Research article

In vivo and *in vitro* antioxidant activity and hepatoprotective properties of polyphenols from *Halimeda opuntia* (Linnaeus) Lamouroux

Ana Mara de Oliveira e Silva[1], Alexis Vidal-Novoa[2], Ana Elsa Batista-González[2], José Ricardo Pinto[3], Dalva Assunção Portari Mancini[3], Wilman Reina-Urquijo[2], Jorge Mancini-Filho[1]

[1]Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, Brasil, [2]Facultad de Biología, Universidad de La Habana, La Habana, Cuba, [3]Instituto Butantan, São Paulo, Brasil

Antioxidant activity and hepatoprotective properties of the aqueous extract and tetrahydrofuran-extracted phenolic fractions of *Halimeda opuntia* (Linnaeus) Lamouroux were investigated in rats with chemically induced liver injury. Total polyphenols were determined by using the Folin–Ciocalteau reagent. Liver damage was induced by CCl_4 and assessed by a histological technique. Reverse transcription/polymerase chain reaction (RT/PCR) analysis showed increased superoxide dismutase (SOD) and catalase (CAT) gene expression and activities in the group treated with free phenolic acid (FPA) fractions of *H. opuntia*, suggesting inducing effects on both enzymes. In addition, rats treated with FPA fractions displayed lower liver thiobarbituric acid reactive substance (TBARS) levels than those observed for rats in the CCl_4-treated group. These data suggest that the phenolic fractions *from H. opuntia* may protect the liver against oxidative stress-inducing effects of chemicals by modulating its antioxidant enzymes and oxidative status.

Keywords: Seaweed, Natural antioxidants, *Halimeda opuntia*, Hepatoprotection

Introduction

Reactive oxygen species (ROS) are by-products of cellular metabolism. However, overproduction of ROS leads to oxidation of biomolecules and consequent cell damage. Antioxidants can alleviate the noxious effects of *in vivo* oxidative stress[1] through inhibiting the generation of ROS, directly scavenging free radicals or increasing the expression of the genes encoding the antioxidant enzymes involved in the elimination of ROS, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx).[2,3]

Seaweeds are commonly consumed as vegetables in Asia and other western countries and have also been used as traditional medicines for the treatment of several conditions.[4,5] Indeed, seaweeds are rich in bioactive compounds that exhibit several phytotherapeutic properties and have excellent potential as functional food ingredients for reducing the incidence of chronic diseases.[6]

In the waters of the Caribbean, seaweeds are exposed to sunlight and oxygen, which leads to the formation of ROS. However, the absence of oxidative damage in their structural and functional components suggests that they have an efficient antioxidant defense system. For this reason, several seaweed extracts have attracted increasing scientific interest.[7]

Significant antioxidant activity of seaweed extracts has been observed *in vitro* and in animal models, indicating great potential for phytotherapeutic, nutraceutical, or both applications. Of the numerous compounds exhibiting these properties, polyphenols are particularly interesting as they can display antioxidant activity at low concentrations.[8,9]

The green seaweeds of the *Halimeda opuntia* species occur widely, mainly in shallow waters of tropical regions. They are harmless, very easy to collect, and a potential source of phytomedicines. *Halimeda* spp. have been investigated for different medicinal properties[10–16] including antioxidant activity.[17,18] Animal

Correspondence to: Ana Mara de Oliveira e Silva, Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, Bloco 14, Cidade Universitária, CEP 05508-900, São Paulo, SP, Brasil. Email: anamara_nut@yahoo.com.br

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studies carried out in our laboratory have shown that *Halimeda incrassata* effectively attenuates oxidative stress in cells by scavenging free radicals, inhibiting lipoperoxidation and exhibiting neuroprotective and hepatoprotective activities.[19–21] The antioxidant activity of *Halimeda* spp. has been attributed to the presence of polyphenolic compounds such as flavonoids, phenolic, and cinnamic acids.[22–24]

In view of these considerations, the aim of this paper was to investigate the antioxidant and hepatoprotective effects of polyphenol-rich fractions from seaweed *H. opuntia* (Linnaeus) Lamouroux on acute liver damage induced by CCl_4 in Wistar rats. The activity of hepatic antioxidant enzymes and their gene expression levels were determined and used to assess these effects.

Materials and methods

Seaweed collection and preparation of hydrophilic fractions

H. opuntia (Linnaeus) Lamouroux was collected in December 2010 in Bajo de Santa Ana, Havana City, Cuba. Specimens were identified and a voucher was deposited for future reference at the Seaweeds Laboratory at the Marine Research Center of the University of Havana.

Freshly collected specimens were washed with distilled water and dried at room temperature (26°C) for 7–10 days. After milling and sieving, the dry powder was extracted with distilled water (1:5 w/v) at room temperature (±22°C) and centrifuged at 800 × g and 4°C for 20 minutes. The supernatant was collected, lyophilized, and kept at −20°C until time of analysis. The yield of extraction (% w/w of seaweed on a dry weight basis) was 4.3%. Polyphenols were extracted according Krygier *et al.*[24] Free phenolic acids (FPAs) were extracted by suspending dry seaweed in tetrahydrofuran. Total phenolic content was determined as in Vidal *et al.*[23] and expressed as milligrams of gallic acid equivalents (GAE)/g dry seaweed.

In vitro antioxidant activity

Reducing power

Reducing power was determined by a slightly modified method described by Oyaizu.[25] Absorbance was measured against a water blank at 700 nm on a VIS-723 G spectrophotometer (Rayleigh, Beijing, China). Absorbance increments were directly proportional to reducing power increments. Reducing power activity was expressed as ascorbic acid equivalents. One equivalent corresponds to 20 μg of ascorbic acid with an absorbance of 0.139.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The free radical scavenging activity of the *H. opuntia* extract was determined using the DPPH assay.[26] Aliquots of the extract (10–40 μg of GAE) were suspended in a methanolic solution of DPPH (60 μM). After 30 minutes, the absorbance was measured against a blank solution at 517 nm. Radical scavenging activity was calculated as follows:

$$\%\text{inhibition (IP)} = [1 - A_{\text{sample}}/A_{\text{blank}}] \times 100$$

where A_{sample} is the absorbance of the sample containing the extract and A_{blank} is the absorbance of the blank solution.

Animals

Male Wistar rats, weighing between 120 and 150 g, were provided by the University of São Paulo Vivarium, and were housed in boxes (six rats each) in a room with controlled lighting (12-hour light/dark cycle) at 25°C and 60% humidity. The rats had free access to water and to a standard food diet according to the Guidelines for Laboratory Animal Care and Use. Animal studies were approved by the Animal Experimentation Ethics Committee of 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 of CCl_4 (1:1 in olive oil). Gallic acid (GA), a known antioxidant compound, was used as reference.

The animals were grouped as follows:

Control: treated daily with vehicle (1.0 ml, per oral (p.o.)) for 20 days.

CCl_4: treated daily with vehicle (1.0 ml, p.o.) for 20 days, followed by treatment with CCl_4 on day 21.

GA: treated daily with GA (100 mg/kg, p.o.) for 20 days, followed by treatment with CCl_4 on day 21.

Ho 20: treated daily with *H. opuntia*-FPA (20 mg/kg, p.o.) for 20 days, followed by treatment with CCl_4 on day 21.

Ho 80: treated daily with *H. opuntia*-FPA (80 mg/kg, p.o.) for 20 days, followed by treatment with CCl_4 on day 21.

At the end of the treatment, blood and liver samples of each animal were collected. Serum was separated and assayed for thiobarbituric acid reactive substance (TBARS) levels. Liver homogenates were prepared and assayed for TBARS and antioxidant enzyme activity.

TBARS assay

TBARS levels, as a marker of lipid peroxidation, were measured in liver homogenates and serum using the method of Ohkawa *et al.*[27] The results were expressed as nanomoles per milligram of protein.

SOD determination

The cytoplasmic SOD activity was determined according to McCord and Fridovich[28] using a reaction mixture containing cytochrome C (100 mM), xanthine (500 mM), ethylenediaminetetraacetic acid (1 mM), and KCN (200 mM) and potassium phosphate buffer (0.05 M – pH 7.8). The results were expressed as units per milligram of protein. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reaction rate by 50% at 25°C and pH 7.8.

Catalase activity (CAT) determination

The CAT activity was determined as described by Beutler.[29] The method is based on the decrease in optical density at 230 nm (molar extinction coefficient – 0.071/mM/ cm) as a result of the decomposition of hydrogen peroxide by catalase at 37°C. The results were expressed as units per milligram of protein. One unit of CAT activity was defined as the amount of enzyme required to hydrolyze 1 mol of hydrogen peroxide per minute at 37°C and pH 8.0.

GPx determination

The GPx activity in the cytosolic fraction was determined by Sies.[30] One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of NADPH per minute at 30°C at pH 7.0.

Reverse transcription/polymerase chain reaction (RT/PCR)

RNA extraction: CAT and SOD gene expression evaluation

RNA was extracted by mixing 100 mg of rat liver and 1000 μl of trizol (Invitrogen, New York City, New York). After the addition of 200 μl of chloroform (Merck, Darmstadt, Hessen, Germany), vortex mixing for 15 seconds, incubation at room temprature for 5 minutes, and centrifugation at 12 000 × g and 4°C for 15 minutes, the supernatant (400 μl) was collected, avoiding the interphase, and mixed with 500 μl of isopropanol by vortexing for 5 seconds. It was then centrifuged at 12 000 × g and 4°C for 5 minutes and the supernatant was discarded. The resulting pellet was washed with 1 ml of ethanol (75%), gently vortex-mixed and centrifuged at 7500 × g and 4°C for 10 minutes. The supernatant was discarded again. The pellet was resuspended in 20 μl of RNAse-free distilled water, incubated at 50°C for 10 minutes, and stored at −70°C.

Reverse transcription

Five micrograms of RNA was added to 1.0 μl of primer (Cu/Zn SOD or CAT), 1.0 μl of dNTP (10 mM), and 4.0 μl of sterile distilled water. The reaction was started by a heating step at 65°C for 5 minutes and then it was quickly chilled on ice. After adding 4.0 μl of 5X First-Strand Buffer (Invitrogen), 2.0 μl of DTT (0.1 M, Invitrogen), and 1.0 μl of RNAseOUTribonuclease inhibitor (Invitrogen), the mixture was incubated at 37°C for 2 minutes. After that 1.0 μl of M-MLV reverse transcriptase (200 U/μl, Invitrogen) was added and the mixture was incubated at 37°C for 50 minutes. The reaction was stopped by a heating step at 70°C for 15 minutes. The PCR product (cDNA) was stored at −70°C.

PCR amplification

Five microliters of cDNA was amplified in a 50.0-μl reaction mixture containing 5.0 μl of Tris (hydroxymethyl) aminomethane–hydrochloride buffer (20 mM; pH 8.4), KCl (500 mM), 1.5 μl of $MgCl_2$ (50 mM), 1.0 μl of dNTP (10 mM), 35.1 μl of diethyl pyrocarbonate, 1.0 μl of primer (SOD or CAT), and 0.4 μl of Taq polymerase (5 U/μl). After an initial denaturation at 94°C for 3 minutes in a thermal cycler (Bio-Rad, Hercules, California, USA), 35 cycles (at 94°C for 45 seconds, at 55°C for 30 seconds, at 72°C for 1.3 minutes, and 72°C for 10 minutes) were carried out. Finally, the mixture was chilled at 4°C. The PCR amplification products were analyzed by electrophoresis on a 2.0% agarose gel (Sigma, St. Louis, Missouri, USA) at 60 V. The gel was stained with 0.5 μg/ml ethidium bromide, visualized on a fluorescence table (Vilber-Lourmat, Marne-la-Vallée, France), and photographed with a digital camera. CAT-262 bp (C to T) and SOD-242 bp (C to T) were genotyped using the following primers (Promega, Madison, AL, USA):

CAT 1 – 5′-GCG AAT GGA GAG GCA GTG TAC-3′

CAT 2 – 5′-GAG TGA CGT TGT CTT CAT TAG CAC TG-3′

Cu/Zn SOD 1 – 5′-TCT AAG AAA CAT GGC GGT CC-3′

Cu/Zn SOD 2 – 5′-CAG TTAGCA GGC CAGCAG AT-3′

Statistical analysis

All experiments were carried out in triplicate and results were expressed as mean values ± standard deviations and the significance level was set at $P < 0.05$. *In vivo* antioxidant activity measurements were compared in terms of mean values using a one-way analysis of variance and the Tukey post-test.

Results and discussion

Antioxidant activity assays

In view of the potential health applications of seaweeds, the genus *Halimeda* has been investigated for numerous health-beneficial properties, including antioxidant activity.[10–16]

The total polyphenolic content of the aqueous extract of *H. opuntia* was 97.2 ± 7.3 μg of GAE/g

dry seaweed. Vidal *et al.*[22] reported similar polyphenolic content (74.3 mg of polyphenols/g dry seaweed) in a study on the antioxidant activity of a water-soluble extract of *H. opuntia* using gas–liquid chromatography. Their research suggests that the antioxidant properties of the seaweed extract are at least partly related to its content of phenolic acids. Yoshie *et al.*[21] have also observed high levels of polyphenols in two *Halimeda* species.

The DPPH scavenging and the reducing power assays are valuable tools for the determination of the antioxidant activity of natural products. Fig. 1 and Table 1 show the reducing power and DPPH results for the aqueous extract of *H. opuntia*, respectively. Both methods delivered similarly high antioxidant activity results, which are in agreement with those observed for aqueous extracts of other *Halimeda* species in studies using different methods.[18–20] As shown in Fig. 1, the aqueous extract at 1 mg of polyphenols/ml showed a significantly lower absorbance value (0.0185) in comparison with that obtained for the positive control (Optical density (O.D.) 0.139 for 20 μg), suggesting that a pure extract would have displayed a potent antioxidant activity. Our results are consistent with the results of Kuda and Ikemori,[31] who observed similar reducing power results for several seaweeds found in Japan, as well as a significant correlation between antioxidant capacity and polyphenolic content.

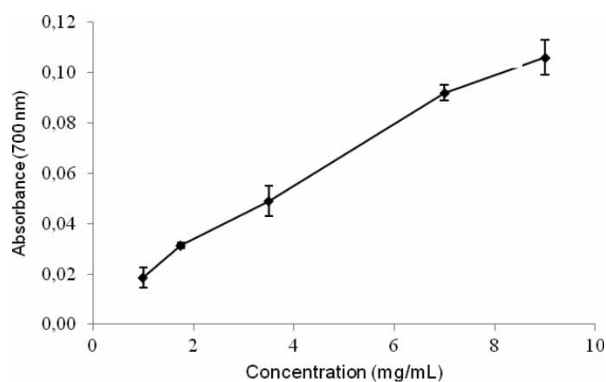

Figure 1 Reducing power versus concentration of polyphenols in the aqueous extract of *Halimeda opuntia*. Results expressed as mean ± standard deviation.

Table 1 Percentage inhibition of DPPH versus concentration of the aqueous extract of *Halimeda opuntia*. Results expressed as mean ± standard deviation.

Concentration (mg/ml)	% Inhibition DPPH
3.0	21 ± 2
4.0	27 ± 2
5.0	32 ± 1
5.5	35 ± 1
6.0	39 ± 2
6.5	44 ± 3
7.0	48 ± 1

Great antioxidant potential has also been reported in studies using polyphenol-rich fractions of *H. incrassata* and *Halimeda monile.*[23,32] Zubia *et al.*[17] reported relatively high antioxidant activity of *H. monile* and *Halimeda tuna* and found a direct relationship between the content of polyphenols and antioxidant capacity in a study of numerous seaweeds including 17 species of *Chlorophyta.* In addition, Senevirathne *et al.*[33] investigated polar fractions of *Ecklonia cava* and observed great reducing ability and significantly high polyphenol content, which are comparable to the results obtained in this study.

Effect of H. opuntia extract on CCl_4-induced liver damage in rats

To investigate the hepatoprotective properties of *H. opuntia*, Wistar rats with CCl_4-induced liver injury were treated with a FPA fraction with a total polyphenol content of 5.92 ± 0.85 μg of GAE/g dry seaweed.

We observed that the rats treated with FPA fraction from *H. monile* or GA proved to be capable of attenuating the changes induced by CCl_4.[34]

TBARS are produced as a result of lipidic peroxidation. As shown in Fig. 2, TBARS levels in serum and liver tissues in CCl_4-treated rats increased, confirming successful induction of oxidative damage.

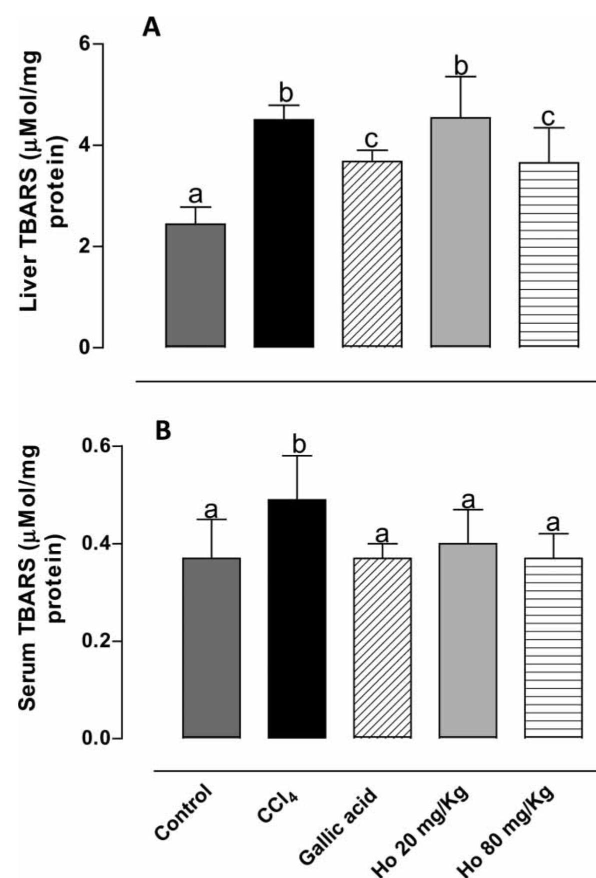

Figure 2 (A) Liver and (B) serum TBARS levels from control, CCl_4-treated, GA-treated, and *Halimeda opuntia*-treated rats. Different letters indicate statistically significant differences, $P < 0.05$.

Pre-treatment with *H. opuntia* (80 mg/kg) led to 20 and 25% reductions in serum and liver TBARS levels, respectively. These results are in agreement with Kim *et al.*,[35] who observed a comparable reduction in hydroperoxide levels in the liver and plasma (30 and 15%, respectively, relative to CCl_4-treated group) in a study of rats fed on *Saengshik*, a non-cooked food containing vegetables and seaweeds. Similarly, a previous study from our laboratory showed that an *H. incrassata* aqueous extract was effective in reducing TBARS levels by 55% in rats with oxidative stress induced by methylmercury.[20]

Of the numerous compounds of the antioxidant defense system in mammals, including low-molecular-weight compounds and enzymes, three stand out as most important: CAT, SOD, and GPx. The activity of these enzymes may be altered by CCl_4 treatment. Punitha and Rajasekaran[36] demonstrated that CCl_4 treatment significantly reduced the activity of the antioxidant enzymes. However, Ozturk *et al.*[37] observed in the CCl_4-treated group significant increases in kidney SOD and CAT activities. In this study, we also investigated the ability of the *H. opuntia* extract to induce antioxidant enzyme activity. Fig. 3 shows the effects of different treatments on the activities of CAT, SOD, and GPx. Treatment with the seaweed led to a significant increase in the activity of all enzymes, which in turn resulted in enhanced antioxidant defense.[38] These results suggest potent hepatoprotective activity of the phenolic fraction of *H. opuntia*. Kim *et al.*[35] observed a comparable rise in SOD activity in rats fed on *Saengshik* for 4 weeks. Mancini-Filho *et al.*[34] reported considerable increase in the activities of SOD and CAT in rats treated with polyphenol-rich fractions (80 mg/kg) of *H. monile*. Batista-Gonzalez *et al.*[32] reported similar results using four-fold lower doses of *E. cava* fractions. High antioxidant enzyme activity has been reported through repeated administration of *Sargassum* extracts.[39,40] Treatment with *Caulerpa prolifera*, *Laurencia obtusata*, and *Porphyra haitanensis* extracts also led to a rise in enzyme activity.[41,42]

Expression levels of antioxidant enzymes by PCR

The levels of CAT and SOD in liver tissues increased after repeated administration of either the *H. opuntia* aqueous extract or GA, whereas they decreased after CCl_4 induction as can be seen in Fig. 4, which shows alterations in catalase gene expression assessed by the RT/PCR technique. Treatment with the *H. opuntia* FPA (band 2) resulted in higher catalase gene expression compared with that observed in the CCl_4-treated group (band 4). A review by Stevenson and Hurst[43] discusses recent evidence that polyphenols also have indirect antioxidant effects through induction of endogenous protective enzymes and these inductive or signalling effects may occur at concentrations much lower than those required for effective radical scavenging.

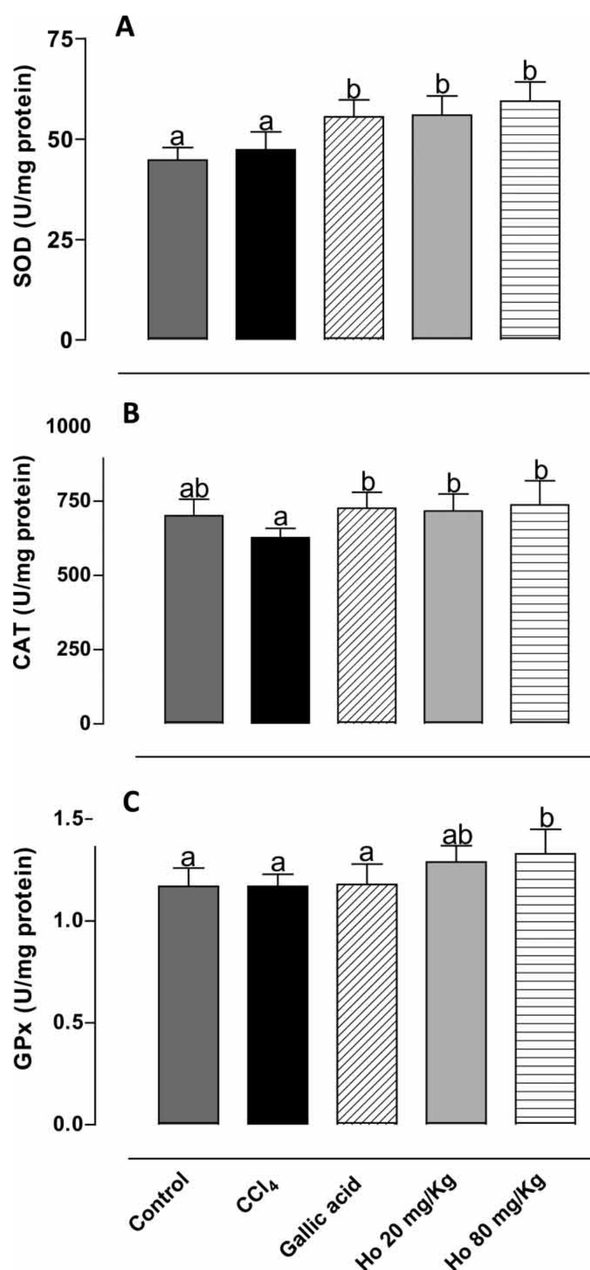

Figure 3 Activity of (A) SOD, (B) CAT, and (C) GPx in liver tissues from control, CCl4-treated, GA-treated, and *Halimeda opuntia*-treated rats. Different letters indicate statistically significant differences, $P < 0.05$.

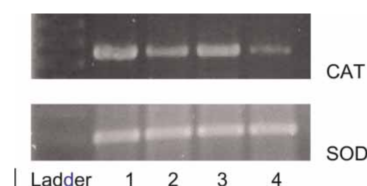

Figure 4 RT-PCR analysis of CAT and SOD expression in liver tissues on agarose gel: (1) control; (2) *Halimeda opuntia* 80 mg/kg, (3) GA, and (4) CCl_4.

Nine phenolic acids including ferulic acid, GA, and p-coumaric acid have been reported to be present in *H.*

opuntia. Yeh[44] suggested that these three phenolic acids modulate phase II antioxidant enzymes and phase II sulphate conjugative enzymes, and seem to selectively induce hepatic mRNA transcripts for Cu, Zn-SOD, GPx, and CAT, probably through up-regulation for gene transcription as well as the Nrf2 transcription factor.

Reduced serum and liver TBARS levels after treatment with *H. opuntia* indicate great antioxidant capacity of the seaweed, which is consistent with observations made in a study of rats treated with natural (phenolic acids from *H. monile*) and synthetic (GA) antioxidants.[34]

Conclusion

Treatment with FPA fractions of *H. opuntia* led to attenuation of liver damage induced by CCl_4 in Wistar rats. Reduced serum and liver TBARS levels suggest hepatoprotective activity of the seaweed. Treatment with synthetic GA led to similar results. Whereas CAT and SOD levels in rat liver tissues decreased as a result of oxidative damage induced by CCl_4, they increased after treatment with either the *H. opuntia* FPA fraction or GA, suggesting great antioxidant capacity of *H. opuntia* and its potential use as a phytodrug, nutraceutical, or both.

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