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Editorial

Originales

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In vitro antiatherogenicity of extracts from Halimeda incrassata seaweed: antioxidant activity and smooth muscle cell migration studies.

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RESUMEN

Objetivos: El objetivo de este trabajo fue evaluar el potencial ateroprotector in vitro del alga *Halimeda incrassata* en la migración de células de músculo liso de ratón y la oxidación de lipoproteínas en relación con su actividad antioxidante.

Material y métodos: La actividad antioxidante fue determinada mediante los métodos de inhibición de radicales DPPH y la Capacidad antioxidante total (ORAC). La actividad inhibitoria de la oxidación de LDL mediada por iones Cu^{2+} se determinó por la cuantificación de TBARS y dienos conjugados. El efecto del extracto acuoso sobre la migración de las células de músculo liso se evaluó en la línea de células de músculo liso aórtica de ratón MOVAS-1.

Resultados: Se demostró el efecto inhibidor del extracto sobre la oxidación de LDL mediada por Cu^{2+}. El extracto del alga causa inhibición dosis-dependiente de la formación de TBARS (IC_{50} = 0,8 mg/mL) y dienos conjugados. Las algas tuvieron una alta actividad antioxidante en los ensayos realizados y podría estar relacionada con el contenido de compuestos fenólicos.

Conclusiones: Los resultados de este trabajo representan un paso más en la caracterización de la acción ateroprotectora de Halimeda incrassata y evidencian sus posibles aplicaciones como nutracéutico y/o fitofármaco.

PALABRAS CLAVES: antiaterogenicidad, *H. incrassata*, actividad antioxidante, células de musculatura lisa.

ABSTRACT

Aim: The aim of this work was to evaluate the *in vitro* atheroprotective potential of the seaweed *Halimeda incrassata* in smooth muscle cell migration and lipoprotein oxidation in relation to its antioxidant activity.

Material and methods: Antioxidant activity was determinate by $DPPH^{\bullet}$ radical scavenging assay and ORAC method. The inhibitory effect of the aqueous extract on LDL oxidation mediated by Cu^{2+} ions was determinate by TBARS and conjugated diene quantification. The effect of the seaweed aqueous extract on smooth muscle cell migration was evaluated in MOVAS-1 mouse aortic smooth muscle cell.

Results: The inhibitory effect of the aqueous extract on lipoprotein oxidation mediated by Cu^{2+} was demonstrated. Seaweed extract caused dose-dependent inhibition of TBARS (IC_{50} = 0.8 mg/mL) and conjugated dienes formation. The seaweed had a high antioxidant activity in the assays performed. The activity could be related to the phenolic content of *Halimeda incrassata*.

Conclusions: In summary, the results of this study represent a further step in the characterization of the atheroprotective action of *Halimeda incrassata* and indicate the seaweed could be used for a nutraceutical and/or phytoterapeutic application.

KEYWORDS: antiatherogenicity, *Halimeda incrassata*, antioxidant activity, smooth muscle cell.

INTRODUCTION

Atherosclerosis is characterized by lipoprotein retention and oxidative modification in the artery wall with subsequent oxidized LDL uptake and foam cell formation. As lesion progresses smooth muscle cells migrate from the tunica media to the arterial intima marking lesion advancement[1]. Oxidative stress is considered a causal factor in all stages of atherosclerosis development, from oxidative modification of lipoproteins to platelet activation and atherothrombosis[2]. Atherosclerosis is also considered a common factor of other diseases related to oxidative stress such as ischemic heart disease and in this field antioxidants have been attractive to decrease oxidative stress associated with plaque formation[2].

Seaweeds are marine organisms that have been consumed as an integral part of the traditional diet in Asian countries and they are becoming increasingly popular in the western world in association to their health boosting properties[3,4]. Seaweeds have relatively high amounts of bioactive phytochemicals such as polyphenols that are consistent with antioxidative properties[3].

Interestingly, the main effect of algae consumption has been associated with cardiovascular and intestinal health with the epidemiological data available indicating a correlation between seaweed consumption and low atherosclerosis-related morbidity and mortality[4]. The mechanisms for cardiovascular protection are beginning to be elucidated and have been so far mainly related to antioxidative, antihypertensive, anti-inflammatory and hypocholesterolemic effects[4].

In a previous study we showed that *Halimeda incrassata* was one of the most active specimens presenting antioxidant activity in an initial screening study using a lipoperoxidation model[5]. The seaweed has also shown antioxidant activity in the mouse hypothalamic cell[6] and exerted neuroprotection[5] and hepatoprotection[7] *in vivo*. In the present study we investigated the antiatherogenic potential of *Halimeda incrassata* in two key events of atherogenesis: lipoprotein oxidation and smooth muscle cell migration in relation to its antioxidant activity.

MATERIALS AND METHODS

Seaweed collection and hydrophilic extracts preparation

The seaweed *Halimeda incrassata* (Ellis) Lamouroux was collected in December 2010 in the Bajo de Santa Ana, La Habana (Cuba). Voucher specimens were authenticated by Dr. A.M. Suárez from Seaweeds Laboratory, at the Marine Research Center. Freshly collected specimens were washed with distilled water and dried at room temperature for 3-5 days. Dry seaweed powder was extracted with distilled water (1:5 w/v) and centrifuged at 800 g and at 4ºC for 20 minutes. Supernatant was recovered, lyophilized and kept at -20ºC until use. Weight yield of the final extract in terms of dry seaweeds was 5.7%.

A polyphenol-rich fraction containing free phenolic acids (FPA) was obtained according to Krygier *et al*.[8] Dry seaweed was extracted with tetrahydrofuran for 3 min, and evaporated to dryness under vacuum at 30ºC and the pellet was resuspended in ethanol. Total phenolic content was determined as in Vidal *et al*.[9] and expressed as mg of gallic acid (GAE)/g of seaweed.

DPPH• radical scavenging assay

The DPPH• radical scavenging method was realized according Goupy *et al*.[10] and the results are expressed as percent decrease from the initial DPPH• radical absorption by the test samples and calculation of the value of 50% inhibitory concentration (IC_{50}).

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was determined according to Gillespie *et al*.[11] using AAPH as a peroxyl radical generator. ORAC values were given as trolox equivalents.

Inhibitory activity on LDL oxidation mediated by Cu^{2+} ions

LDL was isolated from human plasma by sequential ultracentrifugation as in Frostegard *et al*.[12]. LDL was oxidized with 10 µM $CuSO_4$ and degree of oxidation was determined by TBARS and expressed as nmoles MDA equivalents/mg protein.

Conjugated diene formation was also monitored as in Esterbauer *et al*.[13]. Kinetic parameters lag phase, V_{max} and $Diene_{max}$ were determined according to Pinchuk *et al*.[14].

Inhibition of smooth muscle cell migration

Smooth muscle cell (SMC) migration experiments were done with mouse aortic smooth muscle cells MOVAS-1, generously provided by Dr. Mansoor Hussain from the Heart and Stroke Richard Lewar Center of Excellence in Cardiovascular Research (Canada). Cells were cultured in DMEM containing FBS, 2 mM L-glutamine and streptomycin/penicillin in 5% CO_2 atmosphere. All experiments were performed with MOVAS-1 SMC between passages 5 to 15.

In the wound healing assay smooth muscle cells were grown to 90% confluence in DMEM + 10% FBS. After damaging the monolayers with a sterile tip, cells were treated with 5 ng/mL Platelet derived growth factor and seaweed extract

and allowed to migrate for 16 hours. Cells were stained with crystal violet and after taking photographs distance migrated were calculated as described by Ho *et al.*[15].

Transmigration experiments were done as in Goncharova *et al.*[16] with some modifications. Cells were incubated for 16 hours and after swabbing the upper part, membranes were fixed with methanol and then stained with crystal violet. Transmigrated cells were counted under a microscope at 100x; 10 fields were counted/insert.

Statistical analysis

Values are given as mean ± standard deviation (s.d.). For statistical significance an unpaired student´s t test was performed. Data was processed using Microcal Origin 5.0 and GraphPad Prism software (version 3.01 for Windows). $p < 0.05$ was considered statistically significant.

RESULTS

Phenolic content

H.incrassata had a high total phenolic content with 2.8 µg GAE/mg for the lyophilized aqueous extract and 14.1 µg GAE/g of dry seaweed for the FPA fraction.

DPPH· scavenging capacity of the extract

As shown in Figure 1, *H. incrassata* extract presents a concentration dependent free radical scavenging activity, with an IC_{50} of 0.27 ± 0.011 mg/mL.

ORAC

Antioxidant activity by the ORAC assay is shown in Figure 2. The total antioxidant capacity was higher with increasing concentrations of seaweed with an ORAC value of 3960 trolox equivalents/g of dry seaweed.

Figure 1. Antioxidant activity of the aqueous extract of Hi in the DPPH• radical scavenging assay. The assay was performed according to Goupy et al.10.Data are given as mean ± s.d (n=3).

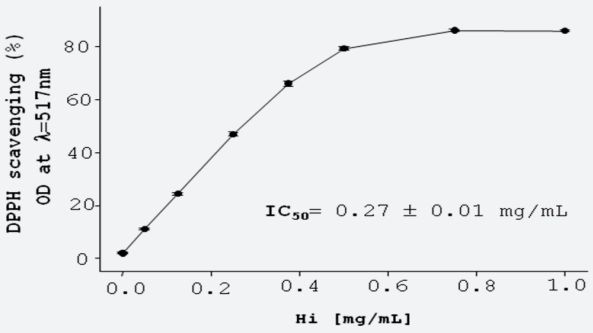
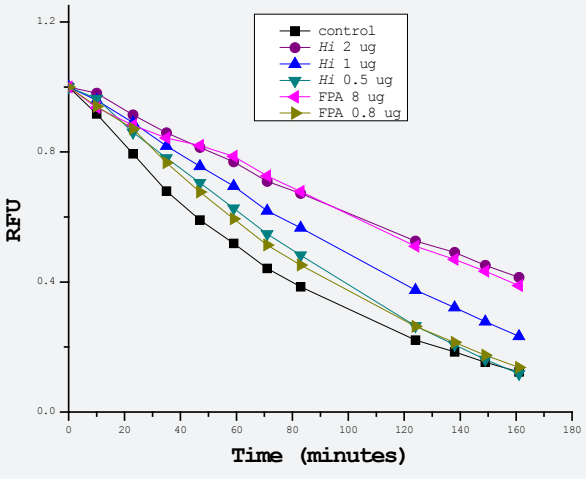


Figure 2. Total antioxidant capacity by ORAC assay as previously described in methods. Data is presented as average RFU where s.d < 10 % of mean RFU.



Inhibition of LDL oxidation

The dose response for inhibition of lipoperoxidation during LDL oxidation mediated by Cu^2 is shown in Figure 3.A with IC_{50} of 0.8 mg/mL while as shown in Figure 3.B conjugated

Figure 3. Effect of Hi on LDL oxidation induced by Cu2+ ions. A. Inhibition of TBARS formation during LDL oxidation. B. Conjugated dienes formation during LDL oxidation. Results are presented as mean ± s.d. (n=3).

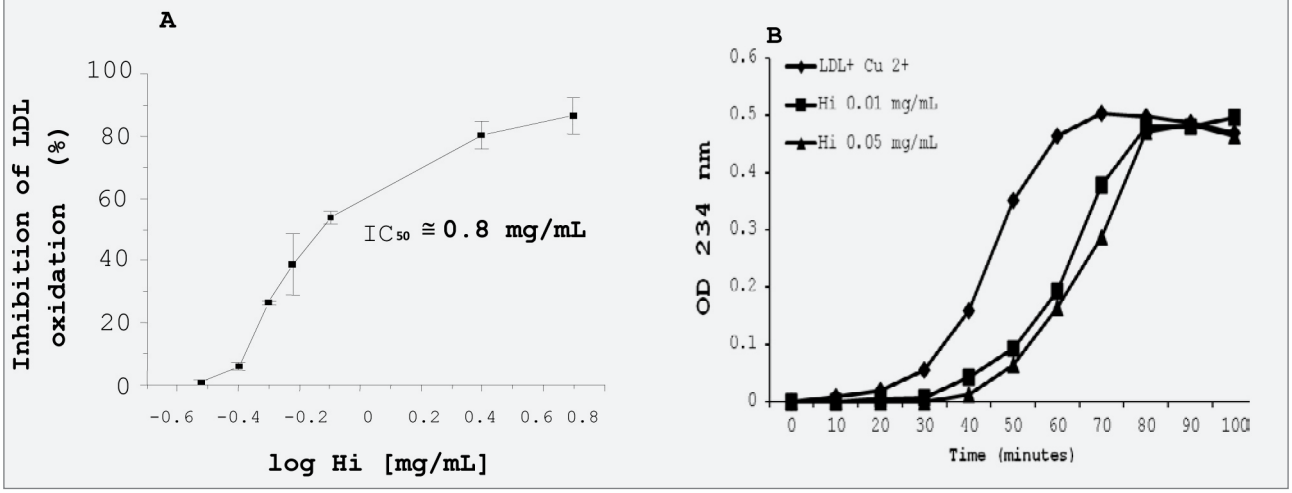


Table 1. Effect of Hi on kinetics of LDL oxidation mediated by Cu2+ ions. Results are presented as mean ± s.d. (n=3).

	Lag phase	V_{max} (μM/min)	Dienes max (μM)
LDL + Cu^{2+}	20.58 ± 2.96	0.57 ± 0.01	16.7 ± 0.55
LDL + Cu^{2+} + Hi 0.01 mg/mL	28.9 ± 6.64	0.49 ± 0.04	16.1 ± 0.28
LDL + Cu^{2+} + Hi 0.05 mg/mL	42 ± 6.50*	0.47 ± 0.07*	16.65 ± 0.35

* Significant differences for $p < 0.05$ vs LDL + Cu^{2+}

Kinetic parameters:
Lag phase: Time of resistance to oxidation of LDL, where conjugated dienes have not formed yet.
Vmax: Maximum rate of conjugated diene formation.
Dienes max: Maximum formation of conjugated dienes.

diene formation. The kinetic parameters lag phase, Vmax and Diene max were presented in Table 1.

Antimigratory activity

The effect of *H. incrassata* on PDGF-BB-induced MOVAS-1 SMC migration is shown in Table 2.

DISCUSSION

Oxidative stress has been critically involved in the progression of atherosclerosis with natural antioxidant extracts being presently studied for potential disease modulation[2]. In this work we further evaluated the *in vitro* antiatherogenic properties of *H. incrassata* and its antioxidant activity in cell free systems.

A high phenolic content was found for hydrophilic fractions from *H. incrassata*, which is similar to the one reported by our group for other seaweeds from the *Halimeda* spp[9]. Likewise Yoshie *et al.*[17] showed that there was a high polyphenols content in the seaweeds *H. opuntia* and *H. macroloba* and identified a variety of phenolic compounds that could be relevant to the antioxidant properties.

lyophilized is in the range of the one reported for other seaweeds in the literature in different regions. Heo *et al.*[18] obtained a 70 % DPPH• scavenging in hydrophilic extracts from *E. cava*; an activity that correlated to phenolic content. Additionally our group informed an IC_{50} of 1,18 mg/mL for the seaweed *Bryothamnion triquetrum*[19] whereas *H. incrassata* - with a significantly higher phenolic content - is about 4 times more effective in DPPH• scavenging.

Likewise Serevinathne *et al.*[20] found an association between antioxidant capacity in DPPH• scavenging and solvent polarity; which also was related to a higher phenolic content in these fractions. The authors found a similar antioxidant activity pattern for other assays such as inhibition of lipoperoxidation.

Together with DPPH• radical scavenging and inhibitory action on lipoprotein oxidation *H. incrassata* also exhibited a high total antioxidant capacity in the range found for other natural extracts[21]. In hydrophilic extracts the activity was similar for the aqueous extract and the polyphenol rich FPA fraction indicating that phenolic compounds are important in the effect observed. Likewise Price *et al.*[22] found significant antioxidant activity by ORAC assay in

Table 2. Effect of Halimeda incrassata seaweed on smooth muscle cell migration in MOVAS-1 mouse aortic smooth muscle cell line. The experiment was performed as previously described by Zargham et al.17
Results are expressed as mean + s.d of each experiment performed in triplicate. Significant differences for $p < 0.05$ compared to PDGF-BB induced migration are indicated in the graphs (*). In transwell experiment 10 fields were counted per insert membrane.

Wound migration assay

	Migrated area (% zero hour)
+ PDGF	100
+ PDGF + *H. incrassata* 1 mg/mL	30.2 ± 2.55 *
+ PDGF + *H. incrassata* 0.5 mg/mL	40.3 ± 4.56 *
+ PDGF + *H. incrassata* 0.1 mg/mL	46.9 ± 1.12 *

Transwell assay

	Transmigrated cells (% of control)
control	100 ± 7.78
PDGF	460.3 ± 37.06
PDGF + *H. incrassata* 1 mg/mL	227.5 ± 21.51 *
PDGF + *H. incrassata* 0.1 mg/mL	262.5 ± 17.68 *

seaweed extracts; where the highest activity correlated to the presence of hydrophilic compounds.

Previous work has shown that the main phenolic compounds in *H. incrassata* are phenolic acids with a majoritary composition of salicylic and lower quantities of ferulic acid that might also contribute to the antioxidant action[23]. Salicylic acid is relevant in plant defence mechanisms and has antinflammatory and antioxidant properties related to lypoxygenase inhibition and to decrease of endothelial cells adhesion molecules expression[24].

LDL oxidation is a key therapeutic target as it is a main emerging risk factor for cardiovascular diseases. Indeed from fatty streak formation to thrombosis in advanced plaques oxidized LDL has a relevant role in atherosclerosis related events such as migration and proliferation of smooth muscle cells and endothelial dysfunction[2]. To help dilucidate the mechanism behind antioxidant action in inhibition of LDL oxidation conjugated diene formation was monitored in presence of seaweed aqueous extract. During LDL oxidation by Cu^{2+} the metal binds to LDL and catalyses the constant formation of free radicals at the expense of the reduction of antioxidants bound to the particle[14]. The variation in oxidation kinetics with increase in the lag phase and decrease in Vmax on addition of aqueous extract could be indicative of a chelating effect of Cu^{2+} or result from the blockage of the sites of binding of cupper to the lipoprotein[14]. Polyphenolic compounds found in the extract could be relevant in this effect since it is known that polyphenols that act through free radical scavenging can also efficiently bind transition metals. The *in vitro* antioxidant activity assays indicate that inhibition of LDL oxidation could be attained potentially by both Cu^{2+} chelating and free radical scavenging, where the phenolic content and antioxidant activity could contribute to this effect.

LDL oxidation *in vivo* is a complex process not fully understood and Cu^{2+} mediated oxidation is a model frequently used to study lipoperoxidation reactions that could take place in the vascular wall[14]. The protective effect in TBARS formation found for *H. incrassata* extract could be associated to the presence of significant amounts of hydrophilic antioxidants involved in scavenging of free radicals formed in the aqueous phase.

Several groups have evaluated the antiatherogenicity of natural extracts against LDL oxidation finding an activity that correlates to phenolic content. The antilipoperoxidative activity found in our study is quite promising as compared to other previously reported natural extracts. For instance a 37 % inhibition of TBARS formation was found in AAPH and 74 % in Cu^{2+} peroxidation by Hseu *et al.*[25]. In that study *T. sinensis* extracts had 6.5 µg GAE; a phenolic content higher than the one needed in our study to reach 50 % inhibition of TBARS formation in both oxidation systems used, adding therefore further evidence to the antioxidant potential of *H. incrassata*.

Antiatherogenic properties of natural extracts in smooth muscle cell biology is another field of relevance in cardioprotection as these are key cell types in the pathogenesis of vascular disease[26]. Their migration from tunica media to subendothelial space marks the transit from the fatty streak to more advanced lesions; since they produce most of extracellular matrix generated during the plaque fibroproliferative response. PDGF secreted locally is the most potent stimulus for smooth muscle cell migration and signalling through its receptor is associated with ROS production resulting from NADPH oxidase activation[27].

Thereafter antioxidants have been of interest for targeting smooth muscle cell migration by several authors. Polyphenol rich natural extracts such as those from tea and cocoa[28] have shown inhibition of smooth muscle cell migration whereas several pure phenolic compounds like the phenolic acid derivative avenanthramide also had antioxidant activity in smooth muscle cells increasing NO production by upregulation of eNOS mRNA[29].

The 43% inhibition of migration in the transwell assay and the decrease migrated area in the wound scratch model found in our study, are consistent with the results for other natural extracts in the literature. Zargham *et al.*[30] had a 62% antimigratory activity in the transwell assay with a tannin extract. Likewise Ho *et al.*[15] evaluated the effect on smooth muscle cell migration of the water extracts from *Nelumbo nucifera* -an aquatic plant used in traditional medicine-, to elucidate the molecular mechanisms of its antiatherogenic action. They found a decrease by 60 % at 0.2 mg/mL in transmigrated cells and decreased inhibition in a wound closure assay, effects the authors discussed that were probably related to the high content of phenolic acids and flavonoids of the extract.

Though the mechanisms of action involved in the antimigratory activity are unknown it could be speculated that it might be related to a direct antioxidant action or to antioxidant enzyme induction capable of modulating migration of these cells.

In the other hand the considerable inhibitory activity of the lyophilized aqueous extract of the seaweed on PDGF-BB induced smooth muscle cell migration adds further evidence to the potential of *H. incrassata* for targeting

atherosclerosis progression and to our knowledge it is the first report of inhibition of smooth muscle cell migration by seaweed extracts.

CONCLUSION

Our previous work has indicated an antiatherogenic effect of the seaweed in atherosclerosis progression in apo E-/- mice[31]. The present study adds evidence to a potential atheroprotective application of *H. incrassata* considering its antioxidant action and its high activity for targeting LDL oxidation and smooth muscle cell migration. Thereafter further studies are needed for elucidating the molecular mechanisms involved in the *in vitro* and *in vivo* effects observed to propose *H. incrassata* extracts for cardiovascular protection.

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