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# Hepatoprotective properties from the seaweed *Bryothamnion triquetrum* (S.G.Gmelin) M.A.Howe against $CCl_4$-induced oxidative damage in rats

[Propiedades hepatoprotectoras del alga *Bryothamnion triquetrum* (S.G.Gmelin) M.A.Howe contra el estrés oxidativo en ratas inducido por $CCl_4$]

**Alexis de J. Vidal Novoa[1*], Ana M. de Oliveira e Silva[2*], Dalva A. Portari Mancini[3], Daylín Díaz Gutiérrez[1], Jorge Mancini-Filho[2]**

[1]Facultad de Biología, Universidad de La Habana, Calle 25 # 455 e/ J e I, Vedado, CP 14 000, La Habana, Cuba.
[2]Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes # 580, Bloco 14, CEP 05508-900, São Paulo-SP, Brasil.
[3]Laboratorio de Virologia, Instituto Butantã, Ave Vital Brasil # 1500, Butantã, CEP 05503-900, São Paulo, Brasil.
*E-mail: alexis.vidal@infomed.sld.cu

**Abstract**

*Context*: Seaweeds are seen as a traditional food and folk medicine by different coastal countries. The red seaweed *Bryothamnion triquetrum* is a widely distributed species that grows in shallow waters, and different authors have demonstrated a possible application of the seaweeds as a source of natural antioxidants and relative diseases.

*Aims*: To evaluate the hepatoprotective properties on $CCl_4$-induced oxidative stress in rats that were associated with the antioxidant activity from the polyphenol-rich fractions of the red seaweed *Bryothamnion triquetrum*.

*Methods*: Polyphenols were determined by Folin-Cioacalteu. Antioxidant activity from phenolic compounds-rich fractions was measured by different assays (DPPH, Reducing power, β-Carotene/linoleic acid assay and Inhibition of lipoperoxidation). Aqueous extract from *B. triquetrum* was administered during 20 days to rats and submitted $CCl_4$-Induced oxidative damage. The peroxidation and hepatic damage (TBARS, ASAT and ALAT), antioxidant metabolite and enzymes (glutathione, catalase and superoxide dismutase) were evaluated. Also, it was evaluated the expression of antioxidant enzymes by RT-PCR.

*Results*: The antioxidant activity determined by different assays with polyphenolic fractions. Free Phenolic Acid was more active: DPPH, 20 µg 87%; Reducing power OD = 0.490, 20 µg ; β-carotene/linoleic acid 1 µg 53%, and inhibition of lipid peroxidation 0.250 µg 100%. Rats treated displayed lower liver TBARS, ASAT and ALAT than $CCl_4$-treated group and catalase activity was increased. It was demonstrated expression of catalase.

*Conclusions*: Data suggest that *Bryothamnion triquetrum* protects the liver against oxidative stress by modulating its antioxidant enzymes and oxidative status with potential use as phytodrug or functional food.

*Keywords*: antioxidant; *Bryothamnion triquetum;* hepatoprotection; seaweeds.

Resumen

*Contexto*: Las algas marinas son consideradas como alimentos tradicionales y fitofármacos en determinados países. El alga marina *Bryothamnion triquetrum* es una especie ampliamente distribuida en aguas poco profundas. Diferentes autores han demostrado su posible aplicación como antioxidante natural.

*Objetivos*: Evaluar las propiedades hepatoprotectoras y protectoras del estrés oxidativo inducido por $CCl_4$ asociadas con la actividad antioxidante de las fracciones ricas en polifenoles del alga *Bryothamnion triquetrum*.

*Métodos:* Los polifenoles fueron determinados por Folin-Cioacalteu. La actividad antioxidante de fracciones ricas en polifenoles se midió mediante: DPPH, capacidad reductora, ensayo de β-caroteno/ácido linoleico e inhibición de la lipoperoxidación. Se administró un extracto acuoso de *B. triquetrum* durante 20 días a ratas y se sometieron a daño oxidativo inducido por $CCl_4$. Se evaluaron la peroxidación y el daño hepático (TBARS, ASAT y ALAT), metabolitos y enzimas antioxidantes (glutatión, catalasa y superóxido dismutasa). También se evaluó la expresión de enzimas antioxidantes mediante la técnica de RT-PCR.

*Resultados*: La fracción de ácidos fenólicos libres (FPA) resultó la más activa: DPPH, 20 µg 87%; capacidad reductora DO = 0,490 20 µg; β-caroteno/ácido linoleico 1 µg 53% e inhibición de la lipoperoxidación 0,250 µg 100%. Las ratas tratadas mostraron niveles más bajos de TBARS hepáticos, ASAT y ALAT que el grupo tratado con $CCl_4$ y aumentó la actividad de catalasa. Se demostró la sobre-expresión de la catalasa.

*Conclusiones*: Los datos sugieren que *Bryothamnion triquetrum* protege al hígado contra el estrés oxidativo al modular sus enzimas antioxidantes y su estado oxidativo con su posible uso como fitofármaco o alimento funcional.

*Palabras Clave*: algas marinas; antioxidante; *Bryothamnion triquetum;* hepatoprotección.

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## INTRODUCTION

Seaweeds are seen as a traditional food diet and folk medicine by different coastal countries. Since ancient times, Asiatic peoples have used seaweeds for various medicinal purposes. The Chinese people have been utilizing seaweeds as phytodrugs and early records of seaweeds appeared in Chinese literature about two thousand years ago in Chinese literature 'Pen Tsae Kan Mu' (Tseng and Chang, 1984). Algae such as *Eisenia* sp. and *Sargassum* sp. are present in Japanese society since the Jomon-pattern (BC 300 - 6000) and Yayoi-pattern eras (BC 300 - AD 400) and in Japanese folk medicine has been used to treat goiter and other glandular problems as long ago as 300 BC, while the seaweed *Digenea simplex* has been used traditionally to parasiticide and *Laminaria* spp., another seaweed native from Japanese coasts and valued as a folk medicine, has been shown to be capable of lowering blood pressure. Seaweeds have recently attracted much attention as a source of antioxidant compounds, due to in part to epidemiological research that strikethrough has supported the existence of an inverse correlation between the incidence of different diseases and consumption of seaweeds (Gomez–Gutierrez et al., 2011).

Seaweeds exhibit different therapeutical properties, and consequently, during the last few years, the interest on the study of seaweeds as sources of bioactive compounds has increased. In addition, epidemiological research has also suggested their positive effects on human health, with an inverse correlation between the incidence of different diseases, several of which relate to oxidative stress, and the consumption of seaweeds. Different researchers have been demonstrated that the addition of seaweeds, in powder or extract form, can improve the nutritional and textural properties of food products with positive effects on different lifestyle diseases such as obesity, dyslipidemia, hypertension, and diabetes (Rasul Suleria et al., 2015, Roohinejada et al., 2017).

Actually, red seaweeds (*Rhodophyta, Ceramiales, Rhodomelaceae*) are the most promising as potential producers of antioxidants. Nogueira et al. (2014) describes more than 60 antioxidants natural products from red seaweeds.

The red seaweed *Bryothamnion triquetrum* is a widely distributed species that grows in shallow waters, exposed to a high incidence of solar light; therefore, it can produce free radicals. The absence of oxidative damage in their structural and physiological components suggests that these organisms present an efficient system of antioxidant defenses and consequently, different authors have demonstrated a possible application of the seaweeds as a source of natural antioxidants (Machu et al., 2015). Species of the genus *Bryothamnion* have shown to possess some therapeutic properties like anti-inflammatory, antinociceptive, antibiotic, tumor markers, antioxidant and relaxing vascular smooth muscle (Pereira de Lira, 2013). In previous research, the antioxidant activity of its aqueous extracts has been investigated in different *in vitro* models of oxidative stress. The antioxidant properties of this algae seem to be explained by its free radical scavenging activity, particularly when related to radical dismutation mechanisms of $O_2^{\bullet-}$, $OH^{\bullet}$ radical quenching and Fe chelation (Vidal et al., 2001; 2017). In previous works, it identified phenolic and cinnamic acids as being the molecules that could explain the antioxidant activity, but further effects should be considered, such as a summation or a synergistic effect of other antioxidant components of the extract (Vidal et al., 2001).

Thus, in view of these previous considerations, the aim of this study was to evaluate the hepatoprotective properties on $CCl_4$-induced oxidative stress in rats that were associated with the antioxidant activity from the polyphenol-rich fractions of the red seaweed *Bryothamnion triquetrum*.

## MATERIAL AND METHODS

### Seaweed collection

The seaweed *Bryothamnion triquetrum* (S.G.Gmelin) M.A.Howe, *Rhodomelaceae*, *Ceramiales*, was collected in July/2017 in Bajo de Santa Ana, La Habana, Cuba (22°57" 00""N and 82°25"00"" W). Specimens were identified in the Seaweeds Laboratory at the Marine Research Cen-

ter of the University of Havana, Cuba (voucher CIM 047).

**Preparation of extracts**

For better understanding of this research, in Fig. 1 are presented a flow diagram with the different extraction processes and possible usages of different fractions.

*Aqueous extract*

The aqueous extract was prepared as follows; freshly collected specimens were washed with distilled water and dried at room temperature (26°C) for 7 – 10 days. After milling and sieving (US Standard Sieve Series No. 40, 35 mesh), 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 min. The supernatant was collected, lyophilized, and kept at −20°C. The yield of lyophilized aqueous extract in terms of fresh seaweed was determined to be 1%. This extract was dissolved in water for animal study.

*Preparation of organic fractions*

For *in vitro* assays of antioxidant activity, the phenolic acids and soluble or insoluble esters were extracted according to Vidal et al. (2011). Dry seaweed (1 g) was extracted six times with tetrahydrofuran (THF, 20 mL) during 3 min, and evaporated to dryness under vacuum at 30°C and after resuspended in 5 mL of methanol; these extracts were analyzed for free phenolic acids (FPA). The supernatant was hydrolyzed with NaOH 4N and posteriorly was extracted with extracted with ethyleter:ethylacetate:THF (1:1:1) and then filtered and corresponding to phenolic acids of soluble esters (PASE). The obtained residue was hydrolyzed by adding NaOH 4N and was extracted with extracted with ethyleter:ethylacetate:THF (1:1:1). The organic phase was rotoevaporated and resuspended into methanol, this was analyzed for phenolic acids of insoluble esters (PAIE).

The total phenolics of the different fractions, in concentration 0.020 - 20 µg, was determined by the Folin-Ciocalteau assay as previously described by Vidal et al. (2017) and expressed as µg of gallic acid equivalents (GAE)/g of sample. The calibration curve was obtained in the range of 0.020 - 50 µg gallic acid/mL.

***In vitro* antioxidant activity**

*DPPH radical scavenging capacity*

The free radical scavenging activity of the polyphenolic fractions was determined using the DPPH assay (Díaz Gutiérrez et al., 2015). Aliquots of algal fractions at different concentrations (2 - 20 µg phenolic compounds/mL) were mixed with a DPPH solution in methanol (60 µM). After 30 min, the absorbance was measured at 517 nm in a VIS-723 G spectrophotometer (Rayleigh, Beijing, China). In this assay was used butylated hydroxytoluene (BHT) as positive control.

The scavenging activity was calculated as follows (1):

$$\% \text{ DPPH scavenging} = (1 - Abs_M/Abs_C) \times 100 \quad (1)$$

where $Abs_M$ and $Abs_C$ are the absorbance of the solution containing the fraction and the blank solution respectively. $IC_{50}$ inhibition values defined as the concentration of the compounds that was able to inhibit 50% of the total DPPH radicals. A lower value of $IC_{50}$ indicates a higher antioxidant activity and vice versa.

*Reducing power*

Reducing power was determined by the method as described by Batista-Gonzalez et al. (2011). This method is based on the ability of the antioxidant compounds to the conversion of the $Fe^{3+}$/ferricyanide complex to the $Fe^{2+}$ form, and the $Fe^{2+}$ concentration could be determined by measuring the enhanced formation of Perl's Prussian blue at 700 nm. Polyphenolic fractions were mixed with a phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1%). The mixture was incubated for 20 min at 50°C. It was then cooled, mixed with trichloroacetic acid (10%) and centrifuged at 800 g for 10 min. An aliquot of the supernatant was mixed with ferric chloride (0.1%) and distilled water. The absorbance was measured at 700 nm in a VIS-723 G spectrophotometer (Ray-

leigh, Beijing, China). Absorbance increments were directly proportional to reducing power increments. In this assay was used ascorbic acid (1 mg) as positive control and Reducing power activity was expressed as ascorbic acid equivalents. One equivalent corresponds to 20 µg of ascorbic acid with an absorbance of 0.139.

*β-Carotene/linoleic acid assay*

A method described by Miller with minor modifications (Barroso de Alencar et al., 2014) was employed. An emulsified mixture containing linoleic acid, Tween 40 and β-carotene was prepared. Distilled water, previously oxygenated, was then added and the solution was vigorously shaken until an absorbance was reached, between 0.6 - 0.7 at 470 nm. Fraction volumes between 1, 5 and 10 µg of phenolic compounds were added to the emulsified mixture. BHT was used as positive control. Their absorbance was monitored at 470 nm every 15 min for 2 h. The antioxidant activity was calculated as follows (2):

$$\text{Antioxidant activity} = 100 - [(A^0_s - A^{120}_s / A^0_c - A^{120}_c) \times 100] \quad (2)$$

where $A^0_s$ and $A^0_c$ are the initial absorbance of the sample and the control respectively, while $A^{120}_s$ and $A^{120}_c$ are the absorbance of the sample and the control tube at 120 min.

## Animals

Male Wistar rats, weighing between 120 g and 150 g, were provided by the University of São Paulo Vivarium, and were housed in boxes (six rats in each box) in a room with controlled lighting (12-h 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 (No.: CEUA/FCF/412) by the Animal Experimentation Ethics Committee of the Faculty of Pharmaceutical Sciences of the University of São Paulo, Brazil. Animals were sacrificed with ketamine (90 mg/kg) and xylazine (10 mg/kg) by via ip.

*Inhibition of spontaneous lipid peroxidation in rat brain homogenates*

Wistar rat brains were promptly excised after decapitation and washed with ice-cold 0.9% NaCl. Tissue homogenates were prepared by using a T10 basic ultra-turrax (IKA, Staufen, Baden-Wurttemberg, Germany) in a phosphate buffer (50 mM, pH 7.4) at a tissue to buffer ratio of 1:5 (w/v). The homogenate was then centrifuged at 800 *g* in a Beckman Centrifuge (Heal Force Beckman GS-GKR, USA) at 4°C for 15 min and the supernatant was collected and stored for a week at -70°C. Lipid peroxidation was determined according to Ohkawa, et al., with modifications (Díaz Gutiérrez et al., 2015). Samples of the brain homogenate (25 µL) were incubated with the same volume of different concentrations of each seaweed fraction at 37°C for 40 min. Then, acetic acid 20% and TBA (0.5%) in acetic acid were added. The mixture was heated in water bath at 90°C for 1 h. Finally, the samples were centrifuged, and the absorbance was measured at 532 nm. A positive control was used in all cases, by measuring the activity of ascorbic acid (1 mg/mL).

The inhibition of lipid peroxidation was calculated as follows (3):

$$\text{Inhibition (\%)} = (1 - M - C_o / C_{40} - C_o) \times 100 \quad (3)$$

where M is the absorbance of the solution containing the fraction, $C_0$ is the initial absorbance of the control solution (without fraction) and $C_{40}$ is the absorbance of the control solution (without fraction) at t = 40 min.

*$CCl_4$-induced oxidative damage in Wistar rats*

*Animal treatment schedule*

Hepatic injury was induced in rats by intraperitoneal administration of 2 mL of $CCl_4$/kg (1:1 in olive oil. Ferulic acid (FA), a known antioxidant compound was used as a reference.

The animals were grouped as follows:

Control: treated daily with vehicle (1.0 mL, 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 72 h and then 24 h prior to sacrifice on day 21.

FA + $CCl_4$: treated daily with ferulic acid (FA - 20 mg/kg, p.o.) for 20 days, followed by treatment with $CCl_4$ on 72 h.

Bt + $CCl_4$: treated daily with an aqueous extract of *B. triquetrum* (200 mg aqueous extract equivalent to 2.6 mg polyphenols)/kg, p.o. for 20 days, followed by treatment with $CCl_4$ on 72 h and then 24 h prior to sacrifice on day 21.

Vidal-Novoa et al. (2017) investigated antioxidant properties of an aqueous extract from *H. incrassata* with *in vitro* technique and in animal model (dose of 300 mg/kg via oral); this extract exhibits values of *in vitro* antioxidant activity comparable to those found with *B. triquetrum* and due to these considerations, the selected dose for this experiment was 200 mg/kg.

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 were determined as described Batista-González et al. (2012). Samples liver homogenate (1 mL) were added to 350 µL of cold acetic acid (20%, pH 3.5). The formation of malondialdehyde (MDA) was observed after the addition of 600 µL of TBA 0.5% dissolved in acetic acid (20%, pH 3.5). The mixtures were incubated at 90°C for 1 h and allowed to cool to room temperature. An aliquot of 50 µL of sodium dodecyl sulfate were then added and the mixture was centrifuged at 500 *g* in a Sorvall RC5C centrifuge (DuPont Company, Wilmington, NC) for 15 min at room temperature. The absorbance was measured at 532 nm and the TBARS content was expressed as mmol MDA/mg protein. TBARS concentrations were calculated using a standard curve for tetramethoxypropane.

*Aspartate transaminase (ASAT) and alanine transaminase (ALAT) activities*

The activity of these enzymes (in serum was determined according to medical laboratory diagnostic test kits (Ref. 108-109, LABTEST, Sao Paulo, Brazil). The results were expressed as international units/mL).

*Glutathione (GSH) analysis*

The glutathione levels were measured according to the method developed by Tietze with minor modifications (Mancini-Filho et al., 2009). Liver homogenate was suspended in ethylenediamine tetraacetic acid (EDTA; $10^{-3}$ M) in 5% trichloroacetic acid and was centrifuged at 17,000 *g* for 15 min at 2°C. The supernatant (200 µL) was mixed with 2.750 µL of phosphate buffer (pH 8.0) and 50 µL of 5,5′-dithiobis (2-nitro-benzoic acid) (10 mM) in acetone. After 15 s, the absorbance was monitored at 412 nm for 5 min in a Spectronic® 20 Genesys spectrophotometer (Spectronic Instruments, Rochester, NY, USA). The total protein concentration was determined by the Bradford method using bovine serum albumin as a standard. and the GSH level was calculated using pure GSH as standard. (50 - 800 µM).

The GSH content was calculated as follows (4):

$$[GSH] = \theta \times F \times 20/V_m \quad (4)$$

where: θ: curve cotangent, F: dilution factor and $V_m$ samples volume.

*Catalase activity (CAT) determination*

The CAT activity was determined as described by Silva et al. (2012). The method is based on the decrement 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. Aliquots of liver homogenates were suspended in Tris–HCl 1 M, EDTA 5 mM (pH 8) and $H_2O_2$ 10 mM. Two dilutions of homogenate (15 or 20 mL diluted to 1 mL) were incubated at 37°C and the absorbance was monitored every minute for 6 min. The results were expressed in

U/mg protein, where one unit of activity (U) corresponds to the amount of catalase that will decompose 1 mmol $H_2O_2$ per min at pH 8 and 37°C.

*Superoxide-Dismutase (SOD) determination*

The cytoplasmic SOD activity was determined according to McCord and Fridovich con minor modifications (Silva et al., 2012) using a reaction mixture containing cytochrome C (100 mM), xanthine (500 mM), ethylenediaminetetraacetic acid (1 mM), KCN (200 mM), and a 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.

## Reverse transcription/polymerase chain reaction (RT-PCR)

*RNA extraction*

RNA was extracted by mixing 100 mg of rat liver and 1000 µL of trizol (Invitrogen, New York City, New York, USA). After the addition of 200 µL of chloroform (Merck, Darmstadt, Hessen, Germany), vortex mixing for 15 s, incubation at room temperature for 5 min, and centrifugation at 12.000 *g* and 4°C for 15 min, the supernatant (400 µL) was collected, avoiding the interphase, and mixed with 500 µL of isopropanol by vortexing for 5 s. It was then centrifuged at 12.000 *g* and 4°C for 5 min 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 min. The supernatant was discarded again. The pellet was resuspended in 20 µL of RNAse-free distilled water, incubated at 50°C for 10 min, and stored at -70°C.

*Reverse transcription*

Five micrograms of RNA were added to 1 µL of primer (Cu/Zn SOD or CAT), 1 µL of dNTP (10 mM), and 4 µL of sterile distilled water. The reaction was started by a heating step at 65°C for 5 min and then quickly chilled on ice. After adding 4 µL of 5X First-Strand Buffer (Invitrogen), 2 µL of DTT (0.1 M, Invitrogen), and 1 µL of ribonuclease inhibitor (Invitrogen), the mixture was incubated at 37°C for 2 min. After that 1 µL of M-MLV reverse transcriptase (200 U/µL, Invitrogen) was added and the mixture was incubated at 37°C for 50 min. The reaction was stopped by a heating step at 70°C for 15 min. The PCR product (cDNA) was stored at -70°C.

*PCR amplification*

Five microliters of cDNA were amplified in a 50 µL reaction mixture containing 5 µL of Tris (hydroxymethyl) aminomethane–hydrochloride buffer (20 mM; pH 8.4), KCl (500 mM), 1.5 µL of $MgCl_2$ (50 mM), 1 µL of dNTP (10 mM), 35.1 µL of diethyl pyrocarbonate, 1 µL of primer (CAT), and 0.4 µL of Taq polymerase (5 U/µL). After an initial denaturation at 94°C for 3 min in a thermal cycler (Bio-Rad, Hercules, California, USA), 35 cycles (at 94°C for 45 s, at 55°C for 30 s, at 72°C for 1.3 min, and 72°C for 10 min) were carried out. Finally, the mixture was chilled at 4°C. The PCR amplification products were analyzed by electrophoresis on a 2% 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) was genotyped using the following primers (Promega, Madison, AL, USA):

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

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

## Statistical analysis

All experiments were subject to statistical analysis. All experiments were carried out in triplicate and the 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.

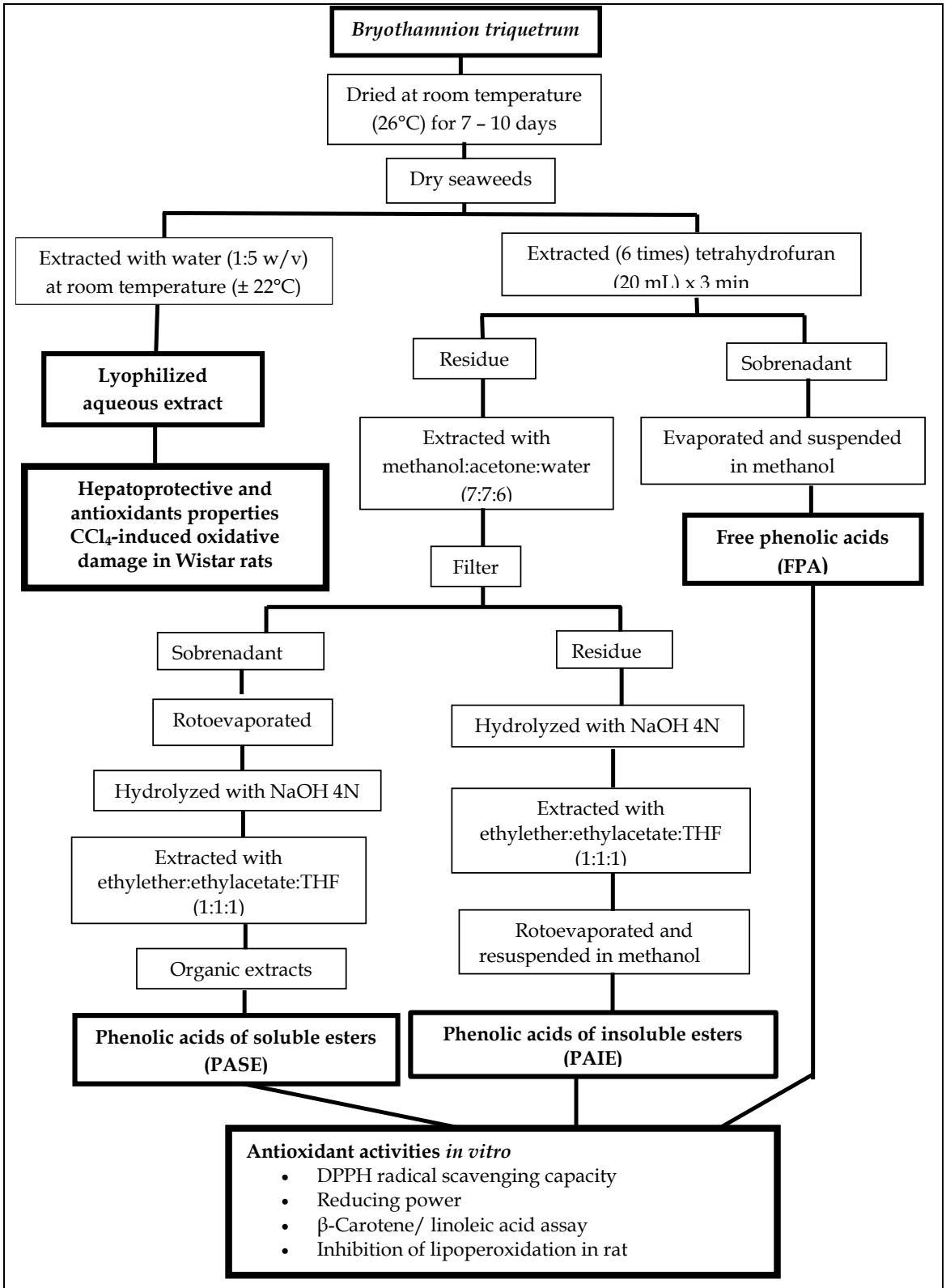


**Figure 1.** Flow diagram of the extraction processes to obtain aqueous and organic extracts (free phenolic acids, soluble esters and phenolic acids of insoluble esters) from the seaweed *Bryothamnion triquetrum* and the antioxidant activity techniques.

## RESULTS AND DISCUSSION

### Total phenolic content

Polyphenolic compounds are commonly found in seaweeds, and different species have reported polyphenols as being responsible for beneficial healing properties. In previous reports, there have been suggestions of a direct relationship between the total phenol content and the antioxidant properties (Machu et al., 2015).

The polyphenolic compounds content in lyophilized aqueous extract from *B. triquetrum* was 8.7 mg/g and in the polyphenolic-rich fractions were 5.52 ± 0.41, 1.41 ± 0.01, and 3.04 ± 0.08 mg gallic acid equivalents/g seaweed dry weight for free phenolic acids (FPA), phenolic acid soluble ester's (PASE) and phenolic acid insoluble ester's PAIE fractions, respectively. Considering that these are fractions rich in polyphenols, and then these results are similar to the polyphenol content (8.08 mg/g dry seaweed) that was found in a lyophilized aqueous extract from *B. triquetrum* (Vidal et al., 2001).

The content of polyphenols in seaweeds can vary by different factors such as place and date of collection, type of extraction and even differences by species including within the same genus (Demirel et al., 2009, Kuda e Ikemori, 2009). Souza et al. (2011) have found significative quantitative differences in the concentrations of phenolic compounds between seaweeds of the genus *Gracilaria*. The phenolic content found in this work is higher than other seaweeds that have been expressed in the literature like *Ulva* spp. (Farasat et al., 2014) while Vidal et al. (2009), Mancini-Filho et al. (2009) and Silva et al. (2012) reported much higher levels of phenolics in rich-phenol fractions from *Halimeda* sp. Different authors have identified phenolic acids as one of the most abundant polyphenolic compounds in seaweeds. In previous work, it had been analyzed the phenolic acids in an aqueous extract from *B. triquetrum*. Vidal et al. (2001) identified and quantified phenolic acids such as t-cinnamic (221.9 µg/g lyophilized), p-coumaric (4187.3 µg/g lyophilized) and ferulic acids (442.3 µg/g lyophilized) and suggested that these compounds were related to the antioxidant properties of the seaweeds. Souza et al. (2011) identified in red seaweeds *G. birdiae* and *G. cornea*, two important antioxidants, apigenin and gallic acid. In addition, in red seaweeds other polyphenolic compounds have been identified with antioxidant activity such as bromophenols, compounds have also been identified in *B. triquetrum* (Vidal et al., 2001).

### Antioxidant activity *in vitro*

#### *DPPH radical scavenging*

Extracts from seaweeds have numerous antioxidant components and it is relatively difficult to measure each antioxidant component separately. For this reason, different methods have been developed and applied in recent years to screen and evaluate the total antioxidant activity of seaweeds. DPPH• radical scavenging has been widely used to study the activity of antioxidant molecules and extracts from seaweeds.

As shown in Fig. 2A, the fractions exhibited a concentration of dependent free radical scavenging activity with $IC_{50}$ values of 2.9 and 4.0 µg of phenolic compounds, corresponding to 0.53 and 1.31 mg/dry seaweed, for the FPA and PAIE fractions, respectively. Vidal et al. (2006) reported an $IC_{50}$ value of 6 mg/mL but investigating a crude aqueous extract very different from the extraction used in this work. The positive control used butylhydroxytoluene (2 mg) showed antioxidant activity of 11.5 %.

However, the maximum scavenging activity for the PASE fraction was significantly lowers (30% at 20 µg). In all fractions, the antioxidant activity increased in a phenolic compound-dependent relationship. Different authors (Balboa et al., 2013; Pinteus et al., 2017) have reported a positive correlation between phenolic content and scavenging capacity with DPPH.

Although in this work, it has studied fractions rich in polyphenols, given the nature and the complexity of extraction and fractions from sea-

weed, it is possible that they contain other compounds that contribute to the enhancement of antioxidant activity (Rasul Suleria et al., 2015). Vidal et al. (2009) reported significantly higher $IC_{50}$ values for FPA and PASE fractions from *H. monile* and *H. opuntia,* when compared with those for similar fractions from *B. triquetrum*; however, PASE from *H. monile* showed a greater antioxidant activity than FPA. Interestingly, a greater phenolic content in *H. monile* was found in FPA. Thus, the specific composition of phenolic compounds in *B. triquetrum* may have played an important role in enhancing the antioxidant activity. In previous work, it was determined that an aqueous extract from *B. triquetrum* contains different molecules that are able to act as antioxidants such as carotenoids and ascorbic acid (Vidal et al., 2006). Ismail and Hong (2002) investigated the antioxidant activity of edible seaweeds (Nori, Wakame, Kumbu and Hijiki) and they reported that the aqueous extracts of Wakame and Hijiki were more effective in scavenging DPPH than their ethanolic extracts. According to the investigators, the differences observed were probably due to the characteristics of the antioxidant components extracted from the matrix of seaweeds.

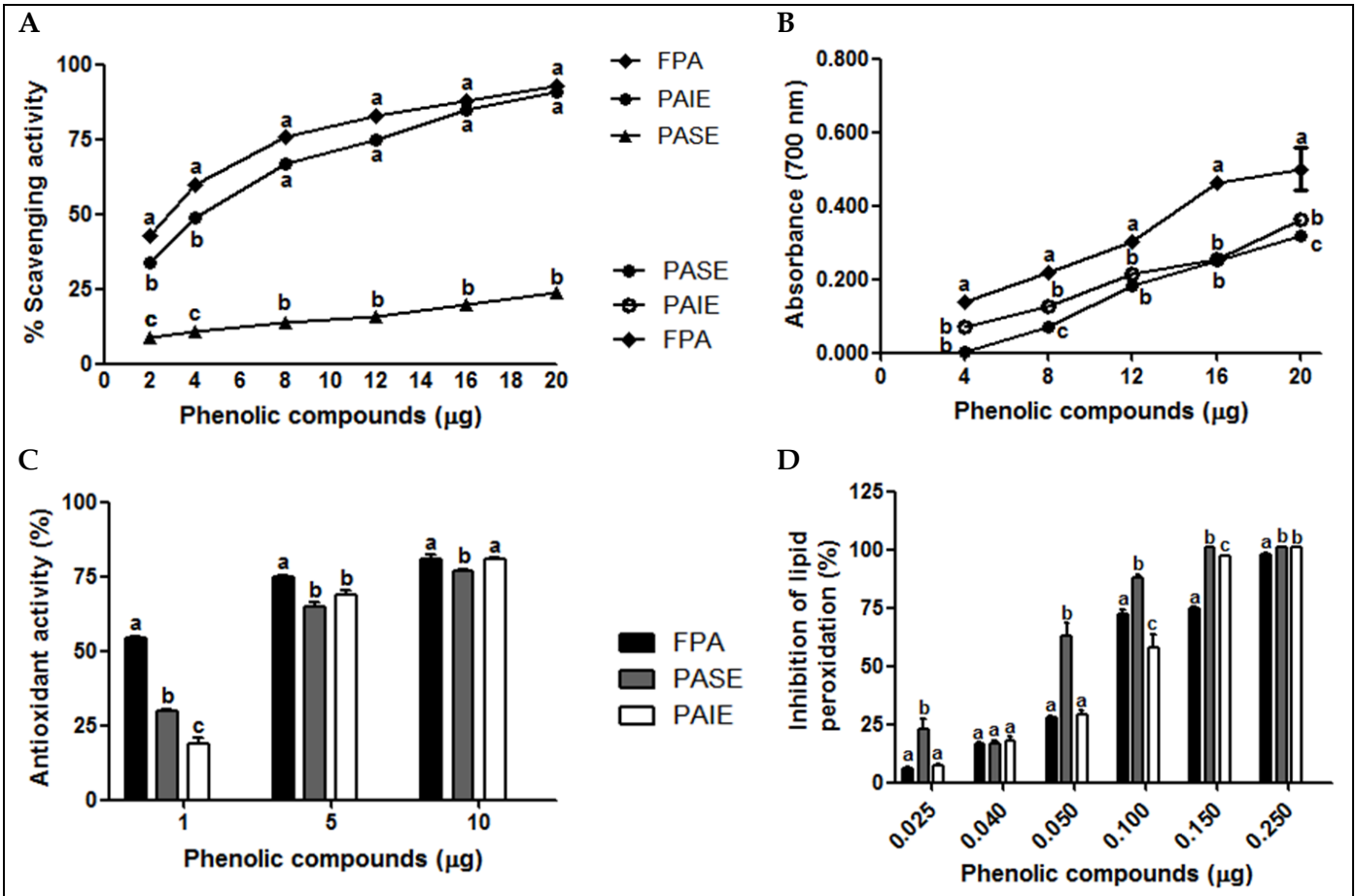


**Figure 2**. Antioxidant capacity *in vitro* of different fractions from *Bryothamnion triquetrum*: Phenolic acid (FPA), phenolic acid insoluble esters (PAIE) and phenolic acid soluble esters (PASE).

Results expressed as mean ± standard deviation. All experiments were realized for triplicate and each point n=3. **A:** DPPH scavenging capacity. In this assay was used as positive control the butyl-hydroxy-toluene (BHT), 2 mg with antioxidant activity of 11.5%. **B:** Reducing power *versus* the content of phenolic compounds (µg phenolic compounds). In this assay was used ascorbic acid (1 mg) as reference compound with an absorbance of 2.824. **C:** Antioxidant activity by β-carotene-linoleic acid assay. The positive control used (BHT, 0.1 and 0.2 mg) showed antioxidant activities of 85 and 94%. **D:** Inhibition of spontaneous lipid peroxidation. As positive control was used ascorbic acid (1 mg/mL) with an inhibition of spontaneous lipid peroxidation value of 73%.

Farasat et al. (2014) studied the antioxidant activity of five edible seaweeds and reported a correlation between the phenolic content and DPPH radical scavenging activity. According to the authors, although many compounds in seaweeds may contribute to their antioxidant activity, the presence of phenolic compounds is essential. In this work, it was observed a significant correlation between DPPH radical scavenging and the phenolic content in the three fractions studied. Senevirathne et al. (2006) have also indicated an association between the phenolic content of seaweeds and DPPH• scavenging. In previous results from our group, *Halimeda* genus has indicated an association of antioxidant activity in DPPH scavenging with a phenolic content (Vidal et al., 2009).

*Reducing power*

The ferric reducing power assay is based on the ability of an antioxidant to reduce $Fe^{3+}$ to $Fe^{2+}$. A higher absorbency, measured at 700 nm, implies a greater reducing power.

As shown in Fig. 2B, the fraction with the greatest phenolic content was FPA, and it showed the highest reducing power, regardless of the amount of phenolic compounds tested. PAIE and PASE fractions had quite similar performances. For all of the fractions, reducing power was directly proportional to phenolic content. The maximum absorbances, 0.5 (FPA), 0.31 (PASE) and 0.37 (PAIE), corresponding to 3.6, 14.2 and 6.6 mg of dry seaweed, respectively, were attained with the highest amount of phenolics (20 µg of GAE). The positive control, ascorbic acid (1 mg), gave an absorbance of 2,824. If considered it is a pure chemical compound, then when comparing this value with the results of the fractions of *B. triquetrum*, it can be concluded that these fractions rich in polyphenol presents a high antioxidant activity.

Senevirathne et al. (2006) reported a high absorbance value for different extracts from *E. cava*. They observed that the level of hydrophilic phenolic content was higher than that of hydrophobics and the reducing ability was significantly dependent on the phenolic content of the fractions.

Sabeena Farvin and Jacobsen (2013) investigated ethanolic and aqueous extracts from 16 seaweeds (red, brown and green) and they observed that the extracts containing the highest levels of total phenolics were also potent in reducing ferric iron, suggesting that algal polyphenols may be the principle constituents responsible for these properties of the extracts. In this work too and it was found a similar relationship.

*Antioxidant activity by β-carotene/linoleic acid method*

The β-carotene-linoleic assay is based on the ability of lipids, such as linoleic acid, to form a peroxyl radical (LOO•) in the presence of ROS and $O_2$, and then this peroxyl radical reacts with β-carotene to form a stable β-carotene radical; but if an antioxidant is present in a testing solution, it reacts competitively with the peroxyl radical, and therefore, antioxidant effects are easily monitored by bleaching the color of a test solution.

As indicated in Fig. 2C, the FPA fraction displayed the highest antioxidant activity with the lowest phenolic content (1 µg of GAE). High activity was observed for the three fractions at the highest phenolic content (10 µg of GAE), with the antioxidant activity of all fractions increased in a phenolic content-dependent relation. The positive control used (BHT, 0.1 and 0.2 mg) showed antioxidant activities of 85 and 94%; when comparing these results with the fractions of phenolic acids of *B. triquetrum*, it could be said that they have a high antioxidant potential.

Ismail and Hong (2002) reported an antioxidant activity within the range of 3% and 57 % for aqueous and ethanolic extracts of Nori (*Porphyra* sp.), Kumbu (*Laminaria* sp.), Wakame (*Undaria* sp.), and Hijiki (*Hijikia* sp.), at 1 mg/mL. In this study, lower amounts of seaweed of 0.18 mg and 0.72 mg resulted in antioxidant activity levels of 20 and 80%, respectively.

Souza et al. (2011) assessed the antioxidant activities of *G. birdiae* and *G. cornea* by the β-carotene-linoleic acid assay, achieving values of β-carotene oxidation inhibition of up to 40% with 5

mg of extracts, values similar to those obtained in this work.

These results suggest that the three investigated fractions [free phenolic acids (FPA): Tetrahydrofuran); phenolic acid insoluble esters (PAIE): methanol: acetone: water; and phenolic acids soluble esters (PASE): ethyleter: ethylacetate: THF)] may contain additionally compounds as phlorotanines and terpenoids with intermedia lipophilic properties capable of donating hydrogen to the linoleic acid free radical.

Demirel et al. (2009) investigated the antioxidant activity of six brown seaweeds using the inhibition of β-carotene bleaching and $ABTS^+$ methods, and they observed a good correlation between the antioxidant activity and the polyphenol content; however, there is no direct correlation between the results of these two trials, which demonstrates the importance of studying this property from different types of tests.

*Inhibition of spontaneous lipid peroxidation in rat brains*

Lipid peroxidation results in the deterioration of fatty acids (PUFA) of membrane phospholipides, especially in the brain, where lipid peroxidation involves Fe-mediated lipid cleavage, resulting in the production of peroxyl and alkoxyl radicals.

Inhibition of lipid peroxidation in rat brains was investigated within a low range of phenolic concentrations (0.025 - 0.25 µg of GAE of phenolic compounds). Overall, the higher the phenolic concentration, the greater the inhibitory effect of the fractions, as shown in Fig. 2D. The $IC_{50}$ values were 0.07, 0.047 and 0.084 µg of phenolic compounds for FPA, PASE and PAIE fractions, respectively. In this work, the positive control (ascorbic acid, 1 mg/mL) gave an inhibitory activity value of 73% of Inhibition of spontaneous lipid peroxidation. Vidal et al. (2001) reported an $IC_{50}$ of 23.9 µg for *B. triquetrum*, a value comparable to that found in this work, considering that these authors studied a crude aqueous extract. Rivero et al. (2003) also determined an $IC_{50}$= 8.5 µg with an aqueous extract of *H. incrassata*. Chakraborty et al. (2013) evaluated the antioxidant activities and the total phenolic contents of brown *Turbinaria* spp. seaweed and they found an $IC_{50}$ = 5.25 µg for a lipid peroxidation inhibitory assay. Phenolic acids of different seaweeds have been correlated with an inhibition of peroxidation, even at very low concentrations. The inhibitory effects of the aqueous extracts of *B. triquetrum* on spontaneous lipid peroxidation were found to be correlated with the presence of different phenolic acids, including p-coumaric, t-cinnamic and ferulic acid (Vidal et al., 2001).

These results show that polyphenolic-rich fractions of *B. triquetrum* are capable of scavenging free radicals and also of inhibiting their generation; the antioxidant activity of the fractions may be phenolic content-related.

Aqueous extracts from *B. triquetrum* showed significant antioxidant activity evaluated in terms of reducing power, free radical scavenging activity, and lipid peroxidation inhibition activity. For all fractions, antioxidant activity increased in a phenolic content-dependent manner; therefore, phenolic compounds must play an important role in their antioxidant activity.

*Antioxidant activity protecting against liver damage in $CCl_4$-induced liver injuries in Wistar rats*

Antioxidant and hepatoprotective properties of an aqueous extract from *B. triquetrum* were investigated in Wistar rats with a $CCl_4$-induced liver injury. Through the ASAT and ALAT activities (Table 1), it was verified there was no damage in the liver from *B. triquetrum*-treated animals, while in Ferulic acid-treated rats, it was observed that an attenuating toxic effect produced by $CCl_4$. In a previous work, Mancini-Filho et al. (2009) reported similar results when investigating polyphenol fractions from *Halimeda monile*. These results are confirmed by Silva et al. (2012), whom investigated the hepatoprotective properties of the polyphenolic fractions from *H opuntia* under an experimental model of $CCl_4$ injury.

In Table 1, it may be appreciated that the TBARS levels in the liver tissues of $CCl_4$-treated rats increased, confirming a successful induction

**Table 1.** Markers of the injury hepatic in serum and oxidative stress in hepatic tissue in Wistar rats treated with $CCl_4$ and *Bryothamnion triquetrum* (Bt) and ferulic acid (FA).

| Groups | Serum | | Liver | |
|---|---|---|---|---|
| | **ASAT (units/mL)** | **ALAT (units/mL)** | **TBARS (nmol/mg protein)** | **GSH (μmol)** |
| Control | 84.36 ± 0.61$^{a}$ | 57.68 ± 2.70$^{a}$ | 0.19 ± 0.02$^{a}$ | 0.10 ± 0.02$^{a}$ |
| $CCl_4$ | 116.30 ± 11.68$^{bc}$ | 90.87 ± 20.08$^{bc}$ | 0.87 ± 0.21$^{b}$ | 0.65 ± 0.11$^{b}$ |
| FA 20 + $CCl_4$ | 112.25 ± 10.22$^{bd}$ | 78.59 ± 13.11$^{ac}$ | 0.56 ± 0.29$^{bc}$ | 0.55 ± 0.12$^{bc}$ |
| Bt 200 $CCl_4$ | 96.03 ± 8.68$^{ad}$ | 62.13 ± 8.41$^{a}$ | 0.45 ± 0.10$^{ac}$ | 0.41 ± 0.12$^{c}$ |

The values represent mean ± SD. In all groups n=10 animals. Different letters in a column indicate statistically significant differences, *p < 0.05.

of oxidative damage. The pre-treatment with *B. triquetrum* (200 mg extract/kg), led to reduction in TBARS liver levels approximately 1.93 times to respect $CCl_4$-control (0.87 ± 0.21 nmol/mg protein for $CCl_4$ control *versus* 0.45 ± 0.10 nmol/mg protein with Bt-treated) while in animals treated with ferulic acid was 1.55 times (0.45 ± 0.10 nmol/mg protein). According to Silva et al. (2012), a pre-treatment with polyphenol-rich fractions from *H. opuntia* ((20 mg/kg, p.o. for 20 days) led to reductions in both serum and liver TBARS. Kim et al. (2008) also observed a comparable reduction in hydroperoxide levels in the liver, relative to a $CCl_4$-treated group, in a study of mice fed on Saengshik, a non-cooked food containing vegetables and seaweeds, during 4 weeks. Other authors (Bupesh et al., 2012) observed a reduction of TBARS levels in liver damage by $CCl_4$ in animals treated with *H. muciformis* (200 mg/kg oral via).

Animals with liver injuries caused by $CCl_4$, had GSH levels increased statistically in respect to all groups. According to Lu (1999), these increased levels may be explained as an adaptive response of rats against the oxidative stress induced by $CCl_4$. However, in *B. triquetrum*–treated rats, it was observed that there were minor values ($p<0.05$.) than those of the $CCl_4$-treated rats; this could be explained by a hepatoprotector effect from the seaweed. Bupesh et al. (2012) also obtained similar GSH levels in both groups. However, Kim et al. (2008), reported lower values in $CCl_4$-treated rats with respect to the control group, while GSH values in the seaweed-treated rats were intermediately between these groups. These seemingly contradictory results in glutathione levels may be explained by the time of induction $CCl_4$ and adaptative response, in addition different functions and the metabolic pathways of this metabolite.

Antioxidant enzymes such as CAT, SOD and GPx are considered as a fundamental defense system in mammals against free radicals. As may be appreciated in Fig. 3, treatment with the seaweed led to a significant increment in the activity of the CAT enzyme, which in turn resulted in an enhanced antioxidant defense; however, an increment in SOD activity was not observed. Additionally, in the animals treated with ferulic acid (20 mg/kg, p.o.) no changes were observed in the antioxidant enzymes studied. These results suggest that the antioxidant and hepatoprotective activities of *B. triquetrum* extract are in agreement with Mancini-Filho et al. (2009) when they reported a considerable increase in the activity of CAT in rats treated with a polyphenol-rich fraction from *H. monile.* A high antioxidant enzyme activity has been reported through the repeated administration of *Padina boergesenii* extracts during $CCl_4$ during induced oxidative damage in Wistar rats (Karthikeyan et al., 2010). Additionally, treatment with *C. prolifera* and *L. obtusata* extracts has also led to a rise in enzyme activity (Abdel-Wahhab et al., 2006). It was reported by Silva et al. (2012) that the ability of the *H. opuntia* extract, to induce the antioxidant enzyme activity of CAT, SOD, and GPx,

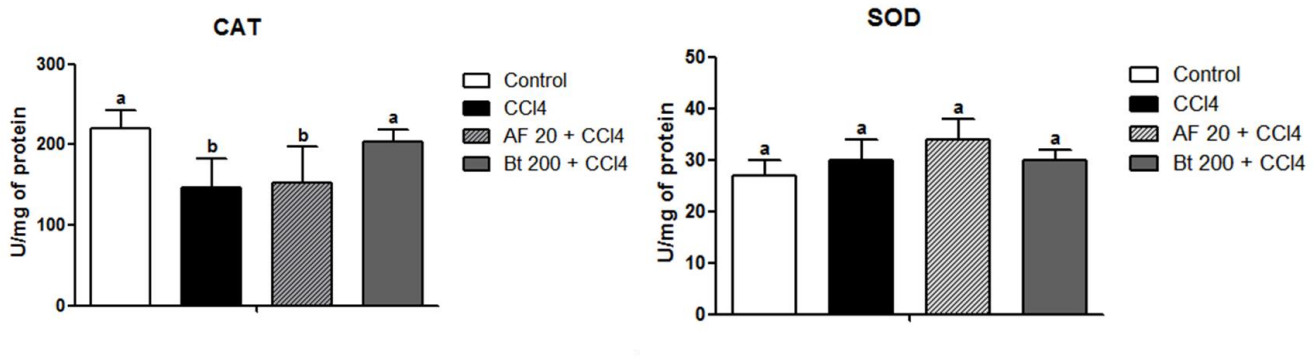


**Figure 3.** Activity of CAT and SOD liver tissues from control ($H_2O$), $CCl_4$-treated, ferulic acid (AF, 20 mg/kg, p.o) + $CCl_4$ - treated, and *B. triquetrum* (Bt, 200 mg aqueous extract equivalent to 2.6 mg polyphenols/kg, p.o.) + $CCl_4$-treated rats. Different letters indicate statistically significant differences, *p<0.05.

which in turn resulted in an enhanced antioxidant defense; they suggested that this was due to a potent hepatoprotective activity of the phenolic fraction of *H. opuntia.*

In summary, the treatment with seaweed led to a significant increment in the activity of the catalase enzyme. It thus proved to be capable of attenuating the changes induced by $CCl_4$, and as such, a decrement in the activities of the enzymes ASAT and ALAT and TABRS hepatic levels. These results suggest a hepatoprotective activity of the seaweed *B. triquetrum*.

*Expression of CAT hepatic enzymes by PCR-RT*

In considering results from the antioxidant enzyme activities, only the expression of catalase by PCR-RT technique has been studied. As can be seen in Fig. 4, the levels of CAT in liver tissues increased significantly with the treatment of seaweed and posterior $CCl_4$ administration, which shows alterations in gene expression.

Treatment with the *B. triquetrum* aqueous extract (band 4) resulted in a higher catalase gene expression when compared with that which was observed in the $CCl_4$-treated group (band 3). Anantachoke et al. (2016) discusses some evidence that polyphenolics found in Thai fruits might exert the antioxidant effect through induction of antioxidant enzymes or these compounds are involved in the regulation of antioxidant enzyme synthesis.

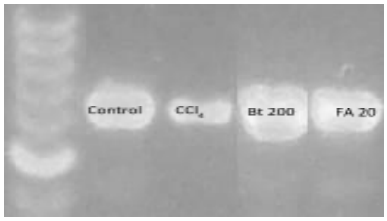


**Figure 4**. Electrophoresis of the CAT enzyme gene of the livers from rat groups.

**1:** Ladder; **2:** Control ($H_2O$); **3:** $CCl_4$; **4:** Bt 200 (*B. triquetrum*, 200 mg aqueous extract equivalent to 2.6 mg polyphenols/kg, p.o.) + $CCl_4$; **5:** FA 20 (ferulic acid, 20 mg/kg, p.o) + $CCl_4$.

Vidal et al. (2001) reported that from the total phenolics content of the aqueous extract of *B. triquetrum*, more than half (60%) corresponds to phenolic acids, and in this fraction, about 86.3% was identified as p-coumaric acid, while a small fraction was associated with ferulic and trans-cinnamic acids. Additionally, Yeh and Yen (2006) demonstrated that ferulic and coumaric acids modulate antioxidant enzymes (CAT, SOD and GPx) and seem to selectively induce hepatic mRNA transcripts for CAT, probably through an up-regulation for gene transcription, as well as the Nrf2 transcription factor. In previous results, Mancini-Filho et al. (2009) reported an over-expression of CAT genes by the treatment with polyphenolic-rich fractions from *Hmonile* while Silva et al. (2012), showed that by using (RT-PCR) increased the CAT gene expression in the group treated with free phenolic acid (FPA) fractions

from *H. opuntia*, suggesting inducing effects on the enzyme.

Aqueous extracts from *B. triquetrum* showed significant antioxidant activity evaluated in terms of reducing power, free radical scavenging activity, and lipid peroxidation inhibition activity. For all fractions, antioxidant activity increased in a phenolic content-dependent manner; therefore, phenolic compounds must play an important role in their antioxidant activity.

## CONCLUSIONS

The present work evidenced polyphenol-rich fractions from the red seaweed *Bryothamnion triquetrum* and it was active as an antioxidant *in vitro* assayed by different methodologies, DPPH radical-scavenging, reducing power, β-carotene/linoleic acid assay and the inhibition of spontaneous lipid peroxidation in rat brain homogenates. On the other hand, results in a hepatotoxicity model of $CCl_4$-induced liver damage in Wistar rats demonstrated the potential of *Bryothamnion triquetrum* as a natural antioxidant. Treatment with seaweed led to the attenuation of liver damage by $CCl_4$. ASAT and ALAT activities diminished and liver TBARS levels suggest a hepatoprotective activity of the seaweed. The CAT level in rat liver tissues decreased as a result of oxidative damage induced by $CCl_4$.

However, with this data comparison, it was possible to suggest that red seaweed *Bryothamnion triquetrum* have hepatoprotective and antioxidants properties with a potential usage as phytodrugs, nutraceutical foods or chemoprevention medicine.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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**AUTHOR CONTRIBUTION:**

| Contribution | Vidal Novoa AJ | de Oliveira e Silva AM | Portari Mancini DA | Díaz Gutiérrez D | Mancini-Filho J |
|---|---|---|---|---|---|
| Concepts or ideas | x | x | | | x |
| Design | x | x | | | x |
| Definition of intellectual content | x | x | | | x |
| Literature search | x | x | x | x | x |
| Experimental studies | x | x | x | x | x |
| Data acquisition | x | x | | | |
| Data analysis | x | x | | | x |
| Statistical analysis | x | x | | x | |
| Manuscript preparation | x | | | | |
| Manuscript editing | x | | | | |
| Manuscript review | x | x | x | x | x |

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