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Article

Inhibition of LDL-oxidation and antioxidant properties related to polyphenol content of hydrophilic fractions from seaweed *Halimeda Incrassata* (Ellis) Lamouroux

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LDL oxidation and oxidative stress are closely related to atherosclerosis. Therefore, natural antioxidants have been studied as promising candidates. In the present study, the LDL oxidation inhibition activity of bioactive compounds from *Halimeda incrassata* seaweed. associated to antioxidant capacity, was evaluated *in vitro*. Experimental work was conducted with lyophilized aqueous extract and phenolic-rich fractions of the seaweed and their effect on LDL oxidation was evaluated using heparin-precipitated LDL (hep-LDL) with exposure to Cu^{2+} ions and AAPH as the free radical generator. *H. incrassata* had a protective effect for hep-LDL in both systems and the presence of phenolic compounds contributed to the activity where phenolic-rich fractions showed significant capacity for inhibition of oxidation mediated by Cu^{2+} ions. The observed effect could be related to the antioxidant potential of polar fractions evidenced by reducing activity and $DPPH^{\bullet}$ radical scavenging. The results obtained *in vitro* further support the antioxidant and LDL oxidation inhibition properties of *H. incrassata* and further knowledge toward future phytotherapeutic application of the seaweed.

Uniterms: *Halimeda incrassata/*antioxidant properties. Antioxidants. Polyphenols. Lipoproteins/ oxidation inhibition.

A oxidação da LDL e o estresse oxidativo estão intimamente relacionados com a aterosclerose. Por isso, os antioxidantes naturais têm sido estudados como candidatos promissores. No presente trabalho foi avaliada *in vitro* a capacidade de inibição da oxidação da LDL pelos compostos bioativos da alga *Halimeda incrassata* em associação à capacidade antioxidante. O trabalho experimental foi conduzido com extratos polares (extrato aquoso liofilizado e frações ricas em fenólicos) e seu efeito na oxidação da LDL foi avaliado usando LDL precipitada com heparina (hep-LDL), oxidada com íons de Cu^{2+} e AAPH, como geradores de radicais livres. A *H. incrassata* apresentou efeito protetor para hep-LDL em ambos sistemas e a presença de compostos fenólicos contribuiu para a atividade em que as frações ricas em fenólicos demonstram capacidade significativa em inibir a oxidação mediada pelos íons de Cu^{2+}. O efeito observado deve estar relacionado com o potencial antioxidante das frações polares medido pela atividade redutora e varredura do radical DPPH. Os resultados obtidos demonstram as propriedades antioxidantes e de inibição da oxidação da LDL da *H. incrassata* e podem contribuir para as evidências de futuras aplicações fitoterapêuticas desta alga.

Unitermos: *Halimeda incrassata/propriedades antioxidantes*. Antioxidantes. Polifenóis. Lipoproteínas/ inibição da oxidação.

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INTRODUCTION

Atherosclerosis is a complex multifactorial disease characterized by modification and oxidation of lipoproteins that, together with a chronic inflammatory response in the artery wall, leads to atherosclerotic lesions (Fernández-Britto, 1998). LDL oxidation and oxidative stress are closely related to atherosclerosis. Therefore, natural antioxidants have been studied as promising candidates (Vogiatzi *et al.*, 2009; Kaliora *et al.*, 2006).

Oxidative modifications have been shown to be involved in all stages of atherosclerosis progression so antioxidants from natural sources are of interest in the quest for antiatherogenic activity. In this respect, edible seaweeds are an excellent source of antioxidants that have been evaluated for cardiovascular health and for their beneficial properties (Jimenez-Escrig *et al.*, 2001; Bocanegra *et al.*, 2009).

Seaweed extracts are presently regarded as an attractive source of natural antioxidants as they have highly developed antioxidant defence systems that have provided evolutionary advantage for sea survive in stressful environmental conditions. Their wide array of antioxidants such as mycosporine-derived aminoacids, polysaccharides, carotenoids, terpenoids and phenolic compounds could be beneficial for human health (Jiménez-Escrig *et al.*, 2001; Kang *et al.*, 2003). Indeed, decreased mortality from cardiovascular diseases in Asian populations could be linked to seaweed consumption (Bocanegra *et al.*, 2009).

It has been shown in previous studies that *H. incrassata* has a high phenolic content (Vidal *et al.*, 2011), together with low amounts of other antioxidants such as ascorbate, β-carotene, chlorophylls and selenium. The quantity of hydrophilic phenolics found in this seaweed lies in the same range as other seaweeds reported in the literature and also studied by our group (Jiménez-Escrig *et al.*, 2001; Yoshie *et al.,* 2002; Vidal *et al.*, 2001). The cardioprotective properties of phenolic compounds from different food types have been described by several authors (Kaliora *et al.*, 2006; Aviram *et al.*, 2008) with particular interest in the unique antioxidant combination found in marine algae that is not found in terrestrial plants (Kang *et al.*, 2003; Chan *et al.*, 2006).

In the present study, the capacity of *H. incrassata* for the inhibition of LDL oxidation owing to its phenolic content and antioxidant activity was evaluated *in vitro*.

MATERIAL AND METHODS

Seaweed collection and hydrophilic extract preparation

The seaweed *Halimeda incrassata* (Ellis) Lamouroux was collected in December/2010 in the Bajo de Santa Ana, Havana City coast, Cuba. Voucher specimens were authenticated at the Seaweeds Laboratory of the Marine Research Center of the University of Havana. Freshly collected specimens were washed with distilled water and dried at room temperature for seven days. An analytical mill and a sieve with a 32 mesh were used for milling and sieving, respectively. The dry powder was used to obtain polar extracts. Dry seaweed powder was extracted with distilled water (1:5 w/v) for 1 hour under agitation at room temperature and then centrifuged at 800 g and 4 ºC for 20 minutes. Supernatant was recovered, lyophilized and kept at -20 ºC until use. Weight yield of the final extract in terms of dry seaweed was 6%. The lyophilized material was dissolved in distilled water at a known concentration for the study.

Polyphenolic rich fractions were obtained according to Krygier *et al.*, (1982). Free phenolic acids (FPA) were extracted six times, each into 20 mL of tetrahydrofurane (THF), using a separatory funnel. The combined extract was then evaporated to dryness under vacuum at room temperature. With the residue of the previous extraction, and through extractions with methanol:acetone:water, the aqueous solution and residue were obtained. The aqueous solution was neutralized and residue dissolved in 20 mL of 2 M NaOH and hydrolyzed for 4 h at room temperature under a stream of nitrogen. After acidification to pH 2 (using 6 M HCl), phenolic acids liberated from esters were extracted five times, each into 20 mL of tetrahydrofurane: ethylether:ethyl acetate, using a separatory funnel. The combined extracts were then evaporated to dryness. The dry residues of of soluble esters of phenolic acids (SEPA) and of insoluble esters of phenolic acids (IEPA) were obtained and dissolved separately in 5 mL of methanol.

Total phenolic concentration

Total phenolic content was determined by the Folin-Ciocalteau assay (Quettier-Deleu *et al.*, 2000) and expressed as µg of gallic acid equivalents (GAE) /gram of sample. The calibration curve was obtained in the range of 100 - 1000 µg gallic acid /mL.

Reducing power

Reducing power of the seaweed phenolic-rich fractions was evaluated according to Oyaizu (1986) with minor modifications. A 1 mL sample (10 - 360 µg GAE) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL 1% $K_3Fe(CN)_6$ and incubated at 50 ºC for 20 minutes. After cooling, 2.5 mL 10% TCA was added and

centrifugation was done at 800 g for 10 minutes. 2.5 mL aliquots were taken from the upper layer and 0.5 mL of 0.1% $FeCl_3$ with 2.5 mL distilled water were added for the final reaction. Absorbance was read at 720 nm on a Rayleigh VIS-723G device against a water blank. Increments in absorbance were considered as increases in reducing power.

DPPH$^{\bullet}$ radical scavenging assay

Free radical scavenging activity of *H. incrassata* extract was examined by the DPPH$^{\bullet}$ assay. DPPH$^{\bullet}$ radical absorbance capacity of *H. incrassata* polyphenol-rich fractions was tested according to Goupy *et al.* (1999). A 0.6 mL volume of the extract (10 - 40 µg GAE) was mixed with 0.6 mL of methanolic solution of DPPH$^{\bullet}$ (60 µM). After 30 minutes, absorbance was measured at 517 nm against a sample without antioxidant. Radical scavenging activity was calculated relative to reference absorption as Percentage Inhibition (PI) (%) = $[1\text{-}A_{sample}/A_{sample\ without\ antioxidant}] \times 100$. Results were given as % antioxidant activity and 50% inhibitory concentration (IC_{50}).

In vitro inhibition of LDL oxidation in cell-free system

Oxidation experiments were conducted with heparin-precipitated LDL (hep-LDL), a model of LDL that has interacted with extracellular matrix and is therefore more prone to oxidation (Upritchard, Sutherland, 1999). Lipoproteins were isolated from normolipemic human serum by the method of Wieland (1983). A 5 mL volume of sodium citrate buffer (64 mmol/L, pH 5.12) containing heparin (50 000 UI/L) was added to 0.5 mL serum. After incubating for 10 minutes at room temperature, the sample was centrifuged at 800 g for 15 minutes. Hep-LDL precipitate was washed 3 times with Hepes buffer (5 mM Hepes, 20 mM NaCl, 4 mM $CaCl_2$ and 2 mM $MgCl_2$, pH 7.2), by centrifuging at 800 g for 15 minutes, hep-LDL was next dissolved in 0.5 mL phosphate buffer, pH 7.4, with NaCl 4 %. The Hep-LDL fraction was then divided into aliquots and kept at 4 ºC. Cholesterol content was determined by enzymatic assay (Boehringer Mannhein Diagnostics) and protein content by the Lowry method (1951).

To evaluate the action of the extracts against LDL oxidation and determine the contribution of the bioactive compounds present in it, experiments were first done with the lyophilized aqueous extract, a more crude extract containing algae component.

The hep-LDL oxidation assay was done using LDL (0.2 µmol cholesterol) diluted in phosphate buffer and incubated in the presence or absence of *H. incrassata* lyophilized aqueous extract for 6 hours at 37 ºC with 10 µM Cu^{2+} or 20 mM AAPH. Inhibitory activity against lipoperoxidation of the phenolic-rich FPA, SEPA and IEPA fractions was evaluated based on Cu^{2+} mediated hep-LDL oxidation. The oxidation was determined by thiobarbituric reactive substances (TBARS) as described in Frostegard *et al.* (1990) and expressed as nmoles MDA equivalents/mg protein, using tetramethoxypropane (TMP) as the standard. Results are expressed as percentage of inhibition of lipoperoxidation.

RESULTS

Phenolic content of hydrophilic fractions of *H. incrassate*

Phenolic content in aqueous extract and fractions FPA, SEPA and IEPA of *H.incrassata* are shown in Table I.

TABLE I - Phenolic content (µg total phenolics/g dry seaweed) in polar fractions obtained from seaweed *Halimeda incrassata.*

Fraction	µg GAE/g dry seaweed
FPA	14.16 ± 0.92
SEPA	9.91 ± 0.81
IEPA	11.06 ± 4.2
aqueous extract	130.98 ± 8.04

Total phenolic content expressed as µg of gallic acid equivalents (GAE)/gram dry seaweed. Legend: FPA - Free phenolic acids; SEPA - Soluble esters of phenolic acids; IEPA - Insoluble esters of phenolic acids. Values expressed as mean ± s.d. (n=3).

Reducing power of polar fractions of *H. incrassata*

There was an increment in reducing power with increasing phenolic content of the polar extracts with values of absorbance - OD of around 0.16 at 30 µg GAE and highest absorbance (nearly 1.2) reached at 360 µg GAE (Figure 1).

DPPH$^{\bullet}$ radical scavenging capacity of polar fractions

The polar fractions had concentration-dependent free radical scavenging activity in the DPPH$^{\bullet}$ assay with more than 50% inhibition at a concentration of 40 µg polyphenols (Figure 2).

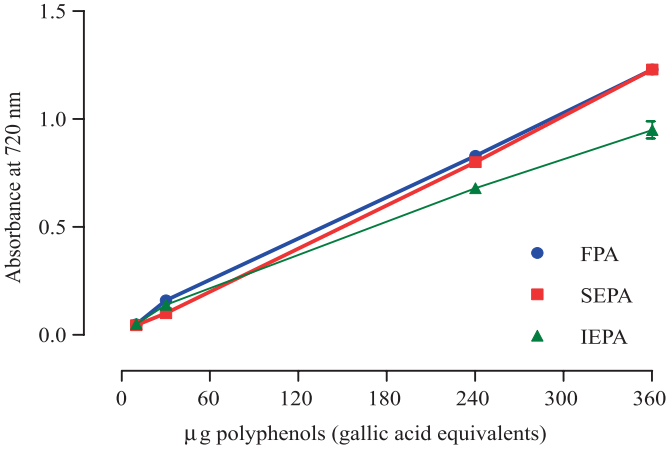

FIGURE 1 - Reducing capacity of polar fractions of *Halimeda incrassata*. Reducing power of the polar fractions was evaluated at concentrations of 10 - 360 µg polyphenols and the assay was performed according to Oyaizu *et al.*, (1986). Values expressed as mean ± s.d. (n=3).

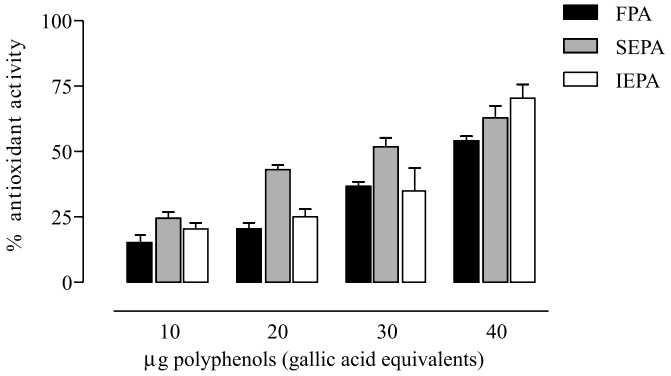

FIGURE 2 - DPPH• radical scavenging activity of polar fractions of *Halimeda incrassata* seaweed. The assay was performed as described by Goupy *et al.*, 1999. Results were given as % antioxidant activity and 50 % inhibitory concentration (IC_{50}). Values represent mean ± s.d. (n=3).

In vitro inhibition of LDL oxidation in cell-free system

Inhibition of LDL oxidation was dose-dependent for both Cu^{2+} ions and AAPH mediated oxidation on addition of *H. incrassata* lyophilized aqueous extract (0.6 - 10 mg/mL) as indicated in Figure 3.

Similarly, the mean inhibitory concentration (IC_{50}) for n-LDL (native LDL) and hep-LDL oxidation mediated by Cu^{2+} and AAPH is shown in Table II.

The three polar fractions rich in phenolic compounds (FPA, SEPA and IEPA) were also active against hep-LDL oxidation mediated by Cu^{2+} ions with more than 50% inhi-

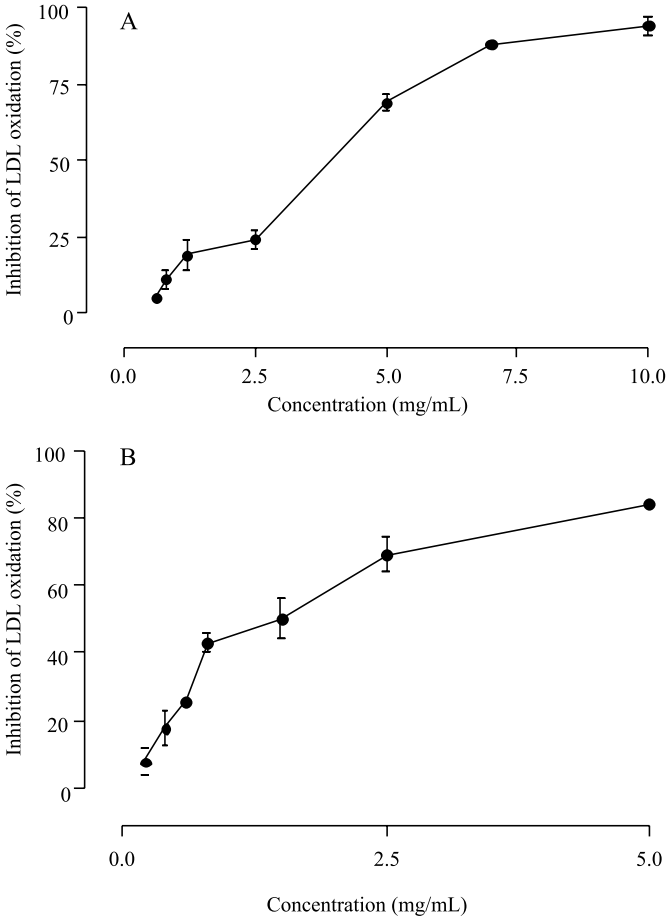

FIGURE 3 - Dose-response curves of the aqueous extract of *Halimeda incrassata* on inhibition of lipoperoxidation in hep-LDL oxidation mediated by Cu^{2+} 10 µM (A) or AAPH 20 mM (B). *H. incrassata* concentration is expressed in log concentration of aqueous extract (1 mg lyophilized is equivalent to 0.02 g dry seaweed containing 2.183 ± 0.134 µg GAE/mg lyophilized aqueous extract. Values represent mean ± s.d. (n=3).

TABLE II - Effect of *Halimeda incrassata* on LDL oxidation in cell free systems.

	50 % mean inhibitory concentration (IC_{50}) of *H. incrassata* (mg/mL)	
Cell-free systems	Cu^{2+}	AAPH
Hep-LDL	3. 747 ± 0.106	1.222 ± 0.1196
n-LDL	0.875 ± 0.0962	0.161 ± 0.014

Results expressed in mg/mL where 1 mg lyophilized is equivalent to 0.02 g dry seaweed containing 2.183 ± 0.134 µg GAE/mg lyophilized aqueous extract and values represent mean ± s.d. (n=3). Legend: Hep-LDL (heparin-precipitated LDL) and n-LDL (native LDL)

bition of TBARS formation at 20 µg polyphenols (Figure 4) indicating that phenolic compounds are relevant in protection against lipoperoxidation exerted by *H. incrassata*.

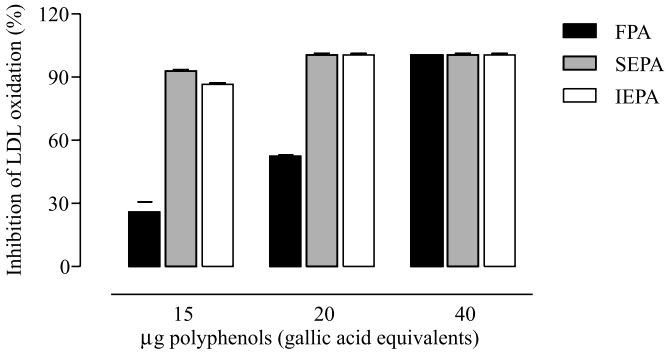

FIGURE 4 - Inhibitory activity of phenolic-rich polar fractions in hep-LDL oxidation mediated by Cu^{2+} done according to Frostegard *et al.* (1990). Oxidation was conducted in the presence of the fractions FPA, SEPA and IEPA from seaweed *Halimeda incrassata* and evaluated by TBARS formation. Values expressed as mean ± s.d.

DISCUSSION

Phenolic content has been correlated to the antioxidant capacity of several seaweed extracts (Jiménez-Escrig *et al.,* 2001; Senevirathne *et al.,* 2006). Values of phenolic contents of *H. incrassata* obtained were 9.99-14.1 and 131 µg GAE/g dry seaweeds for phenolic rich fractions and aqueous extract, respectively. The values for these fractions proved higher than those reported for others varieties of seaweeds, but are consistent with the levels found in *F.vesiculosus* and *B.triquetrum* (Jiménez-Escrig *et al.,* 2001; Vidal *et al.*, 2001). It had been previously reported that FPA, SEPA and IEPA fractions and aqueous extract from *H.incrassata* were rich in phenolic acids (Vidal *et al.*, 2011).

The relevance of hydrophilic phenolics was also emphasized by other authors (Senevirathne *et al.*, 2006) who showed that reducing capacity and DPPH• scavenging increased with solvent polarity and phenolic content in seven fractions of the seaweed *E. cava*. In the present study, a dose-dependent reducing activity and DPPH• scavenging (Figures 1 and 2) was obtained with increasing phenolic content for the evaluated polar extracts ($r^2 = 0.69$ for reducing power and $r^2 = 0.77$ for DPPH• radical scavenging).

Likewise, a similar reducing activity was obtained by Cho *et al.* (2007) with the seaweed *S. siliquastrum* which had an absorbance of 0.8 at a lower phenolic content than the phenolic rich-fractions of our study (240 µg EAG vs 360 µg EAG).

Interestingly, both for reducing capacity and DPPH• radical scavenging (IC_{50}= 29.42 to 39.17 versus 0.75 µg polyphenols, respectively) there was a higher activity for the aqueous extract than for the phenolic-rich fractions showing that other antioxidant components present in *H. incrassata* could have a synergic action with phenolic compounds.

In vitro inhibition of LDL oxidation was evaluated. The assays were done with heparin precipitated LDL, with Cu^{2+} ions and AAPH used as oxidants to model transition metal or free radical mediated oxidation respectively. In LDL oxidation experiments (Figures 3 and 4) there was a more advanced degree of oxidation in the presence of Cu^{2+} than for AAPH as the oxidant (causing formation of 28.4 nmol MDA/mg protein versus 14.5 nmol MDA/mg protein as oxidation products respectively). Indeed oxidation mediated by Cu^{2+} is considered a more aggressive system that transits through Cu^{2+} binding, causing continuous free radical formation with reduction of lipoprotein antioxidants; whereas oxidation by AAPH is a simpler model that ignores the influence of transition metals (Pinchuk and Lichtenberg, 2002). Accordingly, the aqueous extract had inhibitory action in both, where attaining a protective effect required higher doses for transition metal-mediated oxidation compared with free radical oxidation (IC_{50} = 3.747 versus 1.222 mg/mL).

Also, it should be noted that, in comparison to our previous studies on native LDL oxidation, higher doses of aqueous extract were required to protect hep-LDL consistent with their higher susceptibility to oxidation compared to n-LDL (IC_{50} = 0.875 and 0.161 for Cu^{2+} and AAPH as inductor, respectively).

The effect of *H. incrassata* can be compared to that found for other extracts in the literature with 0.5-1 mg/mL needed to inhibit Cu^{2+} ion-mediated LDL oxidation in the case of *P.quinquefolium*, a ginseng variant (Li *et al.*, 1999) and 0.2 mg/mL causing 18.7 % inhibition of TBARs formation for VNP, a commercial product prepared from a mixture of several brown seaweeds (Kang *et al.*, 2003).

The influence of seaweed phenolics on inhibition of lipoprotein oxidation was evaluated (Figure 4). The Cu^{2+} ions-induced hep-LDL oxidation inhibition activity found (100% for 40µg of polyphenols) for the fractions (FPA, SEPA and IEPA) supports the protective effect of the seaweed and indicates that phenolic compounds of H. incrassata contribute to the inhibition of LDL oxidation.

In this study, a positive correlation was confirmed between DPPH• radical scavenging and inhibition of LDL oxidation for phenolic rich fractions ($r^2 = 0.92$ for the FPA fraction) indicating that free radical scavenging is a mechanism involved in inhibition of lipoperoxida-

tion mediated by the seaweed. Our group has previously highlighted the relevance of free radical scavenging for antilipid peroxidative activity (Rivero *et al.*, 2003).

Other authors have studied the action of natural phenolic compounds in the inhibition of LDL oxidation. Aviram *et al.* (2008), studying antiatherogenic properties of pomegranate juice, found that significant inhibition of TBARs formation during LDL oxidation was achieved at 0.5 µg GAE. In another study on the antiatherogenic properties of olive oil polyphenols Rosenblat *et al.* (2007) found 80 % inhibition of oxidation at 1.2 µg GAE for olive oil, whereas when enriched with tea polyphenols the effect obtained was enhanced, correlating with an increase in phenolic content.

CONCLUSIONS

The results of this study indicated that *H. incrassata* polar extracts have properties for inhibition of LDL oxidation that could be related to high antioxidant activity evaluated as reducing capacity and scavenging of free radicals. Seaweed phenolic compounds could be the main components involved in the antioxidant and antilipid peroxidative action, although small quantities of other antioxidants present could have a synergic effect and enhance LDL oxidation inhibition activity. Additionally, these results represent a further step toward its application as a phytotherapeutic for oxidative stress-related conditions.

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