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Antioxidant potential of solvent extracts of *Kappaphycus alvarezii* (Doty) Doty – An edible seaweed

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Abstract

Various solvent extracts of *Kappaphycus alvarezii*, an edible red seaweed (family Solieriaceae) were screened for total phenol content and antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferrous ion chelating activity, reducing power and antioxidant activity assays in a linoleic acid system with ferrothiocyanate reagent (FTC). The total phenol content of different extracts of *K. alvarezii* varied from $0.683 \pm 0.040\%$ to $2.05 \pm 0.038\%$. The radical-scavenging activity of ethanol extract was, as IC_{50} 3.03 mg ml^{-1}, whereas that of the water extract was IC_{50} 4.76 mg ml^{-1}. Good chelating activity was recorded for methanol extract (IC_{50} 3.08 mg ml^{-1}) wherein $67.0 \pm 0.924\%$ chelation was obtained using 5.0 mg ml^{-1} of extract. The reducing power of the samples was in the following order: BHT > methanol > ethanol > ethyl acetate > water > hexane. But, in the linoleic acid system, the ethanol extract proved superior to the synthetic antioxidants butylated hydroxytoluene (BHT). Hence, these extracts could be considered as natural antioxidants and may be useful for curing diseases arising from oxidative deterioration.

Keywords: *Kappaphycus alvarezii*; Antioxidant activity; Total phenolics; Free radicals; Reducing power

1. Introduction

All living organisms contain complex systems of antioxidant enzymes. Some of these systems, e.g. 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 signalling pathways of 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_2O_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 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 radical-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,

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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 of natural origin (Huang & Wang, 2004). An immense number of marine flora and fauna are reported to have a wide spectrum of interesting biological properties. In folk medicine, seaweeds have been used for a variety of remedial purposes, e.g. for the treatment of eczema, gallstone, gout, crofula, cooling agent for fever, menstrual trouble, renal problems and scabies (Chapman & Chapman, 1976).

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, Nagata, & Fan, 1998). Few reports are available on the antioxidant potential of seaweeds (Jimenez-Escrig, Jimenez-Jimenez, Pulido, & Saura-Calixto, 2001). Ismail and 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 in 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 28 000 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

K. 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 °C 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 40 °C. 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 6.0 ml of double-distilled water, 0.1 ml of sample and 0.5 ml of Folin–Ciocalteau reagent were mixed, followed by the addition of 1.5 ml of Na_2CO_3 (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 a gallic acid standard and expressed as a percentage of total phenols obtained on a dry weight basis.

2.4. DPPH radical scavenging assay

DPPH·-scavenging potential of different fractions was measured, based on the 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 by 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 the control, using the following equation:

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

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, Madeira and Almeida (1994). Extracts were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM 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–Fe^{2+} complex formation was determined using the following formula:

$$\% \text{ Inhibition} = \left[1 - A_{1\text{Sample}}/A_{0\text{Control}}\right] \times 100$$

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 contained $FeCl_2$ 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 [$K_3Fe(CN)_6$] (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, 1000*g*). The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (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 the 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 the red colour was measured at 500 nm in the spectrophotometer every two days. The control and standard were subjected to the same procedure except that for the control, 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:

$$\text{Inhibition } (\%) = 100 - [(\text{absorbance increase of the sample}/\text{absorbance increase of the control}) \times 100]$$

The IC_{50} 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, Maurya, de Lampasona, & Catalan, 2006). Hence, the following different 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 focussed on the biological activities of phenolic compounds, which are potential antioxidants and free radical-scavengers (Kähkönen et al., 1999; Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995; Sugihara, Arakawa, Ohnishi, & Furuno, 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, namely 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, Cuvelier, & Benset, 1995). The radical-scavenging activity of *K. alvarezii* extract is shown in Fig. 1 and expressed as percentage reduction of the initial DPPH· absorption by the tested compound. The best radical-scavenging activity could be obtained in

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

Solvents	Total phenol (%)
Acetone	0.963 ± 0.058
n-Propanol	1.40 ± 0.040
Ethyl acetate	1.09 ± 0.597
n-Hexane	0.83 ± 0.048
Chloroform	0.683 ± 0.040
Methanol	1.79 ± 0.77
Ethanol	1.94 ± 0.029
Chloroform:methanol (2:1)	2.05 ± 0.038

Values are means of three replicate determinations; SD, standard deviation.

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 palmata* (dulse) IC_{50} – 12.5 mg ml^{-1} (Yuan, Carrington, & Walsh, 2005a), and purified extract of *Ecklonia cava* IC_{50} – 5.49 × 10^3 μg ml^{-1} (c.f. Suja, Jayalekshmy, & Arumughan, 2005). Ragan and Glombitza (1986) reported the radical-scavenging activity of seaweeds to be mostly related to their phenolic contents. On the other hand, Siriwardhana, Lee, Kim, Ha, and Jeon (2003) and Lu and 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. Metal ion-chelating activity

All the extracts demonstrated reasonable ferrous ion chelating efficacy (Fig. 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 the antioxidant mechanism since it reduces the concentration of the catalyzing transition metal in LPO. It is reported that chelating agents that form σ-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, Harish, & Shivanandappa, 2006). Metal-binding capacities of dietary fibres are well known, e.g. the inhibitory effects on ferrous absorption of algal dietary fibres 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. alvarezii* extracts was concentration-dependent (Fig. 3). As the concentration increased from 0.5 to 5.0 mg ml^{-1}, there was an increase in absorbance with all the solvents except hexane. However, the reducing powers of the samples were found to be in the following order: BHT (0.23–0.879) > methanol (0.07–

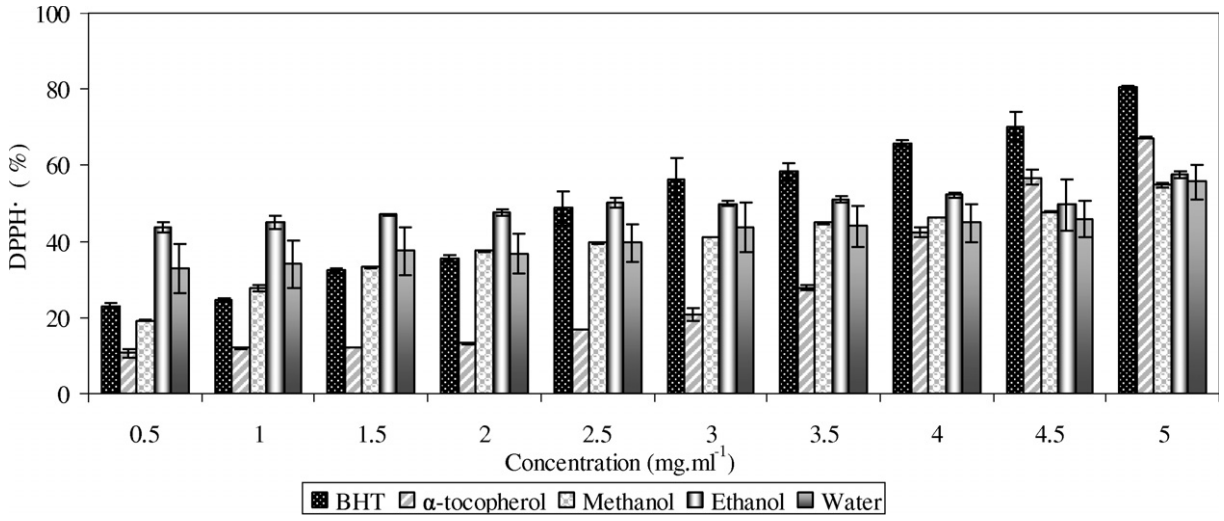

Fig. 1. Antioxidant activities of different solvent extracts of *K. alvarezii* determined as DPPH radical-scavenging activity.

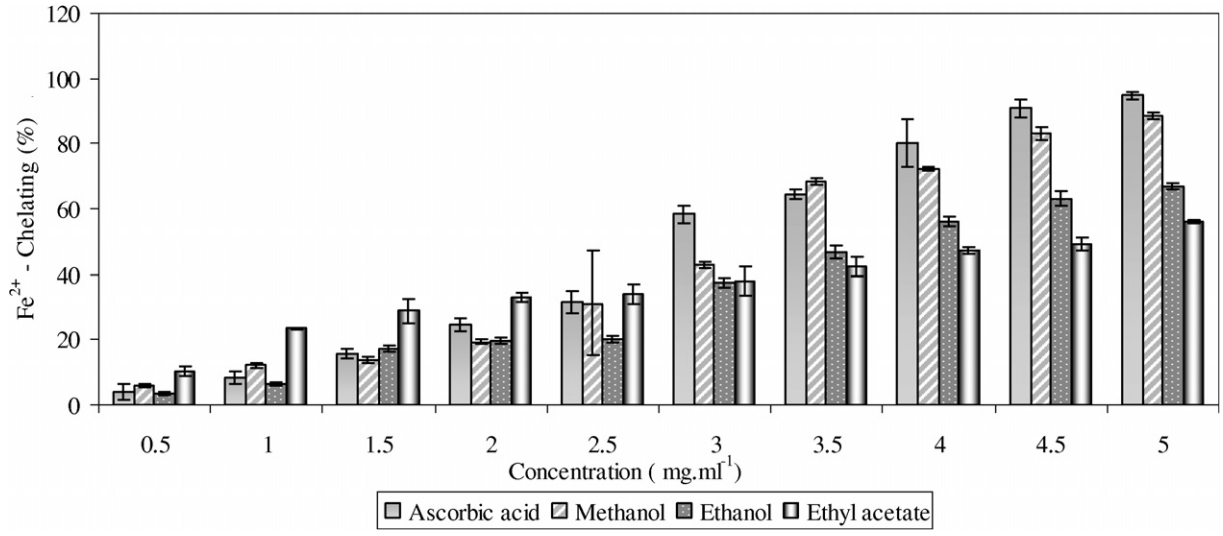

Fig. 2. Ferrous ion-chelating activities of different solvent extracts of *K. alvarezii*.

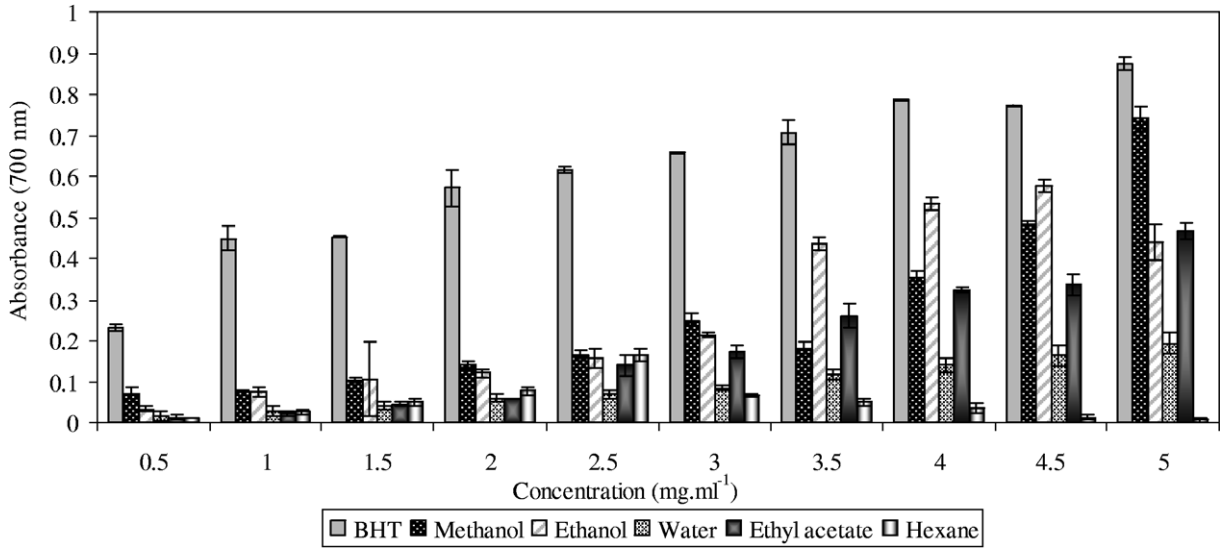

Fig. 3. Reducing powers of *K. alvarezii* extracts, along with a synthetic antioxidant.

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)

Peroxyl 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 the free amino group of proteins, phospholipid, and nucleic acids, leading to structural modifications, 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 Fig. 4. The absorbance ranges 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

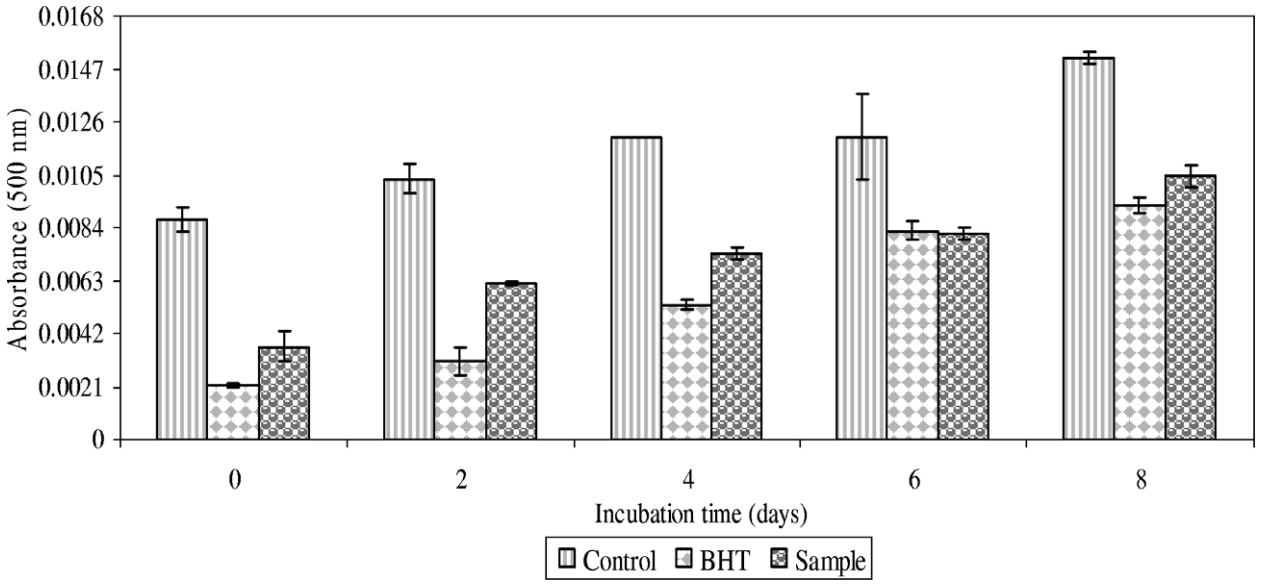

Fig. 4. Inhibitory effect of *K. alvarezii* extract on the primary oxidation of a linoleic acid system, using the ferric thiocyanate method.

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. alvarezi* (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. (2005b). They also reported that phosphorylated and sulfated glucans exhibited better antioxidant ability than did glucans or other neutral polysaccharides, which indicates 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 the presence of ascorbic acid, vitamin A and various phenolics.

4. 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 the linoleic acid system. Thus they could be used in nutraceutical and functional food applications. Since this is a preliminary study, a detailed investigation on the compositions of each component involved is absolutely necessary to establish appropriate applications which may open new frontiers for human consumption of this seaweed world-wide.

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