ANTIOXIDANT ACTIVITY *in vivo* AND *in vitro* OF *Halimeda incrassata* AQUEOUS EXTRACTS[1]

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SUMMARY

The aim of the present paper was to provide the evidences for the antioxidant activity in *Halimeda incrassata* (Ellis) Lamouroux aqueous extracts obtained after simple water extraction of the fresh algae at room temperature (23°C). Previously in the literature, only antioxidant activity associated to carotenoids fractions of seaweeds has been reported. From different species of seaweeds, *Halimeda incrassata* aqueous extract exhibited the highest antioxidant activity on the inhibition of TBARS formed during the spontaneous lipid peroxidation of rat brain homogenates with an IC_{50} of 0.340mg.mL^{-1}. *Halimeda incrassata* aqueous extract (0.5mg.mL^{-1}), was also capable of decreasing the *in vitro* generation of hydrogen peroxide by two distinct metabolic pathways involving glutamic and malonic acids. Also, *Halimeda incrassata* (at doses of 50, 100 and 200mg.Kg^{-1}) showed a neuroprotective effect *in vivo* on the gerbil model of bilateral carotid occlusion because of decreasing the locomotor and exploratory activity induced by ischemia. In summary, *Halimeda incrassata* aqueous extracts exhibit antioxidant properties in different *in vitro* as well as *in vivo* models which could be explained by the presence of several hydrosoluble compounds. Further studies on this way are necessary to elucidate the precise structure of these compounds. Low toxicity of most seaweeds to humans, but particularly of *Halimeda* genus may favor its use as functional food.
Keywords: antioxidant activity;*Halimeda incrassata*; algae; functional food.

RESUMO

ATIVIDADE ANTIOXIDANTE *in vivo* E *in vitro* DE EXTRATOS AQUOSOS DA *Halimeda incrassata*. O presente trabalho teve por objetivo apresentar as evidências da atividade antioxidante de extratos aquosos da *Halimeda incrassata Ellis Lamoroux* obtidos a partir da alga a temperatura ambiente (23°C). A literatura apresenta somente a atividade antioxidante de algas oceânicas associada à frações de carotenóides. Das diferentes espécies de algas oceânicas o extrato aquoso da *Halimeda incrassata* apresentou a atividade antioxidante mais elevada medida pela inibição da formação de TBARS, durante a peroxidação lipídica espontânea de cérebro, com um C.I.50 de 0,340mg.mL^{-1}. O extrato aquoso da *Halimeda incrassata* (0,5mg.mL^{-1}), também diminuiu *in vitro* a formação de peróxido de hidrogênio através de duas vias metálicas envolvendo os ácidos glutâmico e malônico. A *Halimeda incrassata* (nas doses de 50, 100 e 200mg.Kg^{-1}) apresentou efeito neuroprotetivo *in vivo* no modelo gerbil de oclusão carótida bilateral, pela diminuição da atividade exploratória e locomotora induzida pela isquemia. Em resumo, o extrato aquoso da *Halimeda incrassata* apresentou propriedades antioxidantes em diferentes modelos tanto *in vitro* como *in vivo*; as quais podem ser atribuídas à presença de diversos compostos hidrossolúveis. Outros estudos são necessários para elucidar as estruturas destes compostos. A baixa toxicidade para humanos de muitas algas oceânicas, particularmente do gênero *Halimeda incrassata* podem favorecer a sua utilização como alimento funcional.
Palavras-chave: atividade antioxidante; *Halimeda incrassata*; alga; alimentos funcionais.

1 – INTRODUCTION

Seaweeds are traditionally consumed in Asia as sea vegetables, while in Western countries they have been used as sources of gelling or thickening agents. As nutrients, they can be considered low calorie foods, exhibiting high concentrations of minerals Mg, Ca, P, K and I, vitamins, proteins and indigestible carbohydrates but a low content of lipids. A great body of evidences indicates that algal extracts can also display important functional activities, such as antimutagenic, antitumor and antioxidant [9].

Free radicals appear to constitute a particularly promising target for improving the drug treatment of various pathological conditions because reactive oxygen species are now recognized to participate in a growing number of disorders such as cancer, atherosclerosis, and neurodegenerative diseases [6]. In this context, natural antioxidants are receiving increasing attention. Dietary antioxidants from seaweeds are believed to help prevent free-radical mediated diseases [21].

Natural compounds with antioxidative properties have been reported from plant, microbial and animal sources, and have been found within most natural substance classes. Concerning plant metabolites, which are by far the most numerous, flavonoids, tannins, coumarins, lignans and xanthones have been described among others. A great variety of metabolites including in particular, phenazine and carbazole derivatives have been isolated also from *Streptomyteces* and fungal strains [19].

In previous report, the antioxidant activity of algae extracts has been reported to be associated to the carotenoid fraction of the plant. Metabolite antioxidants were isolated with differents chemical structures; MURAKAMI *et al* [17] isolated an indole derivative while fucoxanthin was identified as the major antioxidant molecule in *Hijikia fusiformis*, a Japanese edible seaweed [28]. Two extracts from the brown algae, *Laminaria digitata* and *Himanthalia elongata* exhibited the highest activity

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on the preservation of sunflower oil, on inhibition of methyl linolate oxidation and synergistically enhanced the antioxidant effect of vitamin E, according with results of LE TUTOUR [10], in which other five species, commonly used for food, were analyzed. On the other hand, 27 species of commonly edible seaweeds were examined by YAN *et al.* [29] to test antioxidant activity in 1,1-diphenil-2-picrylhydrazyl (DPPH) assay and deoxyribose assay. The most active species in both systems was *Rhodomela teres* although *Polysiphonia urceolata* and *Sargassum kjellmanianum* were also active. From the red alga *Martensia denticulate* three indole derivatives were isolated (known as denticins A, B and C), and it was showed that these compounds inhibited lipid peroxidation caused by ultraviolet radiation [5]. Additionally, a recently isolated terpenic derivative from the Mediterranean algae of the genus Cystoseira, exhibited singlet oxygen quenching properties comparable to that of alfa-tocopherol and it was inhibitor of methyl linoleate peroxidation while the superoxyde scavenging activity was low [2].

In view of these considerations, the aim of the present paper is to provide evidences for the antioxidant activity of *Halimeda incrassata* aqueous extracts, considering *in vitro* as well as *in vivo* experiments.

2 – MATERIAL AND METHODS

2.1 – Animals

Male Wistar rats 250-300g and female gerbils 45-55g were used. Both species were purchased from the Centro para la Producción de Animales de Laboratorio (CENPALAB, Havana, Cuba). Animals were kept at room relative humidity and temperature, and with free access to water and standard laboratory diet for at least 7 days previous to the experiments.

2.2 – Collection and aqueous extract preparation

Sixteen species of seaweeds were collected on December, 1997, in the area of Havana City coast, Cuba and authenticated in the Laboratory of Seaweeds belonging to Oceanology Institute of the Cuban National Academy of Sciences. Voucher specimens were deposited in this laboratory, and freshly collected specimens were homogenized in destilled water (1:4 $^{w}/_{v}$) and centrifuged at 800G in a Beckman centrifuge at -4°C during 20 minutes. The supernatants were recovered, lyophilized for using in the experiments and kept at -20°C untill used.

2.3 – In vitro *experiments*

2.3.1 – Measurement of thiobarbituric reactive substances (TBARS) after spontaneous lipoperoxidation of rat brain homogenates

- **Rat brain homogenate preparation:** Rat brains were promptly excised after decapitation, weighed and chilled in ice-cold 0.9% NaCl. After washing with 0.9% NaCl solution, tissue homogenates were prepared in a ratio of 1g of wet tissue to 9mL of phosphate buffer (50mM, pH 7.4), by using a Teflon Potter-Elvehjem homogenizer. The homogenates were centrifuged at 800G in a Beckman centrifuge at -4°C during 15 minutes and the supernatants were kept at -70°C in order to be used before 1 week.
- **TBARS measurement assay**: The TBARS measurement assay was developed according to OHKAWA *et al.* [18]. Brain homogenates (25µL) were incubated with the same volume of different seaweed aqueous extracts for the screening (5mg.mL^{-1} concentration in all cases), or different concentrations of *Halimeda incrassata* extracts for dose-response curve (0.05; 0.1; 0.3; 0.5; 0.75; 1 and 5mg.mL^{-1}) or *Halimeda incrassata* fractions obtained after purification steps, at 37°C during 40 minutes in an oscillating bath. Incubations were stopped by the addition of 350µL of cold acetic acid 20% pH 3.5 and malondialdehyde (MDA) formation was followed by the addition of 600µL of TBA 0.5% in acetic acid 20% pH 3.5. The mixtures were incubated at 90°C for 1h, allowed to cool, 50µL of sodium dodecyl sulfate (SDS) were added, and then tubes were centrifuged at 500G in a Kubota 1120 centrifuge during 15 minutes at room temperature. The absorbance was measured at 532nm. All the values are means of three determinations. A positive control was used in all cases, by measuring the activity of boldina (25µM), a major alkaloid present in the leaves and bark of boldo (*Peumus boldus*) which exhibited *in vitro* antioxidant properties in systems undergoing lipid peroxidation [24].

Antioxidant activity was expressed as the percentage of inhibition of TBARS formation related to the control undergoing maximum lipid peroxidation on the assay conditions.

The extract concentration which is needed to achieve the 50% of inhibition of lipid peroxidation (IC_{50}) was calculated by probit transformation of data according to the method of LITCHFIELD AND WILCOXON [12].

2.3.2 – Measurement of antioxidant activity in β-carotene-linoleate model system

The antioxidant activity of aqueous extract of Halimeda incrasata was evaluated using β-carotene-linoleate model system. Aqueous solutions (0.2mL) containing 1 and 2mg of crude extracts were added to a series of tubes containing 5mL of an emulsion of linoleate and β-carotene stabilized by tween 60 prepared as described by MARCO [16]. A controlled experiment was carried out using 0.1mg and 0.2mg of butylated hydroxytoluene (BHT) a synthetic antioxidant. Immediately after the addition of the emulsion to tubes the zero-time absorbance at 470nm was recorded. Samples were kept in a stoppered tubes placed in a water bath at 50°C. The absorbances were measured at 15 minute-intervals for two hours in a Stectronic 20D (Milton Roy Company). The antioxidant activity was

calculated as a percentage of the oxidation of linoleic acid and β-carotene without antioxidant.

2.3.3 – Chemoluminiscent detection of hydrogen peroxide produced by malonic acid and glutamic acid

- **Rat brain homogenates preparation:** For each experiment the entire cortex of two rats were dissected after decapitation. The tissue was chopped in a tissue sectioned (TC-2 Sorvall), transfered to a tube and washed with 5mL of artificial cerebrospinal fluid (CSF): NaCl (252mM), KCl (4.7mM), $CaCl_2.2H_2O$ (2.2mM), $MgCl_2.6H_2O$ (1.7mM), KH_2PO_4 (0.9mM), Na_2SO_4. H_2O (2.0mM) and glucose (10mM) pH 7.4, previously bubbled for 30 min at 37˚C . Then, samples were decanted and renewed by adding 5mL of artificial CSF.
- **Experimental series:** For each experiment (using malonic or glutamic acid as hydrogen peroxide generator) four groups were designed. Experimental procedure is shown in *Table 1*. The mixtures (final volume equal to 2mL) were incubated for 30 minutes at 37°C while bubbling with 100% oxygen. After the incubation period, tubes were centrifuged during 1 minute at 500G and supernatants were stored with 10mL of 100 mM EDTA at 37˚C until used. Aliquots of supernatants were used to protein determination by LOWRY *et al* [15] method.

TABLE 1. Experimental procedure for chemoluminiscent detection of hydrogen peroxide produced by malonic acid and glutamic acid (mL H2O2).

	Negative control	H. incrassata control	Glutamic or Malonic acid	H. incrassata + Glutamic or Malonic acid
CSF (µL)	1500	1300	1200	1000
H. incrassata (5mg/mL) (µL)	-	200	-	200
Glutamic acid (10µM) (µL)	-	-	300	300
Malonic acid (0.2M) (µL)	-	-	300	300
Rat brain homogenate (µL)	500	500	500	500

- **Chemoluminiscent quantification:** For chemoluminiscent detection of H_2O_2, LKB Wallac 1250 Luminometer connected to a LKB Bromma 22101-channel recorder was used. Tubes containing 870µL sodium-phosphate buffer (0.2M) pH 8.9 , 5µL EDTA (10 mM), 25µL of horseradish peroxidase ($2mg.mL^{-1}$) and 50µL Luminol (10mM) were prepared, and the mixture was stirred. Then, 100µL of supernatants (controls or samples prepared as above) were added to this reagent solution and set up in the dark compartment facing the photomultiplier. Reported values are means of four determinations. Antioxidant activity was expressed as function of the ratio: hydrogen peroxide concentration (µM): total protein quantity (µg). The concentration of hydrogen peroxide was determined using a calibration curve of hydrogen peroxide [23].

2.4 – *In vivo* experiments

2.4.1 – Gerbil model of global ischemia

Halimeda incrassata aqueous extract was tested for *in vivo* activity on gerbil model of bilateral carotid occlusion according to THOMAS *et al* [25]. Animals were randomly divided into five groups (4 per group): a naive control group receiving no treatment, a control receiving ischemia but saline solution as previous treatment and 3 groups receiving different concentrations of *Halimeda incrassata* (50; 100 and $200mg.kg^{-1}$) previous to the ischemia [11]. The seaweed extract or the saline solution was given by intraperitoneal injection 30 minutes before occlusion. The animals were anesthetized with intraperitoneal injection of penthobarbital $35mg.kg^{-1}$ and then were subjected to 5 minutes of bilateral carotid occlusion. After the period of ischemia the animals were allowed free access to food and water. At 24 hours post-reperfusion, gerbils were placed in an *open field* (50 x 50 x 50_{cm}) to monitor the exploratory and locomotor activity during 6 minutes according to HIRABAYASHI *et al.* [8]. The results are expressed in terms of count/6 minutes.

2.4.2 – Fractionating of the aqueous extract of *Halimeda incrassata*

The lyophilized extract of *Halimeda incrassata* was dissolved in water and applied to an Amberlite column XAD-2 (BDH) (28 x 2.4_{cm}). The fractionating was performed using 400mL of water, methanol:water (1:1), methanol and acetone. Aqueous fraction of this step was concentrated and applied to a cationic exchange column DOWEX 50WX8-200 of identical dimensions. The elution was achieved using a gradient from 0 to 2 M of ammonium acetate (NH_4Ac) and 23 fractions were collected of 15mL each one. Fractions corresponding to the second peak of the cationic exchange chromatography (60mL of total volume) were concentrated and applied to an anionic exchange column DOWEX 1X8-200. The elution was achieved using a gradient from 0 to 2 M of acetic acid, and 33 fractions were collected of 10mL each one. Finally Thin Layer Chromatography (TLC) on silica gel was performed with the first peak of the anionic interchange chromatography using as solvent chloroform:methanol (9.5:0.5) and phosphomolibdic reactive as revealer. Fractions obtained after each purification step were tested for antioxidant activity, by measuring the TBARS formed after spontaneous lipoperoxidation of rat brain homogenates (previously described on *In vitro experiments*)

2.5 – Statistical analysis

All values were expressed as mean ± standard deviation. The data were analyzed statistically by 1-way ANOVA and different group means were compared by Duncan‘s multiple range test; $p \leq 0.05$ was considered significant in all cases.

3 – RESULTS AND DISCUSSION

Several studies have focused on the antioxidant properties of alga extracts for the last years [2,5,10, 17, 28, 29]. However the antioxidant activity of these extracts has been reported to be associated to the carotenoid fraction of the plant. In this paper we added a new study about the antioxidant capacity of Caribbean seaweed aqueous extracts obtained after simple water extraction of the fresh algae at room temperature.

Sixteen species of seaweeds were used in the screening test. The property of reducing the quantity of TBARS after spontaneous lipoperoxidation of the rat brain homogenates was measured for all aqueous extracts at 5mg.mL^{-1} concentration and the percentages of inhibition of the lipoperoxidation were determined, which is showed in *Table 2*. Seaweeds from *Halimeda* genus showed highest antioxidant activities, but *Galaxaura* genus, as well as *Dictyopteris justis*, *Bryothamnium triquetrum*, and *Caulerpa racinosa* exhibited also effectiveness in the inhibition of spontaneous lipoperoxidation of rat brain homogenates.

The most active genus of all tested species was *Halimeda* spp, which is the most widely distributed and generally found in abundance in areas of high predator activity. *Halimeda* species attain rather high levels of calcification (up to 80% $CaCO_3$) and the coarse texture which result, have been proposed as a major physical adaptation against predation [19]. Several biological properties have been previously reported for species of this genus. For instance, some of them exhibit antimicrobial activities against marine as well terrestrial bacteria and fungi, and some others inhibit cell division of fertilized sea urchin eggs at or below 16µg.mL^{-1} [20]. Additionally there are findings of the presence of a highly bioactive terpenoid (named halimedatrial) with potent fish and larval toxicities, which suggest that halimedatrial probably represents the basis for a chemical defense adaptation of seaweeds in this environment [19].

Even when antioxidant activities were high among *Halimeda* genus (at 5mg.mL^{-1} of crude extract), *Halimeda incrassata* was the most effective. We then decide to continue studying the antioxidant properties of this aqueous extract. Dose-response curve for *Halimeda incrassata* aqueous extract was then obtained and the results are shown in *Figure 1*.

The antioxidant activity of *Halimeda incrassata* was determined in the same experimental model used for screening (*Table 2*) at different concentrations of its aqueous extract (0.05; 0.1; 0.3; 0.5; 0.75; 1 and 5 mg.mL^{-1}. Reported values are means of three determinations. The extract concentration which is needed to achieve the 50% of inhibition of lipid peroxidation (IC_{50}) was calculated by probit transformation of data according to the method of LICHFIELD & WILCOXON [11]. Parameters of linear probit curve used for IC50 determination are showed as insert of *Figure 1*.

The effect of aqueous extracts on the coupled oxidation of linoleic acid and β-carotene was compared to that of BHA and the blank without antioxidants. The samples of aqueous extracts of *Halimeda incrassata* containing 1mg and 2mg showed 68% and 75% respectively of oxidation inhibition while the BHT 0.1 and 0.2mg inhibited the oxidation in 88% and 97% respectivelly.

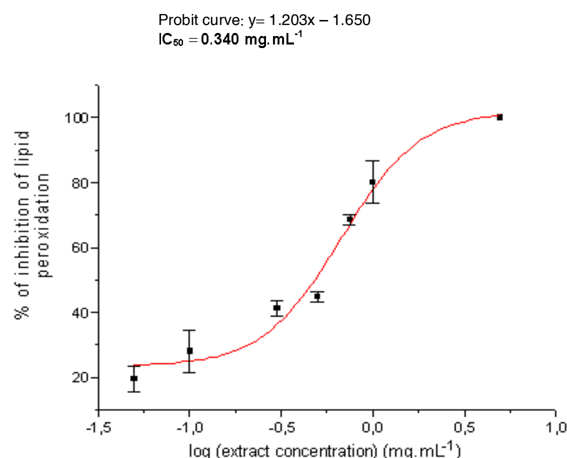

FIGURE 1. Dose response curve for the effect of *Halimeda incrassata* on the TBARS formation after spontaneous lipoperoxidation of rat brain homogenates.

TABLE 2. Screening for antioxidant activity of seaweed aqueous extracts

SPECIES	% of inhibition of lipid peroxidation (% ± sd)
Halimeda incrassata (Ellis) Lamouroux	100.00 ± 0.0
Halimeda copiosa Goreau & Graham	98.63 ± 1.9
Halimeda opuntia (Linnaeous) Lamouroux	98.47 ± 2.7
Halimeda discoidea Descaine	97.57 ± 2.4
Dictyopteris justii Lamouroux	94.33 ± 1.9
Galaxaura oblongata (Ellis & Solander) Lamouroux	88.15 ± 6.0
Galaxaura marginata (Ellis & Solander) Lamouroux	85.60 ± 6.3
Bryothamnium triquetrum (S.G.Gmelim) Howe	85.57 ± 4.1
Caulerpa racemosa (Forsskal) J. Agardh	73.83 ± 6.8
Gracilaria domingensis (Sonder ex Kützing)	70.97 ± 6.5
Bryopsis plumosa (Hudson) C. Agardh	58.93 ± 12.8
Kappaphycus alvarezii (brown) (Doty) Doty	25.4 ± 12.4
Ulva fasciata Delile	16.5 ± 15.4
Kappaphycus alvarezii (red) (Doty) Doty	13.8 ± 8.3
Kappaphycus alvarezii (green) (Doty) Doty	11.7 ± 1.0
Kappaphycus alvarezii (green-brown) (Doty) Doty	9.7 ± 13.7

Once it is showed that *Halimeda incrassata* has the property of inhibiting spontaneous lipoperoxidation of rat brain homogenates, the additional property of inhibiting hydrogen peroxide production by two different metabolic pathways on the rat cortex was examined. The results obtained after testing *Halimeda incrassata* aqueous extract at 0.5mg.mL^{-1} final concentration in two different hydrogen peroxide generator systems (glutamic acid and malonic acid) are presented in *Figures 2* and *3* respectively.

After incubating the previously chopped rat cortex with *Halimeda incrassata* aqueous extract ($0.5 mg.mL^{-1}$) and glutamic acid (1.5µM), a chemoluminiscent method for estimating hydrogen peroxide formation was used previously described on Materials and Methods . A negative control containing only artificial CSF (1) and a control containing only *Halimeda incrassata* aqueous extract (2) were also included *Figure 2*. Reported values are means of four determinations (Different letters on the top of the bars mean statistical differences in a Duncan test with $p \leq 0.05$).

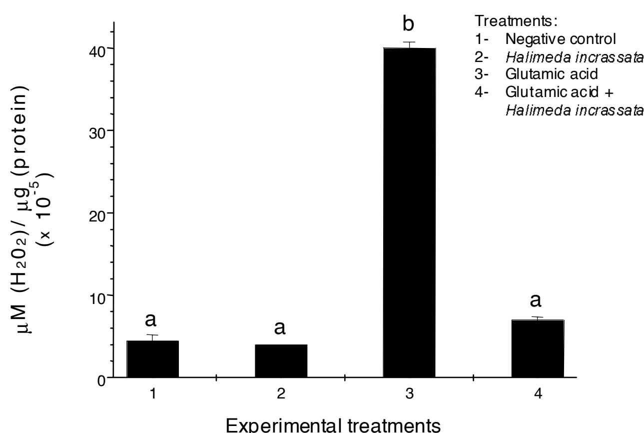

FIGURE 2. Effect of *Halimeda incrassata* on the hydrogen peroxide formation by glutamic acid pathway.

In both cases, significant decrease of hydrogen peroxide generation in presence of *Halimeda incrassata* aqueous extract at $0.5 mg.mL^{-1}$ final concentration was observed, so that it was shown that *Halimeda incrassata* could protect the rat brain tissue to the hydrogen peroxide mediated damage. According to these results, the antioxidant *in vitro* capacity of the extract is not only confined to the inhibition of TBARS formation during the spontaneous lipoperoxidation of rat brain homogenates.

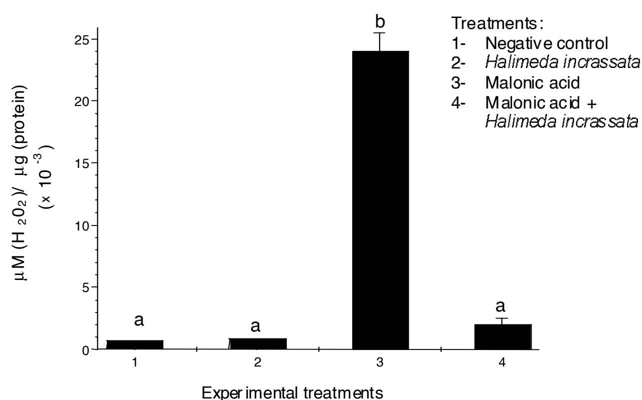

FIGURE 3. Effect of *Halimeda incrassata* on the hydrogen peroxide formation by malonic acid pathway.

Experimental design was identical to the procedure described for *Figure 2*, but malonic acid (29mM) was used in this case for hydrogen peroxide generation. Reported values are means of four determinations. (Different letters on the top of the bars mean statistical differences in a Duncan test with $p \leq 0.05$).

An additional *in vivo* experiment was performed after that, in order to test the ability of *Halimeda incrassata* extract to exert neuroprotection on gerbil brains when exposing the animals to an ischemia-reperfusion injury. As seen in *Figure 4*, ischemia induced an approximate 2-fold increase in locomotor and exploratory activity relative to naive animals, but pre-treatment with 50, 100 or $200 mg.kg^{-1}$ of *Halimeda incrassata* aqueous extract, led to a statistical significant dose-independent lowering of locomotor and exploratory gerbil activity induced by ischemia.

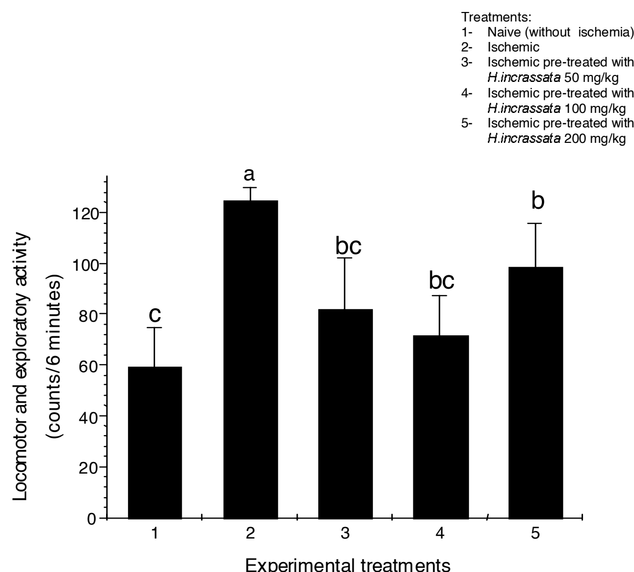

FIGURE 4. Effect of *Halimeda incrassata* on ischemia-induced changes in locomotor and exploratory activity of gerbils.

Rat brain homogenates exposed to oxygen spontaneously exhibit lipid peroxidation by a mechanism which is independent of superoxide and free hydroxyl radical production and whose initiation step may involve and iron-mediated cleavage of lipid hydroperoxides to yield peroxi or alcoxy radicals [1,3,6,7]. In this system, *Halimeda incrassata* aqueous extract effectively inhibited TBARS generation with estimated value of $IC_{50} = 0.340$ $mg.mL^{-1}$. The result could be explained in term of several mechanisms comprising among others, iron quelation, (at the initiation step) or a synergistic increase of vitamin E antioxidant activity (at the propagation step). Molecules of the aqueous extract could be probably capable of transferring electrons and regenerating vitamin E radicals. Consequently, vitamin E antioxidant activity is improved, leading to a diminished radical generation and increased radical scavenging, which inhibit TBARS formation after spontaneous lipoperoxidation of rat brain homogenates.

Similarly, LE TUTOUR [10] suggested that effective principles of two seaweed extracts *Laminaria digitata* and *Himanthalia elongata* react with vitamin E radicals to regenerate vitamin E which could explain the antioxidant

properties of these algal extracts in the preservation of sunflower oil at 75°C. Supporting this idea, the author observed synergistic effects of seaweed extracts with vitamin E on the preservation of methyl linoleate by using kinetic studies at 60°C in heptanol. Vitamin C, phenols, amines and phospholipids are known to synergistically enhance the antioxidative activity of vitamin E, but in case of *Laminaria digitata* and *Himanthalia elongata* extracts, vitamin C amounts were very low.

Apart from the studies carried out in rat brain homogenates undergoing spontaneous lipid oxidation, *Halimeda incrassata* aqueous extract is also capable of inhibiting antioxidative properties in iron-independent models.

In case of hydrogen peroxide production by glutamic acid, the basis of the model lies on the excitotoxicity induced by glutamate in the brain. The process is mediated by glutamate receptors, particularly those of N-methyl-D-aspartate type (NMDA) which, in activated state, can allow and influx of Ca^{2+}, and consequently, initiate a variety of potentially destructive processes. Among other effects, Ca^{2+} can activate phospholipase A_2 which can release arachidonic acid from the membrane phospholipids, and finally arachidonic acid serves as the precursor for prostaglandin biosynthesis, a process considering as an important source of reactive oxygen species, such as hydrogen peroxide [14]. Also Ca^{2+} can lead to calpain activation which can activate xanthine oxidase thus generating hydrogen peroxide by means of the direct xanthine oxidation [22]. A different pathway is used to explain the hydrogen peroxide production by malonic acid. In this case, the inhibition of tricarboxilic acid cycle (succinate deshidrogenase) can increase the leakage of electrons of superoxyde from disrupted mitochondrial electron transport chain [26].

Halimeda incrassata aqueous extract at $0.5 mg.mL^{-1}$ final concentration reduced about 10 folds the hydrogen peroxide generation in the rat brain by the two different pathways already described. This could suggest an additional capacity of the extract for removing hydrogen peroxide, thus potenciating the activity of enzymes involves in this process (catalase or glutathion peroxidase).

Among free radical related diseases, cerebral ischemic stroke is one of the most extensively studied. At the ischemic site, a number of factors involved in cell damage can be the target for neuroprotective drugs, such as antioxidants. In fact, antioxidants have been assessed clinically, and primarily would appear to be useful following sucessful reperfusion. In animal models, antioxidative treatment have consistently reduced infarction size in temporary focal ischemia models, so that it represents a promising alternative for human treatment of ischemia [4]. For these reasons and in view of the above results, *Halimeda incrassata* was tested for the neuroprotective effect on the bilateral carotid occlusion model in gerbils. Neuroprotected groups showed an statistically significant decrease on the locomotor and exploratory activity induced by ischemia, which suggest that aqueous extract of *Halimeda incrassata* could exert an additional *in vivo* protection probably mediated by antioxidant activity.

No seaweed extracts have been reported to exert this *in vivo* neuroprotection until now, although other crude plant extracts are capable of preventing neuronal degeneration associated with brain ischemia in the same model [13].

WEN *et al.* [27] showed that oral administration of red ginseng powder (*Panax ginseng* C.A. Meyer) 5 minutes before of transient forebrain ischemia in gerbil model, prevented the occurrence of ischemia-induced learning disability and hippocampal neuron lost. Neuroprotective molecule was even identified as ginsenoside Rb_1 (protopanaxadiol structure) [28]. Later, LIM *et al* [13] demonstrated that ginsenoside Rb_1 rescued hippocampal neurons from free-radical mediated damage of an aerobic solution of $FeSO_4$ and showed hydroxil radical scavenging activity on Fenton reaction system containing p-nitrosodimethylaniline. So, in that case, neuroprotective properties of red ginseng powder were attributed to the antioxidant molecule ginsenoside Rb_1.

These *in vitro* and *in vivo* evidences for an antioxidant activity of *Halimeda incrassata* aqueous extract suggested the importance of purifying the specific compounds capable of conferring these biological properties to the extract.

The fist developed purification step was a chromatography on Amberlite column XAD-2. For each fraction, antioxidant capacity for reducing TBARS formed after spontaneous lipoperoxidation of rat brain homogenates was determined and results are shown in *Table 3*. The aqueous fraction of this step exhibited the highest antioxidant activity, so that it was applied to a cationic exchange column (DOWEX 50WX8-200). The resultant profile, after determining biological activities of fractions is shown in *Figure 5*.

TABLE 3. Antioxidant activity of fractions obtained after chromatography on Amberlite column XAD-2.

FRACTION	% of inhibition of lipid peroxidation (%)
water	90.6
methanol:water (1:1)	26.0
methanol	31.0
acetone	18.0

Fractions corresponding to the second peak of this chromatogram were then applied to an anionic exchange column DOWEX 1X8-200 and the resultant profile is presented in *Figure 6*.

Fractions corresponding to the first peak of anionic exchange profile were applied on Thin Layer Chromatography (TLC) using silica gel, in which 8 spots were obtained, two of them capable of producing from 50 to 60% of inhibition of TBARS formed during spontaneous lipoperoxidation of rat brain homogenates and three others exhibiting from 40 to 50% of antioxidant activity.

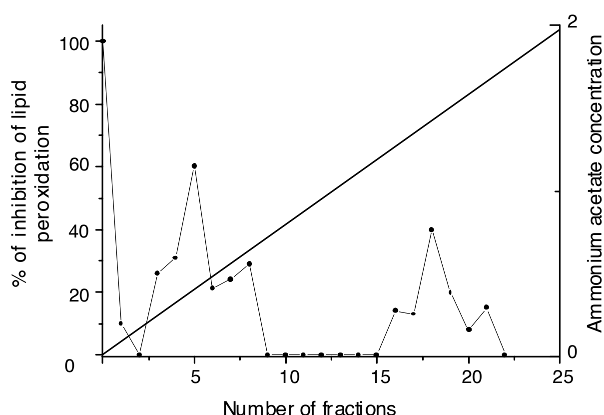

FIGURE 5. Cationic exchange profile on DOWEX 50WX8-200

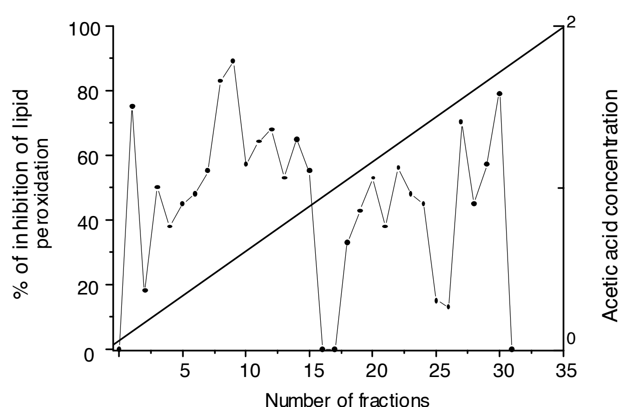

FIGURE 6. Anionic exchange profile on DOWEX 1X8-200.

Taking into account above results, the fractionating of *Halimeda incrassata* aqueous extract was performed. Initially, the extract was applied in an Amberlite XAD-2 column (a non ionic polymeric adsorbent) and the recovered aqueous fraction showed the highest antioxidant activity. Further step involved a cationic exchange chromatography on DOWEX 50WX-200 of the aqueous fraction obtained in adsorption chromatography on Amberlite XAD-2 column. In *Figure 5*, peak exhibiting highest antioxidant activity was then applied to an anionic exchange DOWEX 1X8-200 column and a complex chromatogram with several peaks was obtained (*Figure 6*), which suggest the presence of a mixture of water-soluble metabolites responsible for the antioxidant activity of seaweed extract. Further fractionating step in TLC on silica gel of the first peak of anionic exchange chromatogram was carried out, and additional group of compounds showing antioxidant activity was found. Consequently, this result confirm that antioxidant activity is not explained by the presence of only one metabolite; several hydrosoluble compounds bearing one or two electric charges may act as antioxidants in the complex mixture of *Halimeda incrassata* aqueous extract.

4 – CONCLUSIONS

The results from the present work indicate that even when precise structure of these substances (probably with similar chemical features) is still unclear, it is possible to suggest the existence of several bioactive compounds of water-soluble nature, capable of conferring *in vitro* antioxidant properties to *Halimeda incrassata* aqueous extract as well as *in vivo* neuroprotective effect, which may be useful in protecting to some extent against free-radical mediated diseases. Low toxicity of most seaweeds to humans, but particularly of *Halimeda* genus may favor its use as functional food.

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