

Free Phenolic Acids from the Seaweed *Halimeda monile* with Antioxidant Effect Protecting against Liver Injury

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Phenolic compounds are found in seaweed species together with other substances presenting antioxidant activity. The objective of this work was to evaluate the antioxidant activity of the free phenolic acids (FPA) fraction from the seaweed *Halimeda monile*, and its activity to protect the expression of hepatic enzymes in rats, under experimental CCl₄ injury. The antioxidant activity was measured by the DPPH method. The FPA fraction (80 mg/kg, p.o.) was administered during 20 consecutive days to rats. The peroxidation was performed by thiobarbituric acid reactive substances (TBARS). The SOD and CAT enzymatic expressions were measured by RT/PCR. The histology technique was used to evaluate liver injuries. The expression of both, CAT and SOD genes, was more preserved by FPA. Only partial injury could be observed by histology in the liver of rats receiving FPA as compared with the control group; and CCl₄ administration induced 60% more peroxidation as compared with the rats receiving FPA. These data suggest that FPA could modulate the antioxidant enzymes and oxidative status in the liver through protection against adverse effects induced by chemical agents.

Key words: *Halimeda monile*, Antioxidant Activity, Hepatoprotective Effect

Introduction

Since ancient times, seaweed has been used as a diet component in several parts of the world, mainly in Asian countries. Nowadays, studies have demonstrated the different therapeutic properties of these marine algae, which were verified *in vitro* as well as *in vivo* (Henry *et al.*, 2002; Karakaya, 2004; Shin *et al.*, 2006). It is also reported that seaweed has an efficient antioxidative defense system, which is attributed to adverse environmental conditions, such as solar light, pH value, oxidative stress, and herbivorous fishes present mainly in the tropical seas (Nakai *et al.*, 2006). Within different seaweed species there are varied antioxidant properties, which result from the presence of compounds like carotenoids, mycosporines, and terpenoids; between them are polyphenols such as phenolic acids, phlorotannins, and bromophenols (Hsu, 2008; Kang *et al.*, 2005; Fisch *et al.*, 2003; Rozema *et al.*, 2002).

Some species from the genus *Halimeda* as well as from other genera of green marine algae have

been demonstrated to be phytopharmaceuticals (Ballantine *et al.*, 1987; Dzeha *et al.*, 2003; Gopal and Sengottuvelu, 2008). Previous works have shown the ability of *H. incrassata* to capture free radicals in neuron cells cultivation; its antioxidant effect was verified in rats exposed to oxidative stress induced by intoxication with methyl mercury (Fallarero *et al.*, 2003). The antitumour effect was detected by induction of apoptosis in leukemia cells, using extracts from *H. discoidea* (Harada and Kamei, 1998).

Superoxide dismutase (SOD) and catalase (CAT) along with glutathione peroxidase (GPX) are antioxidant enzymes which play central roles in the organism defense against reactive oxygen species (ROS). SOD, which produces hydrogen peroxide (H₂O₂) from oxyradicals, consists of Cu/Zn isoforms, Mn and extracellular SOD. The activity of CAT, which decomposes hydrogen peroxide, is found at high levels in the liver and other organs or systems (Halliwell, 2006).

Therefore, considering that seaweed is a potential source of bioactive compounds, the present study aimed to evaluate *Halimeda monile* respecting its antioxidant activity expressed as protection of the essential hepatic enzymes SOD and CAT in rats under experimental CCl₄ injury.

Experimental

Seaweed collection

The seaweed *Halimeda monile* was collected on November 2006, in the area of Bajo de Santa Ana, Havana City coast, Cuba. Specimens were authenticated by the Seaweeds Laboratory, Marine Research Center, University of La Habana, Cuba, where a voucher was also deposited.

Preparation of hydrophilic fractions

Fractions containing phenolic acids, free phenolic acids (FPA), their soluble esters (PASE) and their insoluble esters (PAIE) were obtained according to Krygier *et al.* (1982).

Determination of total phenolic contents

Total phenolic contents were determined according to Swain and Hills (1959) and expressed as mg of gallic acid/g of sample.

Identification and quantification of phenolic compounds

Phenolic acids were studied according to Vidal *et al.* (2001). Different seaweed fractions were analyzed by gas chromatography on a GC Shimadzu 17A instrument equipped with an FID and a DB-5 fused silica capillary column [J & W Scientific, 5% phenyl-methylpolysiloxane (30 m × 0.25 mm × 0.25 μm)]. Helium was used as the carrier gas. Peak areas were computed by a GC Workstation Class-GC-10 (Shimadzu) integrator.

Measurement of antioxidant activity in vitro: β-carotene/linoleic acid and DPPH methods

The antioxidant activity of the different fractions was evaluated according to Miller (1971). A mixture of β-carotene and linoleic acid, subjected to oxidation by oxygenated water, was used with sample volumes between 100 and 200 μL.

The DPPH radical scavenging method is based on the reduction of the methanolic DPPH radi-

cal in the presence of a hydrogen-donating antioxidant (Brand-Williams *et al.*, 1995). The results were expressed in terms of percent decrease from the initial DPPH radical adsorption by the test samples and calculation of the value of 50% inhibitory concentration (IC₅₀).

Animals

Male Wistar rats from the University of São Paulo, weighing 120–150 g, were maintained under controlled diet, with cycles of 12 h of light/obscurity, at 25 °C and 60% humidity. The rats had free access to water and to a standard food diet according to the care guidelines for laboratory animals used in research. These animals were kept into four boxes, with six rats each. Animal studies were approved by the Institutional Ethical Committee for Animal Experimentation from the Faculty of Pharmaceutical Sciences of the University of São Paulo, Brazil.

Animal treatment schedule

Hepatic injury was induced in rats by intraperitoneal administration of a single dose of 3 mL CCl₄ (mixed 1:1 with olive oil). Gallic acid (GA), a known antioxidant compound, was used as reference. The animals were grouped as follows:

Group I: Control, treated daily with vehicle (1.0 mL, p.o.) for 20 d.

Group II: Treated daily with FPA (80 mg/kg, p.o.) for 20 d, followed by treatment with CCl₄ on day 21.

Group III: Treated daily with GA (100 mg/kg, p.o.) for 20 d, followed by treatment with CCl₄ on day 21.

Group IV: Treated daily with vehicle (1.0 mL, p.o.) for 20 d, followed by treatment with CCl₄ on day 21.

At the end of the treatment, blood samples and liver of each animal were collected. Serum was separated and used for the assay of thiobarbituric acid reactive substances (TBARS).

Liver homogenate

The liver extract from the rat groups was obtained from livers previously washed in 0.9% NaCl solution. Then, 1 g from the major lobule was maintained in the bath, and the homogenates were prepared using a Potter-Elvehjem homogenator in phosphate buffer (PBS, 50 mM, pH

7.4). Then these homogenates were centrifuged at $800 \times g$ in a Beckman centrifuge at 4°C for 15 min. The supernatants were kept at -20°C until the assays were carried out.

Determination of superoxide dismutase (SOD) activity (Mc Cord and Fridovich, 1969)

The cytoplasmatic SOD activity was evaluated using 100 mM cytochrome C, 500 mM xanthine, 1 mM EDTA and 200 mM KCN in 0.05 M potassium phosphate, pH 7.8. The xanthine oxidase (same volume in the blank) was placed in a glass tube along with 15 μL of the cytosolic fraction from each liver tissue. The results were expressed as U/mg protein. One unit (U) was the enzyme activity that induced 50% of inhibition of the xanthine reaction at 25°C , pH 7.8.

Determination of catalase (CAT) activity (Beutler, 1975)

The activity was evaluated by decomposition of hydrogen peroxide caused by the enzyme CAT through the decrease of the optic density at 230 nm (coefficient of the molar extinction $0.0071 \text{ mm}^{-1} \text{ cm}^{-1}$) at 37°C . One U of CAT corresponded to the enzyme activity that hydrolyzed 1 mol of H_2O_2 per min at 37°C , pH 8.0. The activity was expressed as U/mg protein.

TBARS assay

As a marker of lipid peroxidation, the TBARS contents were measured in liver homogenates and serum using the method of Ohkawa *et al.* (1979). The results were expressed as nmol/mg protein.

Glutathione (GSH) analysis (Ellman, 1959)

The hepatic total GSH content was measured as the change in absorbance that was monitored at 410 nm for 5 min, and the GSH level was calculated using pure GSH as standard.

RT/PCR

RNA extraction: CAT and SOD gene evaluation

RNA was extracted from rat liver homogenate utilizing a mixture of 250 μL sample and 750 μL trizol reagent (Invitrogen). The extract was kept at room temperature for 5 min with addition of 200 μL chloroform (Merck). The samples were mixed by vortexing for 15 s and kept at room temperature for 5 min. After this, they were cen-

trifuged at $12,000 \times g$ for 15 min at 4°C . 400 μL of the supernatant were removed, avoiding the interphase, and mixed with 500 μL of isopropanol by vortexing for 5 s. These samples were centrifuged at $12,000 \times g$ for 5 min at 4°C discarding the supernatant. To the resulting pellet, 1,000 μL of ethanol (75%) were added and gently mixed, followed by centrifugation at $7,500 \times g$ for 10 min at 4°C discarding the supernatant. Finally, 20 μL of distilled water, RNase-free, were added and incubated at 50°C for 10 min. This material was stored at -70°C .

Reverse transcription

5 μL of RNA were added to 1.0 μL of primers (SOD or CAT), 1.0 μL of 10 mM dNTP, and 4.0 μL of sterile distilled water. The reaction started by heating at 65°C for 5 min; then it was quick chilled on ice. Next, 4.0 μL of 5X first strand buffer (Invitrogen), 2.0 μL of 0.1 M DTT (Invitrogen), and 1.0 μL of RNase out ribonuclease inhibitor (Invitrogen) were added and incubated at 37°C for 2 min. After that, 1.0 μL (200 U) of reverse transcriptase (M-MLV RT-Invitrogen) was added and incubated at 37°C for 50 min. The reaction was stopped by heating at 70°C for 15 min. The PCR product (cDNA) should be stored at -70°C .

PCR reaction for amplification

5 μL of cDNA were amplified in a volume of 50.0 μL containing 5.0 μL 20 mM Tris-HCl (hydroxymethyl aminomethane-hydrochloride) buffer, pH 8.4, 500 mM KCl, 1.5 μL 50 mM MgCl_2 , 1.0 μL 10 mM dNTP, 35.1 μL diethyl pyrocarbonate (DEPEC), 1.0 μL of primers (SOD or CAT), and 0.4 μL (5 U/ μL) Taq polymerase. This reaction mixture was warmed by a thermal cycler (Bio-Rad) at 94°C for 3 min and 35 cycles of 45 s at 94°C , 30 s at 55°C , 1.3 min at 72°C , and 72°C for 10 min. Finally, the temperature was cooled at 4°C for indeterminate time. The PCR-amplified product was analyzed by a 2.0% agarose gel (Sigma) electrophoretic run (60 V). The bands, stained with 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide, were documented by fluorescent table (Vilber-Lourmat) and photographed by a digital camera (Sony). The bands were revealed with -262 bp (C to T) from the primers used to CAT genotyping and $+242 \text{ bp}$ (C to T) from the primers used to SOD genotyping (Promega, Madison/USA): Primer SOD 1 – sequence (5' to 3'): TCT AAG AAA CAT GGC GGT CC.

Primer SOD 2 – sequence (5' to 3'): CAG TTA GCA GGC CAGCAG AT.

Primer CAT 1 – sequence (5' to 3'): GCG AAT GGA GAG GCA GTG TAC.

Primer CAT 2 – sequence (5' to 3'): GAG TGA CGT TGT CTT CAT TAG CAC TG.

Histopathological studies

Small pieces of liver, fixed in 10% neutral buffered formalin, were processed for embedding in paraffin. Sections of 5–6 μm were cut and stained with hematoxylin and eosin.

Statistical analysis

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

Results and Discussion

Through the enzymatic assays carried out with *Halimeda monile*, the presence of phenolic acids was verified in the fractions by means of determination of the antioxidant activity by β -carotene/linoleic acid and DPPH methods (Figs. 1 and 2).

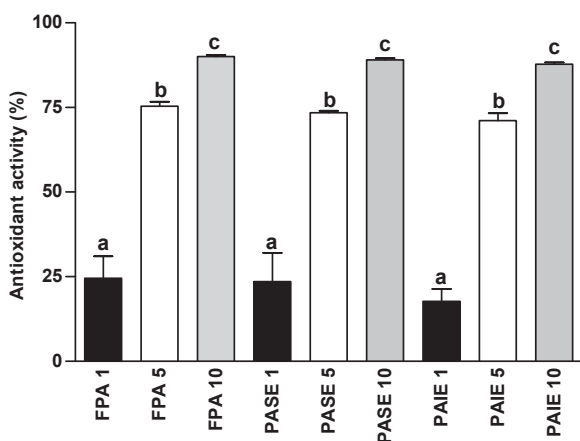


Fig. 1. Antioxidant activity (referring to every 1, 5, and 10 μg of phenolic compounds) measured in fractions containing free phenolic acids (FPA) and soluble and insoluble phenolic acid esters (PASE, PAIE) obtained from the seaweed *Halimeda monile*. Different letters mean statistical differences, $p < 0.05$.

The content of free phenolic acids (FPA) was 27.76 mg/g dry seaweed, and the main components were salicylic, cinnamic, gallic, and caffeic acids (data not shown) (Hsu, 2008; Kang *et al.*, 2005). The activity levels were very similar among the different polar fractions, such as FPA, soluble phenolic acid esters (PASE) and insoluble phenolic acid esters (PAIE) (Fig. 2).

Through the determination of SOD enzyme activity, it was possible to verify the occurrence of statistical differences in this enzyme activity levels between animals receiving or not the *Halimeda monile* extract. So, we have evidenced a hepatoprotective effect on the three study arms treated with phenolic compounds from *H. monile*, previous to CCl_4 administration (Halliwell, 2006) (Fig. 3).

This hepatoprotective effect was also verified through evaluation of the CAT activity and GSH levels in the FPA (II) and GA (III) groups. In fact, these groups receiving phenolic compounds presented results with statistical differences in the values of enzymatic activity (Figs. 3a, b, c), in relation to the control group.

TBARS were indicative of oxidative stress inducing injury in rat livers by CCl_4 . In liver as well as in serum, the TBARS revealed hepatoprotection in the groups treated with GA and FPA antioxidants (Figs. 4a, b) (Ohkawa *et al.*, 1979).

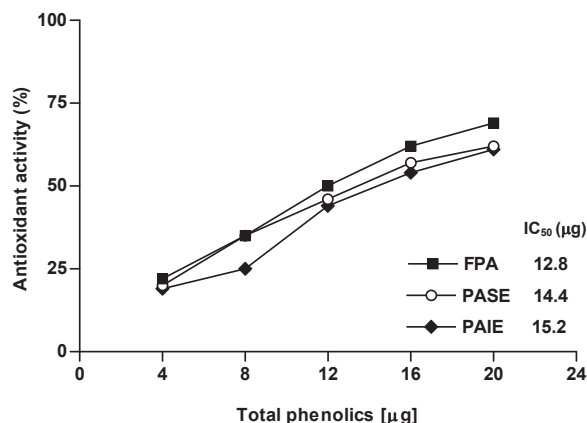


Fig. 2. Antioxidant activity of different polar fractions of *Halimeda monile* determined by the DPPH assay.

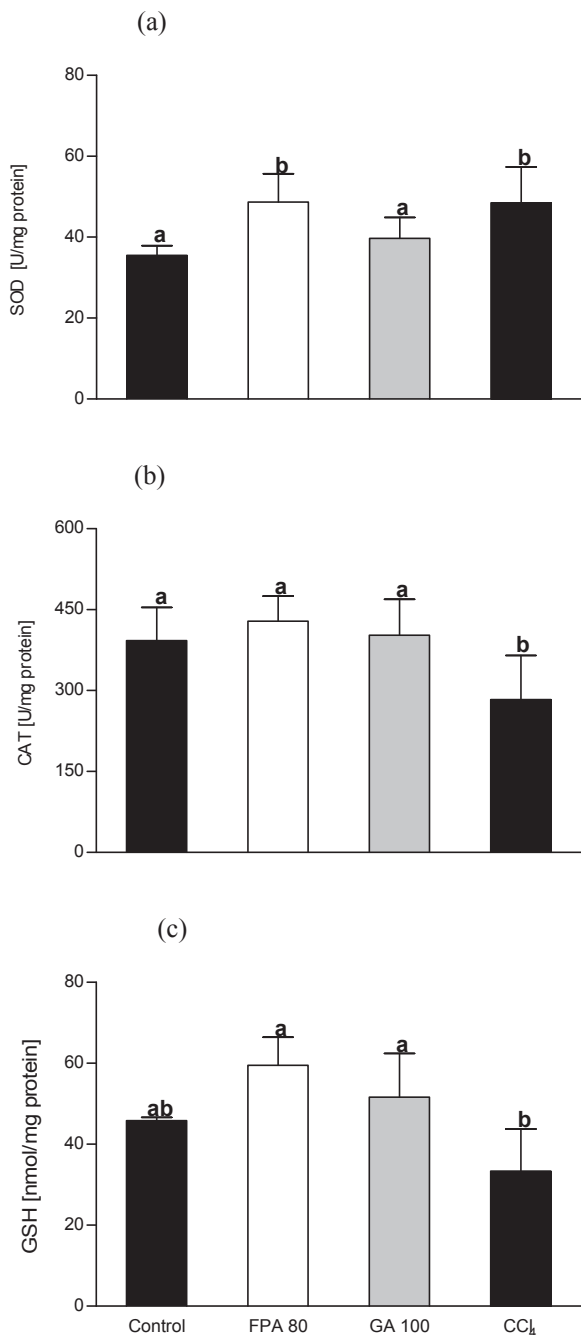


Fig. 3. Activity of the enzymes SOD (a) and CAT (b) and GSH (c) from the livers of rats treated or not with FPA (80 μ g/kg) or GA (100 μ g/kg) antioxidants and CCl₄ injury. Different letters mean statistical differences, $p < 0.05$.

Using molecular evaluation, it was observed that livers collected 24 h after CCl₄ administration preserved the CAT and SOD genes, when the rats were previously treated with GA and FPA antioxidants. In rats with liver injuries caused by CCl₄ and without any previous treatment, the absence of electrophoretic bands corresponding to GA and FPA genes was observed (Figs. 5a, b) (Halliwell, 2006).

From previous evaluation of liver injuries caused by CCl₄, which was reported to occur in a range of 6 to 12 h after CCl₄ administration to rats, and the restoration that starts after 48 h (Tanigushi *et al.*, 2004), it was possible to suppose

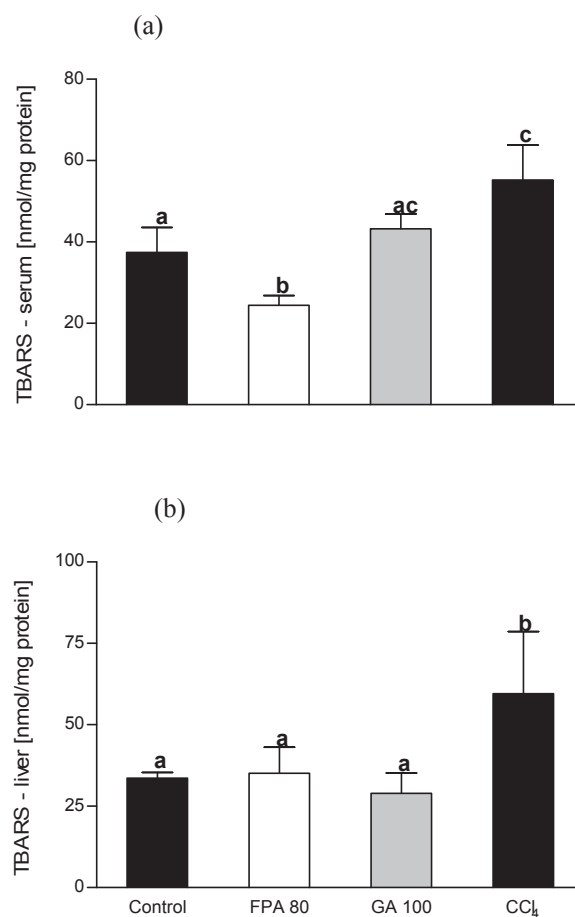


Fig. 4. TBARS measured in both serum (a) and liver (b) from rat groups. Different letters mean statistical differences, $p < 0.05$.

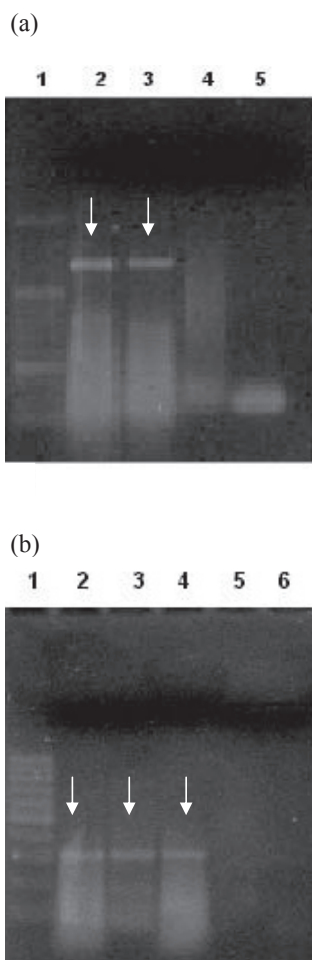


Fig. 5. Electrophoresis of the CAT (a) and SOD (b) enzyme gene of the livers from rat groups. (a) 1, Ladder (50 bp); 2, FPA; 3, GA; 4, CCl_4 ; 5, H_2O . (b) 1, Ladder (100 bp); 2, control; 3, FPA; 4, GA; 5, CCl_4 ; 6, H_2O .

that the treated rats had a faster recovery from liver injury at 24 h than expected.

The investigation in order to define the molecular mechanism of both, CAT and SOD gene expression in the rat groups, has evidenced by RT/PCR techniques the occurrence of both, alteration and restoration of the enzyme genes. Visualization by electrophoretic bands showed the presence or absence of the SOD and CAT genes expressed from livers originated from both rat groups, with previous antioxidant treatment (natural and synthetic) and under CCl_4 action only. The normal control rat groups conserved CAT and SOD genes in the molecular evalua-

tion (Gopal and Sengottuvelu, 2008; Asha *et al.*, 2007).

Observing the histology of a liver cut from rats of both groups (data not shown), an hepatoprotective effect is visualized, in which natural antioxidants (like FPA) could be induced in the rat group treated with repeated dosages of these antioxidants, before CCl_4 administration. The controls for rats, either normal or injured by CCl_4 , gave support to the differences between damaged and undamaged liver tissue.

Based on data from the present study with the seaweed *Halimeda monile*, a source of natural antioxidants (cinnamic, gallic, and caffeic acids detected in the respective extract) was demonstrated (Fallarero *et al.*, 2003; Vidal *et al.*, 2001). Previous studies showed that gentisic acid, ferulic acid, gallic acid, and *p*-coumaric acid modulate phase II antioxidant enzymes and phase II sulfate conjugative enzymes. These phenolic acids seem to selectively induce hepatic mRNA transcripts for CuZnSOD, GPX, and catalase, probably through upregulation of gene transcription as well as the Nrf2 transcription factor (Yeh and Yen, 2006).

The antioxidant property of *Halimeda monile* has shown its ability to help in the rat liver recovery from injuries caused by CCl_4 . The TBARS levels detected in both, liver and serum from the rats, were indicative of such injuries. By elevation of the hepatic GSH levels, it was possible to measure in live organisms the mechanism for injury repair and the corresponding inactivation. This situation was observed in rats treated with natural antioxidants originated from the phenolic compounds of *Halimeda monile*, and also in rats treated with the synthetic antioxidant gallic acid.

Through the CAT, SOD and GSH assays, it was verified that antioxidant enzymes increased their levels during dosage repetition of both *Halimeda monile* extract and gallic acid, while CCl_4 depressed these enzyme levels in the liver of untreated rats that were observed for gene expression alterations using RT/PCR techniques (Gopal and Sengottuvelu, 2008; Asha *et al.*, 2007). Furthermore, the liver tissue presented more structural preservation in rats treated with FPA, than in rats injured by CCl_4 , when the specimen were examined by histology. Partial injury could be observed by histology in the liver of rats with the same treatment, as compared with both, the

control group and the CCl₄ group, which induced 60% more peroxidation.

Therefore, with this data comparison, it was possible to conclude that phenolic compounds from the seaweed *Halimeda monile* with antioxidant activity can reinforce the mechanism of liver repair regulated by hepatic enzymes and by the scavenging action on free radicals (direct action);

so, diets containing seaweed with active phenolic compounds are important for human health maintenance.

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