1. Introduction

All living organisms contain complex systems of antioxidant enzymes and chemicals. Some of these systems, like the thioredoxin system, are conserved throughout evolution and are required for life. Antioxidants in biological systems have multiple functions, including defending against oxidative damage and participating in the major signaling pathways of the cells. One major action of antioxidants in cells is to prevent damage caused by the action of reactive oxygen species. Reactive oxygen species include hydrogen peroxide (H$_2$O$_2$), the superoxide anion (O$_2^-$), and free radicals such as the hydroxyl radical (·OH). These molecules are unstable and highly reactive, and can damage cells by chemical chain reactions such as lipid peroxidation, or formation of DNA adducts that could cause cancer-promoting mutations or cell death. In order to reduce or prevent this damage all cells invariably contain antioxidants.

Lipid oxidation by reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals, and hydrogen peroxide causes a decrease in nutritional value of lipids, in their safety and appearance. In addition, it is the predominant cause of qualitative decay of foods, which leads to rancidity, toxicity, and destruction of biochemical components important in physiologic metabolism. Free radicals-mediated modification of DNA, proteins, lipids, and small cellular molecules are associated with a number of pathological processes, including atherosclerosis, arthritis, diabetes, cataractogenesis, muscular dystrophy, pulmonary dysfunction, inflammatory disorders, ischemiareperfusion tissue damage, and neurological disorders such as Alzheimer’s disease (Frlich & Riederer, 1995).

Antioxidants are classified by the products they form on oxidation (these can be antioxidants themselves, inert, or pro-oxidant), by what happens to the oxidation products (the antioxidant may be regenerated by different antioxidants or, in the case of "sacrificial" antioxidants, its oxidized form may be broken down by the organism) and how effective the antioxidant is against specific free radicals. Several synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) are commercially available and currently used. However, these antioxidants have
been restricted for use in foods as they are suspected to be carcinogenic. Some toxicological studies have also implicated the use of these synthetic antioxidants in promoting the development of cancerous cells in rats. These findings, together with consumers’ interests in natural food additives, have reinforced the efforts for the development of alternative antioxidants from natural origins (Huang & Wang, 2004). An immense number of marine flora and fauna are reported to have wide spectrum of interesting biological properties. In folk medicines seaweeds have been used for a variety of remedial purposes for the treatment of eczema, gallstone, gout, crofula, cooling agent for fever, menstrual trouble, renal problems, scabies, etc. (Chapman & Chapman, 1976).

Apart from the nutritional qualities the seaweeds are now being considered to be a rich source of antioxidants (Nagai & Yukimoto, 2003). Marine algae, like other photosynthesizing plants, are exposed to combination of light and high oxygen concentrations that lead to the formation of free radicals and other strong oxidizing agents (Dykens et al., 1992). The absence of oxidative damage in the structural components (polyunsaturated fatty acids) (Matsukawa et al., 1997) and stability to oxidation during storage (Ramarathnam et al., 1995) in the seaweeds forms a source to use them as antioxidants.

Seaweeds are rich in polysaccharides, minerals, proteins and vitamins. Documented antioxidant activity would elevate their value in the human diet as food and pharmaceutical supplements (Yan et al., 1998). Few reports are available on the antioxidant potential of seaweeds (Jimenez-Escrig et al., 2001). Ismail & Hong (2002) reported antioxidant activity of four commercial edible seaweeds namely Nori (Porphyra sp.), Kumbu (Laminaria sp.), Wakame (Undaria sp.) and Hijiki (Hijikia sp.).

The Rhodophyta (red algae) are a distinct eukaryotic lineage characterized by the accessory photosynthetic pigments phycoerythrin, phycocyanin and allophycocyanins arranged in phycobilisomes. They contain a large assemblage of species that predominate the coastal and continental shelf areas of tropical, temperate and cold-water regions. Red algae are ecologically significant as primary producers, providers of structural habitat for other
marine organisms, and they play an important role in the primary establishment and maintenance of coral reefs. Some red algae are economically important as providers of food and gels (Wilson, 2000). For this reason, extensive farming and natural harvest of red algae occur in numerous areas of the world. *Kappaphycus alvarezii*, an economically important red tropical seaweed which is highly demanded for its cell wall polysaccharide, is the most important source of kappa carrageenan. The world production of *Kappaphycus species* is approximately 28000 tons per annum. This seaweed accounts for the largest consumption worldwide (McHugh, 1987). It is easily accessible in huge amounts for food and pharmaceutical applications. The present study deals with antioxidant properties of *K. alvarezii*.

2. Materials and Methods

2.1. Collection of samples

*Kappaphycus alvarezii* was collected from a cultivation site at Port Okha (L 22° 28.528’ N; L 069° 04.322’ E) located on the North West coast of India during April 2006. The sample was thoroughly washed with seawater to remove epiphytes and dirt particles followed by shade drying for two days. It was then brought to the laboratory, oven dried at 70 ºC for 4 h to obtain a constant weight and pulverized in the grinder (size 2 mm). This sample was used for determination of phenolic content as well as for antioxidant studies. The chemicals used in these studies were of analytical grade.

2.2. Preparation of Extracts

The pulverized moisture free sample (20 g) was extracted with 200 ml of individual solvents using a Soxhlet extractor. The extraction was repeated many times to obtain a sizable quantity of extract. Consequently, the extract was concentrated in a rotary evaporator at 40ºC. Different solvents were used for the preparation of extracts to determine the antioxidant efficacy of *K. alvarezii*. All the experiments were conducted in triplicate.
2.3. Determination of total phenol

Total phenolic content was estimated by Folin–Ciocalteau method (Singleton & Rossi, 1965). To 6.0ml double distilled water, 0.1 ml sample and 0.5 ml Folin–Ciocalteau reagent was mixed followed by the addition of 1.5 ml Na₂CO₃ (20 g 100 ml⁻¹ water) and the volume was made up to 10.0 ml with distilled water. After incubation for 30 min at 25 °C, the absorbance was measured at 760 nm and the total phenolic content was calculated with gallic acid standard and expressed as percentage of total phenols obtained on dry weight basis.

2.4. DPPH Radical Scavenging Assay

DPPH scavenging potential of different fractions was measured based on scavenging ability of stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals by *K. alvarezii* antioxidants. The ability of extracts to scavenge DPPH radicals was determined according to the method of Blois (1958). Briefly, 1 ml of 1 mM methanolic solution of DPPH was mixed with 1 ml of extract solution (containing 0.5-5.0 mg ml⁻¹ of dried extract). The mixture was then vortexed vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and activity was expressed as percentage DPPH scavenging activity relative to control using the following equation:

\[
\% \text{ Radical scavenging activity} = \left[ \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right] \times 100
\]

2.5. Ferrous ion chelating activity

Iron chelating abilities of methanol, ethanol and ethyl acetate extracts of *K. alvarezii* were used for the present investigation. The chelating of ferrous ions by the extracts and standards was estimated by the method of Dinis et al. (1994). Extracts were added to a solution of 2mM FeCl₂ (0.05 ml). The reaction was initiated by the addition of 5mM ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured at 562 nm. The percentage inhibition of ferrozine – Fe²⁺ complex formation was determined using the following formula:
\[
\% \text{ Inhibition} = \left[1 - \frac{A_{1\text{Sample}}}{A_{0\text{Control}}} \right] \times 100
\]
Where \(A_0\) was the absorbance of the control and \(A_1\) was the absorbance in the presence of the sample extracts and standards. The control contains \(\text{FeCl}_2\) and ferrozine, with complex formation molecules.

2.6. Reducing power

Extracts of \(K.\) alvarezii were prepared using methanol, ethanol, water, ethyl acetate and hexane. The reductive potential of extracts was determined by the method of Oyaizu (1986). The different concentrations of extracts (0.5-25 mg ml\(^{-1}\)) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide \([\text{K}_3\text{Fe(CN)}_6]\) (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then subjected to centrifugation (10 min, 1000g). The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and \(\text{FeCl}_3\) (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reductive potential.

2.7. Antioxidant activity in linoleic acid system with ferrothiocyanate reagent (FTC).

Ethanolic extract of \(K.\) alvarezii was subjected to the assay adopted by Osawa and Namaki (1983). The extract (4 mg) was dissolved in 99.5% ethanol and mixed with 2.5% linoleic acid in 99.5% ethanol (4.1 ml), 0.05 M phosphate buffer (pH = 7, 8 ml) and distilled water (3.9 ml) and kept in screw cap containers under dark conditions at 40 °C; 0.1 ml of this solution was added to 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. After 3 min, 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture, the absorbance of red colour was measured at 500 nm in the spectrophotometer, for every two days. The control and standard were subjected to the same procedure except for the control, where there was no addition of sample and for the standard 4 mg of sample was replaced with 4 mg of Butylated hydroxy toluene (BHT) used as a positive control. Absorbance was measured at intervals of 2 days. The percent inhibition of linoleic acid peroxidation was calculated as:
Inhibition (%) = 100 - [(absorbance increase of the sample/absorbance increase of the control) × 100].

The IC50 value represented the concentration of the compounds that caused 50% inhibition. All experiments were carried out in triplicate.

2.8. Statistical analysis

For the extract, three samples were prepared for each experiment. The data were presented as mean ± standard deviation.

3. Results and Discussion

3.1. Antioxidant activity

The antioxidant activity is system dependent. Moreover it depends on the method adopted and the lipid system used as substrate (Singh et al., 2006). Hence, different following methods have been adopted in order to assess the antioxidative potential of *K. alvarezii* extracts.

3.2. Total phenol content

A number of studies have focused on the biological activities of phenolic compounds, which are potential antioxidants and free radical scavengers (Rice-Evans et al., 1995; Marja, Ka¨hko¨nen et al., 1999; Sugihara et al., 1999). The total phenol content was maximum when a mixture of chloroform and methanol (2:1) was used (2.05 ± 0.038 %) followed by ethanol (1.94 ± 0.029 %), methanol (1.79 ± 0.77 %), n-propanol (1.40 ± 0.040 %) and ethyl acetate (1.09 ± 0.597 %). Extracts obtained using other solvents, viz. acetone, n-hexane and chloroform, showed < 1% total phenol content (Table 1).
3.3. Scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH)

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical is a stable radical with a maximum absorbance at 517 nm that can readily undergo reduction by an antioxidant. Because of the ease and convenience of this reaction, it has now widespread use in the free radical scavenging activity assessment (Brand-Williams et al., 1995). The radical scavenging activity of *K. alvarezii* extract is shown in Figure 1 and expressed as percentage reduction of the initial DPPH absorption by the tested compound. The best radical scavenging activity could be obtained in the ethanol extract (IC$_{50}$ 3.03 mg.ml$^{-1}$) followed by methanol (IC$_{50}$ 4.28 mg.ml$^{-1}$). Extracts obtained using water also showed equivalent scavenging activity (IC$_{50}$ 4.76 mg.ml$^{-1}$). These values were lower than those obtained using BHT (IC$_{50}$ 2.83 mg ml$^{-1}$), but the IC$_{50}$ values of the methanol and water extracts were comparable with α-tocopherol (IC$_{50}$ 4.55 mg.ml$^{-1}$). The extracts of *K. alvarezii* showed better radical scavenging activity than did the extract of *Palmaria pamata* (dulse) IC$_{50}$ - 12.5 mg ml$^{-1}$ (Yuan et al., 2005), and purified extract of *Ecklonia cava* IC$_{50}$ - 5.49 x 10$^{-3}$ µg.ml$^{-1}$ (c.f. Suja et al., 2005). Ragan & Glombitza (1986) reported the radical scavenging activity of seaweeds to be mostly related to their phenolic contents. On the other hand, Siriwardhana et al., (2003) and Lu & Foo (2000) reported a high correlation between DPPH radical scavenging activities and total polyphenolics $r = (0.971)$. In the present study the linear regression analysis of DPPH scavenging (i.e EC$_{50}$ values) with the total phenol content (gallic acid equivalents) gave an $r$ value of 0.937, showing statistically significant correlation. *K. alvarezii* is the main industrial source of carrageenan (having alternating D-galactose 4-sulphate and 3, 6- anhydro D-galactose residues), which may also contribute to the antioxidant potential of this seaweed. Components such as low molecular weight polysaccharides, pigments, proteins or peptides also influence the antioxidant activity (Siriwardhana et al., 2003).

3.4. Ferrous ion-chelating activity

All the extracts demonstrated reasonable ferrous ion chelating efficacy (Figure 2). The ascorbic acid extract demonstrated best ferrous chelating efficacy (IC$_{50}$ 2.88 mg.ml$^{-1}$) followed by methanol, ethanol and ethyl acetate (IC$_{50}$ 3.08, 3.83 and 4.38 mg.ml$^{-1}$...
respectively). Iron is known to generate free radicals through the Fenton & Haber–Weiss reaction. Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalyzing transition metal in LPO. It is reported that chelating agents that form $\sigma$-bonds with a metal, are effective as secondary antioxidants since they reduce the redox potential thereby stabilizing the oxidized form of the metal ion (Srivastava et al., 2006). Metal binding capacities of dietary fibers are well known, e.g. the inhibitory effects on ferrous absorption of algal dietary fibers such as carrageenan, agar and alginate, were reported (Harmuth-Hoene & Schelenz, 1980). In this present study the carrageenan might have caused the decrease of ferrous ion in the assay system.

3.5. Measurement of reducing potential

The reducing power of *K. alvarezi* extracts was concentration dependent (Figure 3). As the concentration increased from 0.5 to 5.0mg.ml$^{-1}$, there was an increase in absorbance with all the solvents except hexane. However the reducing power of the samples were found in the following order: BHT (0.23-0.879) > Methanol (0.07-0.74) > Ethanol (0.333-0.44) > Ethyl acetate (0.013-0.467) > Water (0.017-0.193) > Hexane (0.017-0.16). It is believed that antioxidant activity and reducing power are related. Reductones inhibit LPO by donating a hydrogen atom and thereby terminating the free radical chain reaction (Srivastava et al., 2006).

3.6. Antioxidant activity in a linoleic acid system with ferrothiocyanate reagent (FTC).

Peroxy radicals are formed by a direct reaction of oxygen with alkyl radicals. Decomposition of alkyl peroxides also results in peroxyl radicals. Peroxyl radicals are good Oxidizing agents, having more than 1000 mV of standard reduction potential (Decker, 1998). They can abstract hydrogen from other molecules with lower standard reduction potentials. This reaction is frequently observed in the propagation stage of lipid peroxidation. Cell membranes are phospholipid bilayers with extrinsic proteins and are the direct target of lipid
oxidation (Girotti, 1998). As lipid oxidation of cell membranes increases, the polarity of lipid phase surface charge and formation of protein oligomers increase; and molecular mobility of lipids, number of SH groups, and resistance to thermal denaturation decrease. Malonaldehyde, one of the lipid oxidation products, can react with free amino group of proteins, phospholipid, and nucleic acids leading to structural modification, which induce dysfunction of immune systems. The antioxidant effects of K. alvarezii extract and BHT on the peroxidation of linoleic acid were investigated and the results are presented in (Figure 4). The absorbance range recorded for control, BHT and sample were 0.0087-0.0151, 0.0021-0.0093 and 0.0037-0.0104 respectively. The ethanolic extract of K. alvarezii showed higher inhibitory effect than did the positive control BHT. This might be due to the presence of ascorbic acid and vitamin A (β-carotene) content in the extract of K. alvarezii (Fayaz et al., 2005).

Algal polysaccharides play an important role as free-radical scavengers in vitro and antioxidants for the prevention of oxidative damage in living organisms. Their activity depends on several structural parameters such as the degree of sulfation (DS), the molecular weight, the sulfation position, type of sugar and glycosidic branching. Moreover, some reports reveal that the sulfate and phosphate groups in the polysaccharides lead to differences in their biological activities. In vitro antioxidant activity of κ-carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives have been reported by Yuan et al., (2005). They also reported that phosphorylated and sulfated glucans exhibited better antioxidant ability than did glucans and other neutral polysaccharides, which indicated that polyelectrolytes, such as glucan sulfate or phosphate, might have increased scavenging activity. Moreover, the sulfate content of polysaccharides from Porphyra yezoensis was reported to contribute to the antioxidant activity. The cell wall of K. alvarezii is known to be constituted of carrageenan, a sulfated polysaccharide, which may contribute to its antioxidant potential in addition to presence of ascorbic acid, vitamin A and various phenolics.
3.7. Conclusion

In the present investigation the various solvent extracts of *K. alvarezii* exhibited excellent scavenging effect (%) by DPPH assay, reducing power, ferrous ion chelating activity and antioxidant property in linoleic acid system. Thus, they could be used in nutraceutical and functional food applications. Since this is a preliminary study, a detailed investigation on composition of each component involved is absolutely necessary to establish appropriate applications which may open new frontiers for the human consumption of this seaweed world wide.

Table 1
Percent phenol content of *K. alvarezii* in various solvents

<table>
<thead>
<tr>
<th>Solvents</th>
<th>% Total phenol*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>0.963 ± 0.058</td>
</tr>
<tr>
<td>n-Propanol</td>
<td>1.404 ± 0.040</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>1.091 ± 0.597</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>0.830 ± 0.048</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.683 ± 0.040</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.793 ± 0.770</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.940 ± 0.029</td>
</tr>
<tr>
<td>Chloroform Methanol(2:1)</td>
<td>2.050 ± 0.038</td>
</tr>
</tbody>
</table>

*Values are means of three replicate determinations; SD, standard deviation.*
Figure 1. Antioxidant activities of different solvent extracts of *K. alvarezii* determined as DPPH· radical-scavenging activity.

Figure 2. Ferrous ion-chelating activities of different solvent extracts of *K. alvarezii*. 
Figure 3. Reducing power of *K. alvarezii* extracts along with synthetic antioxidants.

Figure 4. Inhibitory effect of *K. alvarezii* extract on the primary oxidation of linoleic acid system, using the ferric thiocyanate method.
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