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Antioxidant in vitro activity of extracts of some green seaweed (Chlorophyta) from southern Brazilian coast

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Abstract

The antioxidant activity of four species of green seaweeds of the phylo Chlorophyta (Codium decorticatum, Enteromorpha intestinalis, Ulva fasciata and Chaetomorpha anteninna) collected at the sea-coast of the state of Santa Catarina in Brazil was evaluated by means of the inhibition of peroxidation of linoleic acid converted into emulsion. Both ethereal and methanolic extracts were obtained from dried biomasses by sequential extraction procedures in concentrations of 0.01%. The most efficient species towards lipid peroxidation were E. intestinalis and C. anteninna with inhibition yields above 70% .The capacity of methanolic extracts to quench hydrogen peroxide was also estimated. Mean values varied from 1.26 to 20.01%. Chlorophylls a, total carotenoids and phenolic compounds were also quantified in the biomasses. Results indicated that studied green seaweeds are a very promising source of biologically active compounds with antioxidant properties.

Keywords: Sea algae; Antioxidants; Phenolic compounds; Carotenoids; Chlorophylls.

1. Introduction

Despite the high content of polyunsaturated fatty acids, marine algae are stable against oxidation during storage (Ramarathnam *et al.*, 1995). Like any photosynthetic organism, algae are exposed to high amounts of light and high concentrations of oxygen, a combination that causes free radicals, as well as other potent oxidants. The absence of oxidative damage in the structural polyunsaturated fatty acids of algal membranes suggests that these foods have antioxidant compounds and mechanisms (Matsukawa *et al.*, 1997).

Algae are also rich in polysaccharides and minerals, however, few algae have been widely

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used as edible plants. The genus Enteromorpha (Chlorophyta) has potential for feeding, being found abundantly in the Brazilian coast. The finding of antioxidant activity could potentially raise its value as a human or additive food and expand its consumer market. In Brazil, in particular, algae are poorly exploited commercially, being almost restricted the exploration of some genera of red algae (Oliveira, 1998).

Yan *et al.* (1998) working with several algal species tested the methanolic extracts of two species of ulva, U. lactuca and U. pertusa. The results obtained with the DPPH method (azo-radical 2,2-diphenyl-1-picrylidrazyl) showed activity of 8% for U. lactuca and 15% for U. pertusa against the formed root. The authors also found radical sequestering activity, to a lesser extent, in the other extracts tested. In the same study, two species of Enteromorpha, E. intestinalis and E. prolifera, were tested and the methanolic extracts presented radical sequestering activity of 15% and 7%, respectively.

Biochemical studies focusing on Brazilian green algae are restricted to biochemical components with a nutritional character. Caldas (1986) evaluated the changes in the centesimal composition of the species U. fasciata, from the island of São Luiz (Brazil), in response to seasonal variations. Reports involving bioactive compounds are scarce, especially with regard to antioxidants. Freitas (1986) studied the pharmacotherapeutic activity on cardiac metabolism using methanolic extracts of two species of the Chlorophyta phylum, obtaining positive results.

The study objective was to evaluate the amount of total phenolics, total carotenoids, chlorophyll a and antioxidant potential of some species of green algae, noting its value as raw material for encapsulated with bioactive characteristics or other types of products, in which the presence of antioxidants effective for both the food and pharmaceutical industries.

2. Materials and methods

2.1. Reagents and solvents

All organic solvents (ethyl alcohol, ethyl ether, hexane and acetone) had purity of about 99% or greater. Linoleic acid was purchased from Fluka, and hydroxybutyl anisole (BHA) from Merck. All other commercial reagents were used without prior purification.

2.2. Collection of samples

The algae were collected in the beaches of Canasvieiras and Ponta das Canas, north coast of Santa Catarina Island, in July and August 2002, under low tide. Based on the greatest abundance, they were selected in diverse points of the algal communities, to guarantee sample variability. The species were identified according to conventional methods in the Marine Algae Laboratory of the Federal University of Santa Catarina and are deposited in the Herbarium of said institution. The tests were carried out with four species of Chlorophyta: Ulva fasciata Delile, Codium decorticatum (Woodw.) M. Howe, Enteromorpha intestinal is and Chaetomorpha anteninna (Bory) Kutz.

2.3. Preparation of the samples

The samples were cleaned, washed with potable water to remove the salt and then rinsed with distilled water. Afterwards, they were dried in greenhouse; brand FR Santer, with forced air circulation at 35, 0 ± 0.5 °C until complete

drying (approximately 24 hours). After drying, the samples were stored in a freezer at -18.0 ± 0.5 ° C, under a nitrogen atmosphere, for further analysis.

2.4. Obtaining extracts

The extracts were obtained by sequential extraction of the samples. 5.0 g of the dry biomass were weighed on a scale of the Metler Toledo brand model AB204-S, crushed in a Walita brand household processor, the particle size being 1.75 to 3.00 mm. The sequential extraction was followed by ethyl ether followed by methanol under mechanical stirring under full light for two hours. The extracts were filtered on Whatman paper no.1 and concentrated under nitrogen atmosphere. The determination of the dry weight was performed by gravimetry, taking a 5 mL aliquot in porcelain crucible, previously tarred, followed by oven drying at 105.0 ± 0.5 ° C until constant weight (AOAC, 1990).

2.5. Evaluation of the antioxidant activity by the Ferric Thiocyanate method

The antioxidant activity of ethereal and methanolic extracts was evaluated according to the methodology described by Mitsuda *et al.* (1967), and Kikuzaki and Nakatani (1993), with modifications. Mixture containing 1 mL of the algal extract, 1.1 mL of 2.51% (w / v) linoleic acid in ethanol (99.0% v / v), 2 mL of 0.05 M phosphate buffer (pH 7.0) and 0.9 mL of distilled water was placed in an amber glass vial with a screw cap and stowed in a forced air circulation oven at 50.0 ± 0.5 °C. To 0.1 mL of this solution was added 5 mL of 75% (v / v) ethanol, 0.1 mL of 30% (w/v) ammonium thiocyanate and 0.1 mL of 0.02 M ferrous chloride. After, exactly 3 minutes of reaction at room temperature (25.0±0.5 ºC), the absorbance at 500 nm was performed on a Hewlet Pakard model 8452A UV-VISIVEL spectrophotometer, which was repeated every 24 hours until that the purple color of the control reached a maximum value. The concentration of the extracts and the synthetic antioxidant hydroxybutylanisole, BHA, was 0.01% (w/v). The percentage of inhibition in lipid oxidation was calculated according to the algebraic expression: % inhibition = {[abs. mean of control - abs. final sample mean]/abs. final control mean} x100.

2.6. Evaluation of the hydrogen peroxide sequestration power of methanolic extract

The ability of extracts to sequester hydrogen peroxide was determined according to the method of Ruch *et al.* (1989) and Yen and Chen (1995). A solution (4 mM) of hydrogen peroxide was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined spectro-photo-metrically at the wavelength of 230 nm using molar absorptivity of 81-1cm-1 (Yen and Chen, 1995). To the extract (10-400 mL, in 4 mL of distilled water) was added hydrogen peroxide solution (0.6 mL). After 10 minutes of reaction, at room temperature (25.0 ± 0.5 °C), a spectrophotometer reading at a wavelength of 230 nm was performed against white solution containing the extract in buffer without hydrogen peroxide.

2.7. Determination of total phenolic compounds

The content of total phenolic compounds was determined, using protocol according to Budini *et al.* (1980) for extraction and protocol similar to that of Amerine and Ough (1976) for

quantification. 0.5 g of the dried and milled biomass was weighed and 2 M HCl solutions in a 1:10 (w/v) ratio were added, heating for 30 minutes in a water bath at (95.0 ± 0, 5 °C). The mixture was then filtered on Whatman paper No. 1 to a 100 mL volumetric flask. The volume was completed. Exactly 1.0 mL of the extract was transferred to a 100 mL volumetric flask, followed by the addition of 60 mL of distilled water and 5 mL of the Folin-Ciocalteu reagent. After one minute, 15 mL of 20% sodium carbonate solution was added, the volume was completed and allowed to stand for two hours under the light at 24.0 ± 0.5 °C. The absorbance at 756 nm was determined in a spectrophotometer. The calibration curve was constructed with gallic acid, R^2 = 0.99, in a concentration range of (0.5-5.0) mg / mL gallic acid.

2.8. Determination of chlorophyll a and b

Chlorophyll extraction was carried out using 1.0 g of fresh biomass in 50 mL of acetone under maceration for 24 hours at 5.0 ± 0.5 ° C under light to avoid photodamage . The extracts were then centrifuged at 2000 rpm in a Fanem model 204 spinner for 5 minutes and the supernatant analyzed in a spectrophotometer at wavelength of 662 nm for chlorophyll a and 646 nm for chlorophyll b. The Ecm1% extinction coefficient values for chlorophyll a and b in acetone were quoted in Jones *et al.* (1962).

2.9. Determination of total carotenoid pigments

The procedure of extraction of the carotenoid pigments followed the protocol according to Kurilich and Juvik (1999). The absorbance was read in a spectrophotometer at the wavelength of maximum absorption. Each carotenoid has its point of maximum absorption in a given solvent, which can be obtained in tables for identification of carotenoids (Mosquera and Isabel, 1997; Davies, 1976). The concentration was expressed in terms of the majority carotenoid pigment, based on chromatographic data obtained in the laboratory (Rf value for thin layer chromatography and light absorption spectra). These data were compared with reference values found in literature for carotenoids (Mosquera and Isabel, 1997; Davies, 1976). The values of the extinction coefficients Ecm1% in ethanol were consulted in the carotenoid identification tables found in Davies (1976).

2.10. Statistical analysis

Statistical analysis was performed using the Statistical program, version 5.0 (1986-1996). Analysis of variance (ANOVA) was applied, with differences were determined by the Tukey test. Differences between means at a level of 5% ($p < 0.05$) were considered significant. All analyzes were performed in triplicate.

3. Results and Discussion

The mean absorbance values for the peroxidation of linoleic acid determined by the ferric thiocyanate method at 50 ° C after the addition of ethereal extracts are shown in Figure 1. The values for the methanolic extracts are shown in Figure 2. In the stages the ethereal extracts of the species U. fasciata and E. intestinalis showed a pro-oxidant effect in relation to the control (Figures 1 and 2). The same effect can be observed for the methanolic extracts of algal species U. fasciata and C. decorticatum in relation to the control in Figure 2.

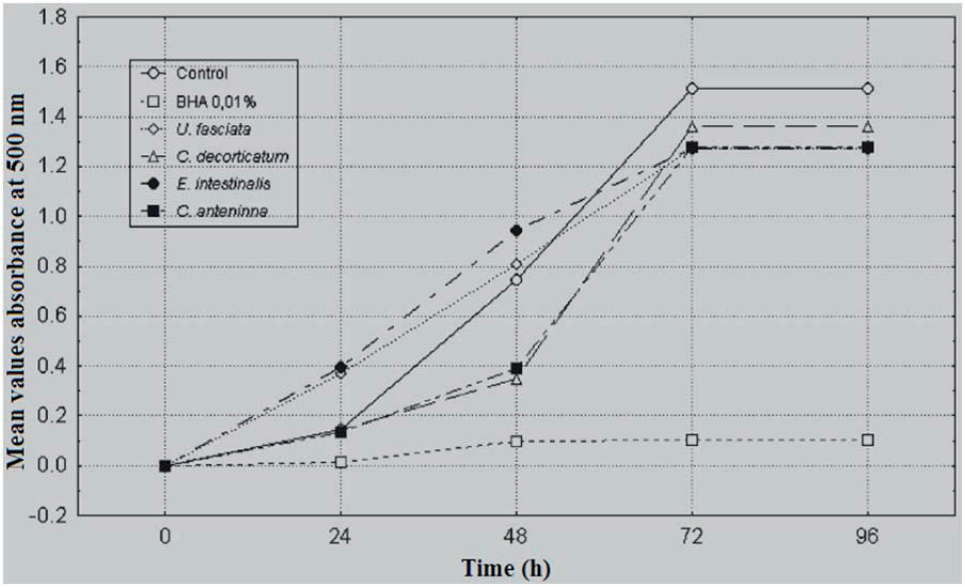

Figure 1. Mean absorbance values of the samples containing the ethereal extracts from seaweed (0.01%) obtained in Test ferric thiocyanate at times 0, 24, 48, 72 and 96 hours

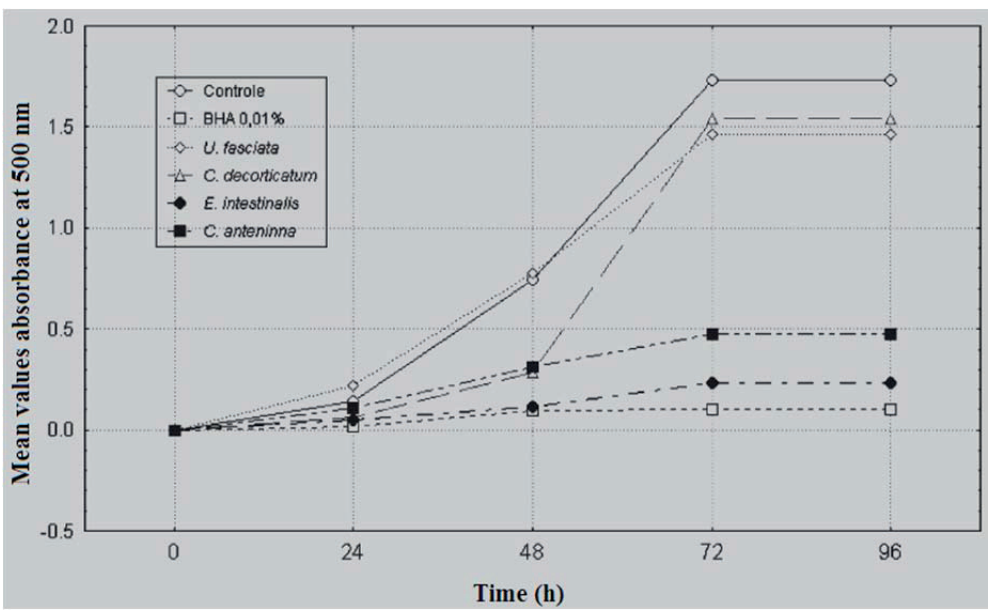

Figure 2. Mean absorbance values of the samples containing the methanolic extracts from seaweed (0.01%) obtained in Test ferric thiocyanate at times 0, 24, 48, 72 and 96 hours.

The antioxidant activity of the algal extracts was compared with the activity of the commercial antioxidant BHA. It was found that all ethereal extracts provided inhibition of lipid peroxidation of less than 50%. The maximum average value of 39, 43% was of the algal species C. anteninna, whereas among the methanolic extracts, the most effective were the species E.

intestinalis and C. anteninna. There are data in the literature that correlate antioxidant activity of plants with anti-inflammatory properties (Recio *et al.*, 1995; Sadique *et al.*, 1987). Thus, plants containing antioxidant activity, such as algae in this study, could be used in the therapy of antiinflammatory processes. At the concentration tested, no extract exceeded the synthetic antioxidant efficiency of 92.45% (Figure 3). In general, natural antioxidants are not as effective at deactivating free radicals as in vitro and synthetic tests such as BHA. This reaffirms the need for a constant search for more effective natural antioxidants, especially for industrial purposes.

The antioxidant effectiveness of natural sources has been related by several authors to phenolic compounds, which occur naturally in terrestrial and aquatic plants (Hayase and Kato, 1984). These compounds are mainly responsible for the antioxidant activity of plants, playing a key role in the inhibition of autoxidation in oils (Ramarathnam *et al.*, 1986). The natural occurrence of substituted phenols and polyphenols in algae has been scientifically disseminated and expanded over the years (Pedersen, 1978). Although this class of compounds includes effective antioxidants (Denisov, 1987), little is known about the antioxidant activity of algal extracts. Of the 21 marine algal species studied by Fujimoto and Kaneda (1980), about 60% presented antioxidant activity to some degree. The mean values of the total phenolic compounds found for the species tested, values obtained by the Folin-Ciocalteu spectrophotometric method (Amerine and Ough, 1976), and percent inhibition of linoleic acid peroxidation are shown in Figure 3.

The highest percentage of inhibition (75.75%) was obtained by the methanolic extract of the species E. intestinalis, whose biomass contained one of the highest total phenolic values (610.31 mg/100 g). Although the biomass of U. fasciata presented a statistically significant amount of phenolic compounds (635.53 mg / 100 g) to that of E. intestinalis, the antioxidant effectiveness between the methanolic extracts of the two species presented significant differences ($p < 0.05$). This result suggests distinctions in the chemical composition between the phenolic compounds of such algae, differences in the solubility of these compounds by the solvent used or, finally, the involvement in the antioxidant activity of other antioxidant compounds, present in the algal extracts, in addition to phenolics, such as carotenoids and chlorophylls. In agreement with the previous hypothesis, Nakamura *et al.* (1996) isolated in species of the genus Enteromorpha a relatively hydrophobic antioxidant, identified as pheophytin a, which constitutes a chlorophyll without magnesium. Figure 3 shows the different antioxidative efficiencies between ethereal and methanolic extracts, the latter being more effective. Yan *et al.* (1998) evaluated the antioxidant activity of 27 algae species by DPPH (2,2-diphenyl-1-picrylhydrazyl) using four organic solvents by sequential extraction. The authors observed large differences in radical sequestering activity among different species of algae. In this study and others (Anggadiredja and Andyani, 1997), the methanolic extract generally presents the highest values of antioxidant activity in many algal species, suggesting that methanol is a more effective solvent during the preparation of algal extracts, especially if the study aims to investigate their antioxidant activity.

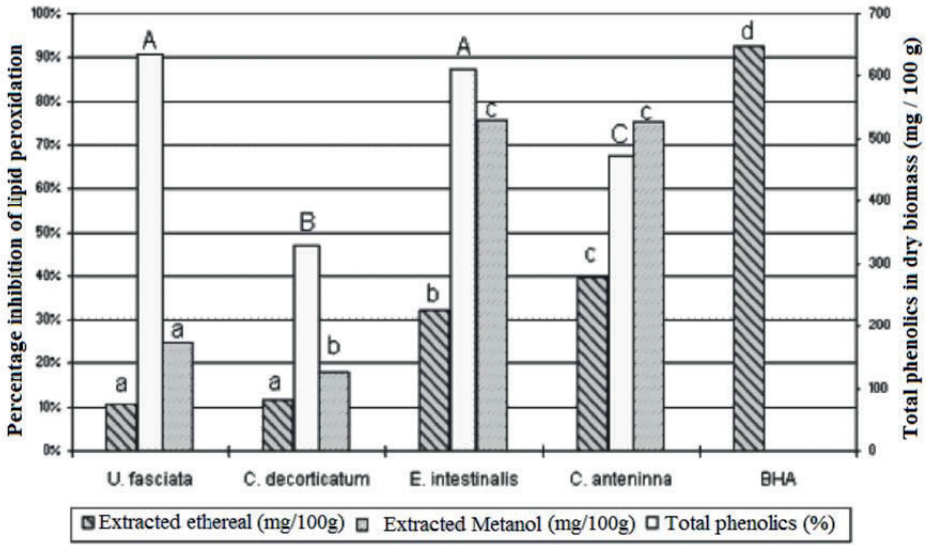

Figure 3. Mean values for lipid peroxidation inhibition percentage of linoleic acid provided by the addition of ethereal and methanolic extracts, together with mean values for total phenolic contents of dry biomasses. The values followed by equal letters did not differ statistically ($p<0.05$)

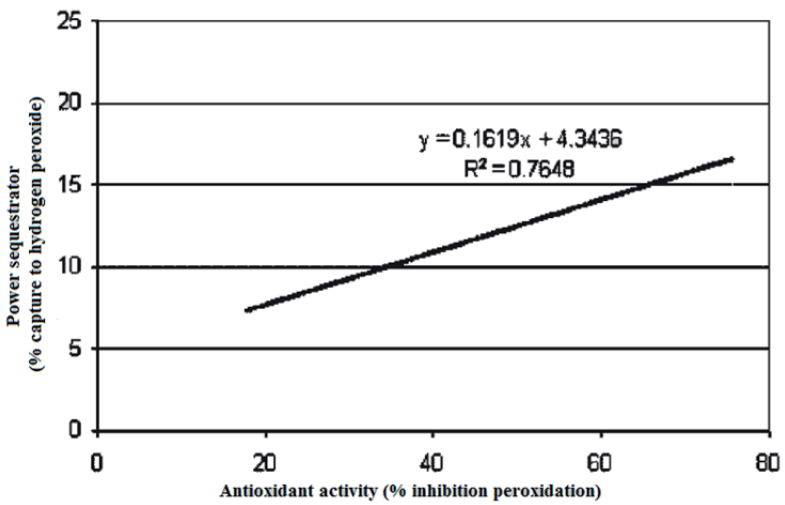

Figure 4. Regression of the antioxidant activity versus the sequestering power of the extracted methanolic

Algae methanolic extracts were able to sequester hydrogen peroxide, but the effect was greatly reduced varying between 1.26 and 20.01%. The most effective extracts were C. anteninna (1.26% to 20.01%) and E. intestinalis (1.18% to 13.16%) for the volumes tested (10 µL-400 µL). High ability to sequester hydrogen peroxide is related to the presence of antioxidants, in the extract, able to react with free radicals. This relationship can be observed in Figure 4. Linear regression suggests an increase in the sequestering power with an

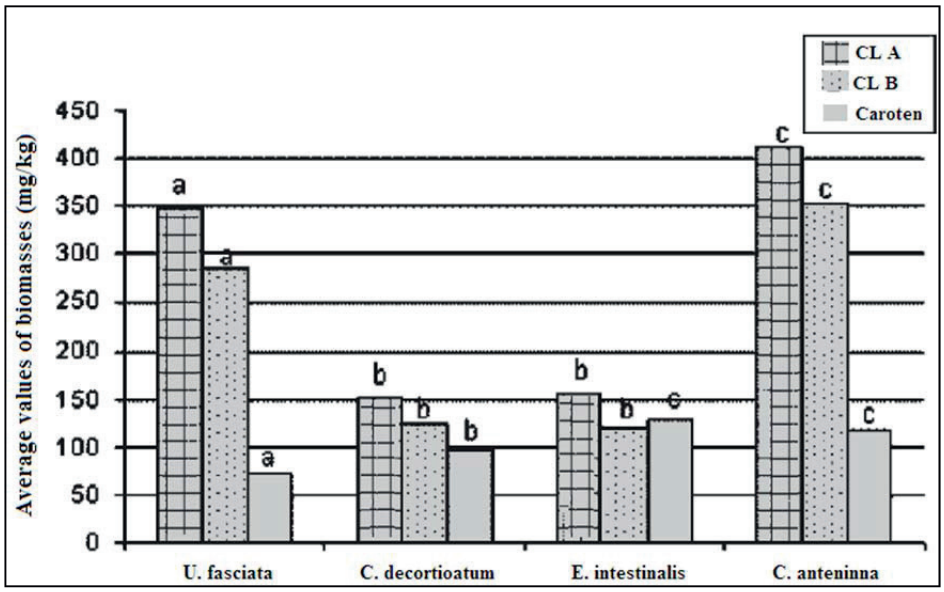

Figure 5. Mean values of chlorophyll and carotenoid pigment contents found in biomasses. Total carotenoids were expressed as lutein. Columns of the same color whose top letter are the same do not differ significantly ($p < 0.05$)

increase in antioxidant activity ($R^2 = 0.7648$).

The Chlorophyta phylum has chlorophyll a and b, xanthophylls (mainly lutein) and carotenes (mainly b-carotene). Carotenoids and chlorophyll a are known to act as antioxidants in in vitro tests (Naguib, 2000; Cahyana *et al.*, 1993). Figure 5 shows the mean values of such compounds obtained by spectrophotometric methods for algal biomass. The values for chlorophyll a varied between 151.48 and 411.51 mg/kg, being the largest of the species C. anteninna and the smallest of the C. decorticatum species. The mean values for the total carotenoid content varied between 72.27 and 129.04 mg / kg, being the highest content of E.intestinalis and the lowest of the U. fasciata species. Algae extracts are a mixture of several compounds with antioxidant potential. Le Tutour *et al.* (1998), studying the ability of five algal species to sequester peroxyl radicals in a model system, observed that the antioxidant activity of the five extracts examined was apparently not due to their tocopherol content alone, many other lipid soluble compounds generally found in algae (Ito and Hori, 1989) and identified in algal extracts, such as carotenoids and chlorophyll, can act as antioxidants.

Endo *et al.* (1985) reported that chlorophyll an exhibits some antioxidant activity in the dark, highlighting the importance of the porphyrin ring as an essential structure for this effect. In general, porphyrin derivatives play some antioxidant activity (Cahyana *et al.*, 1993). Le Tutour *et al.* (1996) studied the synergistic effect of adding chlorophyll a to vitamin E in the preservation of methyl linoleate in a model system. Chlorophyll acted synergistically, providing a 24% increase in inhibitory effect at a concentration of 0.1 mmol L-1. Lutein, the main carotenoid of the Chlorophyta class, has activity, as well as β-carotene (Burton

and Ingold, 1984; Mortensen *et al.*, 1997). Data in the literature report that the green alga Enteromorpha spp. contains greater amounts of β-carotene than that of yellow-green vegetables. Amounts range from 22 to 25 mg/100g (Ito and Hori, 1989). B-carotene reacts with lipid peroxides (Tsuchihashi *et al.*, 1995). Mortensen *et al.* (1997) evaluated the mechanisms and reaction rates of lutein and other carotenoids with generated radicals of pulse radiolysis and concluded that the rate-of-capture mechanism of a given carotenoid is strongly dependent on the nature of the oxidant (radical) species and carotenoid structure.

Conclusions

Green algae have several compounds of great interest to the food, cosmetics and pharmaceutical industries. Compounds such as carotenoids, chlorophylls and phenolics were abundantly found in the species analyzed. It was observed better antioxidant activity in methanolic extracts in relation to ethereal extracts. Although the values for the percentage inhibition of lipid peroxidation of methanolic extracts, mainly in two species, E. intestinalis and C. anteninna, were higher than 70%, none of the extracts in the tested concentration showed higher efficiency than the synthetic antioxidant.

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