

Antioxidant activity and possible bioactive components in hydrophilic and lipophilic fractions from the seaweed *Halimeda incrassata*

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Article

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Abstract: Hydrophilic and lipophilic fractions, obtained from the marine algae *Halimeda incrassata* (J.Ellis) J.V.Lamouroux, Halimedaceae, were studied by using the β -carotene-linoleate assay system. In case of hydrophilic fractions, the total phenolic compounds were quantified and two of their components were identified as salicylic and ferulic acids. From the lipophilic fraction, fatty acid composition was studied. The highest antioxidant activities values were found on the polar fractions containing phenolic acids. The total phenolics content on the hydrophilic fractions was 255 μ g of gallic acid equivalents/g of fresh seaweed. To further characterize *H. incrassata* chemical composition, the total lipid content was quantified (7.4 mg per gram of dried algae) as well as the saturated and unsaturated fatty acids ratio (1:1.46). In summary, this paper adds more convincing evidences in support of the antioxidant abilities of the lyophilized aqueous extract of *Halimeda incrassata* and it also relates this bioactivity, for the first time, with particular phenolic components of the extract. Altogether, these results represent another step towards the use of this natural product as drug candidates.

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Introduction

In the last ten years, the interest on the study of seaweeds as sources of bioactive compounds has increased. Such tendency could be at least partially explained on the basis of their chemical components, which exhibit different beneficial properties for the human health (Shin et al., 2006).

It is well known that algae contain minerals, polysaccharides, amino acid derivatives, carotenoids and phenolic compounds. These can display antioxidant properties at very low concentrations, which has been exploited on the food industry to protect foods from oxidation process (Yuan & Walsh, 2006). In addition, epidemiological research has supported the existence of an inverse correlation between the incidence of cardiovascular diseases and consumption of phenolic-rich foods (Bocanegra et al., 2009).

Different seaweed extracts have received increased attention, due to their potent pharmacological

effects, particularly *in vivo* hypolipidemic (Ara et al., 2002) antioxidant (Yuan & Walsh, 2006), immunological (Saker et al., 2004) and antitumoral activities (Lee & Sung, 2003). Similarly, *in vitro* studies have confirmed the antioxidant properties of seaweed extracts (Nakai et al., 2006) which has suggested their possible uses as nutraceuticals and/or phytodrugs.

Some groups have studied the ability of seaweed extracts to inhibit lipid peroxidation or to scavenge free radicals (Fayaz et al., 2005; Dutra et al., 2007). Different compounds such as carotenoids, mycosporine-related aminoacids and terpenoids together with phenolic compounds as cinnamic acids, phlorotannins, and bromophenols has been identified among the principal entities responsible for these properties in marine algae (Shick & Dunlap, 2002; Fisch et al., 2003).

The green seaweed *Halimeda incrassata* (J.Ellis) J.V.Lamouroux, Halimedaceae, is a specie that grows in shallow waters, and thus it is exposed

to a combination of light and oxygen that leads to the formation of free radicals. However, the absence of oxidative damage on the structural components of seaweeds and their stability to oxidation during storage indicate that their cells should have protective antioxidative defense systems. *Halimeda incrassata* aqueous extract has been investigated over the past years by our group as a source of natural antioxidants and neuroprotectant compounds. Indeed, several lines of results have documented the ability of this natural product to target free-radical mediated processes on in vitro and in vivo experimental models (Rivero et al., 2003; Linares et al., 2004; Vidal et al., 2009). However, in spite of previous efforts, important information is still lacking in relation to: i) the polar components of this aqueous extract underlying its antioxidant and neuroprotective properties and ii) the antioxidant properties of non-polar fractions obtained from *Halimeda incrassata* and its possible bioactive components.

Thus, in view of these previous considerations, the aim of this study was to evaluate the antioxidant capacity of the hydrophilic and lipophilic fractions obtained from the green seaweed *Halimeda incrassata*, and to identify and quantified the metabolites contained on such fractions.

Materials and Methods

Seaweeds collection

The seaweed specie (*Halimeda incrassata* (J.Ellis) J.V.Lamouroux, Halimedaceae) was collected on August (2008), in the area of Bajo de Santa Ana, Havana City west coast, Cuba. Specimens were authenticated by Dr. A.M. Suárez from Seaweeds Laboratory, at the Marine Research Center of the University of Havana, where a voucher CIM (Suárez) 1973 was also deposited.

Hydrophilic fractions preparation

Fractions containing phenolic acids either in free form or as soluble esters were obtained according to Vidal et al. (2009). Dry seaweed (1 g) was extracted six times with tetrahydrofuran (THF, 20 mL) during 3 min, and evaporated to dryness under vacuum at 30 °C and after resuspended in 5 mL of methanol; these extracts were analyzed for free phenolic acids (FPA). The remaining residue was extracted with methanol:acetone:water (7:7:6) and then filtered. The obtained supernatant was hydrolyzed by adding NaOH 4N and the solution was adjusted to pH 2. The obtained phenolic acids were extracted with ethyl ether:ethylacetate:THF (1:1:1). The organic phase was

rotoevaporated and resuspended into methanol, this was analyzed for phenolic acids soluble esters (PASE).

Determination of total phenol concentrations

Total phenolics was determined as in Vidal et al. (2009) and expressed as mg of gallic acid/g of seaweed.

Identification and quantification of phenolic compounds

Phenolic and cinnamic acids were evaluated according to Vidal et al. (2009). Different seaweed fractions were analyzed by Gas Chromatography on a GC Shimadzu 17 A equipped with a FID and a DB-5 fused-silica capillary column (J & W Scientific 5% fenil-methylpolisiloxane (30 m x 0,25 mm x 0,25 µm). Helium was used as the carrier gas. Peak areas were computed by a GC Workstation Class-GC-10 (Shimadzu) integrator.

Lipophilic fractions preparation

The freshly collected specimens were washed with distilled water and dried at room temperature for several days. The dried seaweed were milled into powder and then sieved. Lipophilic fractions were obtained with methanol, and later with chloroform, then the solution was filtered. The remaining residue was mixed with chloroform: methanol (3:1) and KCl 0.88% was further added. Organic later was finally evaporated to dryness. The obtained fatty acids were methylated according to the procedure described by Hartman & Lago (1973) using heptadecanoic acid as internal standard. The mixture of NH₄Cl in methanol: H₂SO₄ was used as methylating agent. In all fractions, total solutes were quantified by gravimetric analysis.

Identification and quantification of fatty acids

Aliquots of methylated fatty acids obtained by the *Halimeda incrassata* were studied by Gas Chromatography according to AOAC method (Cunniff, 1995) using an GC Shimadzu 17 A equipped with a FID and a Supelcowax 10 fused-silica capillary column (Supelco 30 m x 0,25 mm x 0,25 µ, USA). Helium was used as the carrier gas. Peak areas were computed by a GC Workstation Class-GC-10 (Shimadzu) integrator.

Measurement of antioxidant activity with β-carotene/linoleic acid assay

The antioxidant activity of the different fractions was evaluated according to Vidal et al.

(2009), using a mixture of β -carotene and linoleic acid from Sigma[®]. Initial absorbance at 470 nm was taken at zero time and the absorbance measurements were repeated at 15 min intervals during 120 min. Butylated hydroxytoluene (BHT Sigma[®]) was used as positive control.

Statistical analysis

All experiments were carried out by triplicate and results were expressed as mean value \pm standard deviation. In antioxidant activity measurements, the group's comparisons were performed by one-way Analysis of Variance (ANOVA) and Tukey post test. $p < 0.05$ was always considered statistically significant.

Results and Discussion

Seaweeds have recently attracted much attention as a source of bioactive compounds due to, at least in part, to the growing body of epidemiological studies supporting the beneficial effects of seaweed consumption in a variety of pathological conditions.

Phenolic compounds represent a large and heterogeneous group of biologically active substances, widely occurring in vegetable products and seaweeds, with important roles on cell metabolism and physiology. Their biological activities, including antioxidant properties, have suggested their positive effects on the human health (Bravo, 1998).

The phenolic content measured on the hydrophilic fractions obtained from the seaweed *Halimeda incrassata* are shown in Table 1. Both fractions exhibits high value of total phenolic compounds, from which more than half (63%) corresponds to free phenolic acids, while 37% represents PASE compounds. In the free phenolic acids fraction, about 32% was identified as salicylic acid, while a small fraction was associated to ferulic acid. In the PASE fraction, same compounds were identified, and salicylic acid was also found as an important component, representing 27% of the total PASE content.

Values of phenolic contents reported here for hydrophilic fractions of *H. incrassata* are higher than those referred for a variety of seaweeds, but

Table 1. Content of total phenolic compounds (μg of phenolics/ g of fresh seaweeds), yield (%) and phenolic acids identified on the fractions of free phenolic acids and soluble esters of phenolic acids obtained from the seaweed *Halimeda incrassata*.

Fraction	Phenolic compounds $\mu\text{g/g}^*$	Yield (%)	Salicylic acid ($\mu\text{g/g}$ of fresh seaweed)*	Ferulic acid (ng/g of fresh seaweed)*
FPA	160 \pm 15	0.016	51 \pm 4	7,5 \pm 0.2
PASE	95 \pm 6	0.0095	26 \pm 5	8 \pm 0.2

*mean \pm standard deviation

corresponds to the level found in *Fucus vesiculosus* and *Bryothamnion triquetrum*. A relation between phenolics content and reactive oxygen species (ROS) scavenging abilities was observed for the hydrophilic fractions of *H. opuntia* and *H. monile* and this finding is an agreement with previous reports made for edible seaweed extracts (Vidal et al., 2009)

The antioxidant activity of the polar fractions obtained from the seaweed *Halimeda incrassata* is shown in Figure 1. The different hydrophilic fractions from the extracts exhibit a high antioxidant activity. The result regarding the high antioxidant activity displayed by the hydrophilic fractions of *H. incrassata* is also in agreement with earlier reports. The polar fractions of *H. incrassata* may contain others bioactive compounds which to could be directly responsible for the antioxidant activity, as carotenoids and polysaccharides (Rivero et al., 2003; Linares et al., 2004).

Next step was directed towards the isolation of free and esterified phenolic acids fractions and two different phenolic acids: ferulic and salicylic acids were identified as bioactive components. Phenolic and cinnamic acids have been considered responsible for antioxidant properties of plants and algae extracts. Although ferulic acid levels are very low in *H. incrassata*, the free radicals scavenging properties of this cinnamic acid have been very well documented even at very low concentrations (Nardini et al., 1995). Salicylic acid is widely found on plants, and several lines of evidences support its involvement on the plant defense mechanisms against oxidative stress (Rao et al., 1997). Different authors and also our group have referred the presence of salicylic or ferulic acids in microalgae and macroalgae displaying antioxidant properties (Fallarero et al., 2006).

Apart from salicylic and ferulic acids, other phenolic compounds could be related to the antioxidant properties of *H. incrassata*. Some studies have indicated the presence of significant amounts of simple bromated polyphenolic compounds in *Halimeda* spp and have also

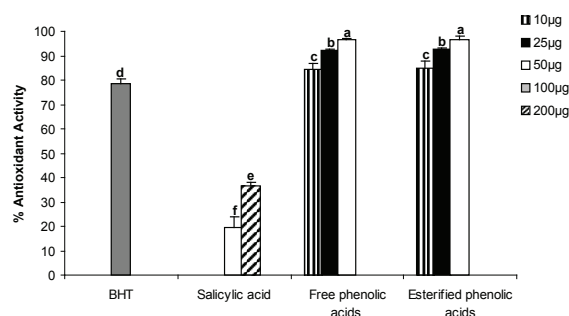


Figure 1. Antioxidant activity (referred to μg of phenolic compounds) measured on fractions containing free (FPA) and esterified phenolic acids (PASE) obtained from the seaweed *Halimeda incrassata*. Different letters means statistical differences $p < 0.05$.

identified and quantified different flavonoids and catechins in *H. opuntia* and *H. macroloba* (Yoshie et al., 2002).

The lipid content in *H. incrassata* was determined to be low (7.4 mg of lipid fraction/g of dried algae). Fatty acid composition analysis (Table 2) demonstrates that saturated: unsaturated ratio in *H. incrassata* is high (1:1.46), considered as a significant nutritional feature for this algal product. Fatty acids have been traditionally regarded as “target” molecules on oxidative stress situations, and the formation of new lipid radicals during lipid peroxidation processes contributes to the oxidative damage and exacerbates free radical toxicity. However, authors have suggested that fatty acids may also exert antioxidant functions, in marine algae and natural products (Huang & Wang, 2004).

Table 2. Fatty acid composition (mg of fatty acid/g of esterified lipids) of *Halimeda incrassata*.

Fatty acid	Formulae	Content (mg/g)*
Tridecanoic	C13:0	35.4±0.7
Myristic	C14:0	18.6±0.6
Myristoleic	C14:1	19.9±1.4
Palmitic	C16:0	219.9±15.8
Stearic	C18:0	22.1±0.8
Oleic	C18:1	285.8±5.8
Eicosenoic	C20:1	30.9±0.7
Eicosatrienoic	C20:3 n-3	58.3±1.2
Arachidonic	C20:4	21.8±0.7
Docosapentaenoic	C20:5	26.8±0.1
Lignoceric	C24:0	23.6±2.3
Nervonic	C24:1	22.1±0.4
TOTAL	Saturated	319.6
	Unsaturated	465.6

*mean±standard deviation

The lipophilic fractions obtained from *H. incrassata* exhibit antioxidant activity, although lower to the results registered for the hydrophilic fractions with values between 19.2 to 37.3% of antioxidant activity (data not shown).

The antioxidant activity of the lipophilic fractions could apparently be related to the content of unsaturated fatty acid, as in Huang & Wang (2004), who have also found a direct relationship between antioxidant activity and unsaturated fatty acids content. In current research, antioxidant activity exerted by lipophilic fractions may occur during initiation reactions of lipid peroxidation, as a fatty acid could accept a free radical involved on the initiation of lipid peroxidation. Thus, the overall scenario indicates that fatty acids cannot be regarded as an efficient long-term

defensive system against oxidative stress, combined to the profound metabolic disturbances that could be also implicated as result of lipid oxidative degradation (Aruoma, 2003).

In summary, the hydrophilic fractions obtained from the green seaweed *Halimeda incrassata* display a potent antioxidant activity as assessed by the β -carotene-linoleate assay, which could be at least partially explained by the presence of salicylic and ferulic acids, although the involvement of other phenolic compounds is not excluded. The fatty acid content on *H. incrassata* is low, but the content of unsaturated fatty acids is relatively high. Although the lipophilic fraction was shown to exert antioxidant activity, finally this fraction will act with pro-oxidant effect. Thus, current results support the notion that marine algae *H. incrassata* is a source of hydrophilic antioxidants, which could be further recommended for the prevention of oxidative-stress related disturbances, and can be of usefulness either as dietetic supplements or as food ingredients.

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