on the anti-oxidant activity of its algal content i.e. sulfated polysaccharide and mycosporines, are widely accumulating. These researches encouraged us to evaluate the anti-oxidant potential and thus we found that *Porphyra vietnamensis* is a rich source of natural antioxidants.
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