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Effect of a Crude Sulfated Polysaccharide from *Halymenia floresia* (Rhodophyta) on gastrointestinal smooth muscle contractility

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ABSTRACT

The aim of this work was to study the effect of Halymenia floresia *(Hf) on duodenum contractility, and on experimental protocols of gastric compliance (GC) in rats. Fraction Hf2s exhibited a concentration-dependent myocontractile effect (EC_{50} 12.48 µg/ml), and an inhibitory effect after consecutive washing. The contractile response promoted by Hf2s in the duodenum strips was completely inhibited by verapamil, and the effects were prevented in the presence of Ca2+-free medium. The pretreatment with atropine prevented the Hf2s myocontractile effect. Hf2s was also capable to decrease the GC (from 3.8±0.06 to 3.4±0.13 ml, P<0.05), which did not return to basal levels after more 50 min of observation. These results indicated that the algal polysaccharide possessed* in vitro *and* in vivo *gastrointestinal effects.*

Key words: *Halymenia floresia*, sulfated polysaccharides, gut motility

INTRODUCTION

Seaweeds are traditionally consumed in the Orient as part of the daily diet. Some researchers describe them as the foods without toxic and/or anti-nutritional effects, being thus considered as alternative sources for food industry (Campo et al., 2009). These are known to possess good nutritional value as source of proteins, carbohydrate, minerals, carotenoids and vitamins (Marinho-Soriano, 2006; Pires et al., 2008). Marine algae are also considered as low-calorie food, leading to reduction in body weight control and total cholesterol and LDL-C, and prevention

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of gastrointestinal diseases when consumed (Carvalho et al., 2009). However, there are only a few carried out so far describing their composition. They contain polysaccharides that may bind to metals through their carbonyl, hydroxyl or sulfated groups, and that can also exhibit toxic properties (Langwinski and Patiño, 2001). According to Andrade et al. (2010), the brown algae *Padina gymnospora*, when exposed to heavy metal-contaminated environments, changes its polysaccharide content. As a result, this species overproduces cell wall polysaccharides as a defense mechanism.

Sulfated polysaccharides (SPs) make up a group of biopolymers that occur in a great variety of marine organisms (Stephen, 1995; Hayashi et al., 1996; Mourão and Pereira, 1999; Aquino et al., 2005; Pomin and Mourão, 2008; Robic et al., 2009; Rodrigues et al., 2009a; Mestechkina and Shcherbukhin, 2010; Rodrigues et al., 2010). In red seaweed, SPs are known as sulfated galactans, and are found in carrageenans and agarans (Melo et al., 2002; Marinho-Soriano, 2006; Pomin and Mourão, 2008; Campo et al., 2009; Silva et al., 2010; Rodrigues et al., 2011a). These highly charged macromolecules (presence of sulfate groups) exhibit chemical structures of complex and heterogeneous nature, playing an important role in ionic, mechanical and osmotic functions, and are constituents of the extracellular matrix of marine algae (Kloareg and Quatrano, 1988). Seaweed SPs are also considered non-toxic, non-dependent, low cost, and do not leave any residue. They do not cause negative impacts to the environment and to the consumer (Becherè, 2000; Campo et al., 2009). Nevertheless, the adverse effect level of SPs depend the administered dose (Li et al., 2005).

In recent years, SPs have been extensively studied as anticoagulant and antithrombotic agents (Farias et al., 2000; Matsubara et al., 2001; Mourão, 2004; Pereira et al., 2005; Pushpamali et al., 2008; Azevedo et al., 2009; Rodrigues et al., 2009a; Rodrigues et al., 2009b; Rodrigues et al., 2010; Mestechkina and Shcherbukhin, 2010; Rodrigues et al., 2011a). Their biological actions suggest that each SP may be dependent of a structural requirement (Mourão and Pereira, 1999; Pereira et al., 2005). SPs are reported to have a large number of biological properties. For example, a highly sulfated heteropolysaccharide (known as ulvan), extracted from the green marine algae *Ulva pertusa* exhibited strong *in vitro* antioxidant activity (Zhang et al., 2003). Talarico et al. (2004) reported that the sulfated galactans obtained from red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata* showed antiviral properties against four serotypes of dengue virus in different host cell types. Fonseca et al. (2008), investigating the effects of a SP isolated from *Gelidium crinale* (Rhodophyta) on a venous thrombosis model, reported that it could be considered as a valuable tool for better comprehension of the physiopathology of thromboembolic diseases. A SP isolated from the red marine algae *Champia feldmannii* demonstrated to be an important edematogenic agent (Assreuy et al., 2008). When its *in vitro* and *in vivo* antitumor properties were evaluated, the results showed that the compound was also capable of inhibiting the development of sarcoma 180 tumor, suggesting an immunostimulant. This molecule induced a discreet hyperplasia of lymphoid follicles of the spleen white pulp of treated mice. Histopathological analysis of liver and kidney showed that both organs were also moderately affected by *C. feldmannii* SP treatment (Lins et al., 2009). Recently, a crude polysaccharide from the cell-wall from *Turbinaria ornata* (Phaeophyta) presented *in vivo* anti-inflammatory activities (Ananthi et al., 2010).

Halymenia floresia (Clemente) C. Agardh is a red marine algae belonging to the Halymeniaceae family, found at Mucuripe Beach, coast of Ceará State, Brazil. Morales et al. (2006) identified a toxic effect in the antibacterial activity of secondary metabolites extracted from this species. Recently, the *H. floresia* SPs have been extracted and studied as anticoagulant agents (Amorim et al., 2011; Rodrigues et al., 2011b). Based on the fact that no biological study about their effects *in vivo* and *in vitro* in models of gastrointestinal has, to our best knowledge, yet been reported, we decided to investigate the effects of a crude soluble SP fraction of this species on rat duodenum contractility *in vitro*, and in gastrointestinal property *in vivo*.

MATERIAL AND METHODS

Animals

Male *Wistar* rats (250-350g) from the Animal House of the Federal University of Ceará were

randomly selected and kept under a 12-h light/dark cycle, in temperature-controlled rooms, and were fed with water and food *ad libitum*. All the procedures and animal treatments used in this study were approved by the Institutional Animal Care and Used Committee of the Federal University of Ceará, Fortaleza-CE, Brazil, in accordance with the international guidelines (NIH publications No. 85-23, revised 1985), previously approved by the 125/07 protocol.

Drugs and reagents

The reagents used in the study included NaCl, KCl, $MgCl_2$, $CaCl_2$, NaH_2PO_4, $NaHCO_3$, glucose, ketamine, xylazine (Vetec Química Farm. Ltda, São Paulo, Brazil), atropine, verapamil, urethane (Sigma Chemical Co., St. Louis, MO, U.S.A or Sigma Aldrich Chemie, Steinheim, Germany).

Extraction of SPs

The *H. floresia* SPs were successively extracted as described elsewhere (Amorim et al., 2011). Crude fractions Hf1s, Hf2s and Hf3s were obtained, respectively, at different temperatures 25 or 80°C (twice). Briefly, the algae were submitted to mechanical stirring for 24 h at room temperature in water at 1.5% (w/v). The residue was removed by centrifugation (5.000 × *g* for 15 min at 4°C). The supernatant was precipitated with absolute EtOH (1:3, v/v), centrifuged, re-dissolved in distilled water, dialyzed against water, freeze-dried and denominated Hf1s. The algal residue was re-extracted but this time at 80°C for 4 h, followed by centrifugation under the same conditions. The hot extraction was repeated once more, using the second extraction residue. The supernatants were precipitated with absolute EtOH (1:3, v/v), and denominated Hf2s and Hf3s for the second and third extractions, respectively.

Agarose gel electrophoresis

The crude fractions Hf1s, Hf2s and Hf3s were analyzed by 0.5% agarose gel electroforesis according to Dietrich and Dietrich (1976). Samples of each crude SP fraction (30 µg) were applied to a gel and run for 1 h at 110 V in 0.05 M 1.3 diaminopropane-acetate buffer (pH 9.0). SP on gel were fixed with 0.1% *N*-cetyl-*N*-*N*-*N*-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue and discolored with an acetic acid: absolute ethanol: distillated water solution (0.1:0.45:0.45).

Molar mass distribution

The peak molar masses (M_{pk}) were estimated by gel permeation chromatography (GPC) with a Shimadzu equipment at room temperature using an Ultrahydrogel linear column (7.8 × 300 mm), flow of 0.5 mL/min, 0.5% polysaccharide concentration (Hf1s, Hf2s and Hf3s) and 0.1 M $NaNO_3$ as solvent. A differential refractometer and an ultraviolet photometer (at 280 nm) were used as detectors, and the elution volume was corrected to the internal marker of ethylene glycol at 11.25 mL. Pullulan samples (Shodex Denko) of M_w 5.9×10^3, 1.18×10^4, 4.73×10^4, 2.12×10^5 and 7.88×10^5 g/mol were used as standards (Melo et al., 2002).

Experimental protocols

***In vitro* duodenum contractility**

Tissue preparations: Male *Wistar* rats (250–350 g, n=6) were initially killed by cervical dislocation. A 2-cm long whole segment of the proximal rat duodenum was removed and placed into a Petri dish containing Tyrode's solution (composition in mmol/l: NaCl: 136.9; KCl: 2.68; $MgCl_2$: 1.05; $CaCl_2$: 1.8; NaH_2PO_4: 0.42; $NaHCO_3$: 11.9; glucose: 5.55). After the dissection of mesentery muscle, the luminal contents were washed with physiological solution. The duodenal segments were placed under 1-g resting tension in a glass organ bath filled with 10 ml of Tyrode´s solution. The solution was maintained at 37°C (pH 7.4) and bubbled continuously with air. Longitudinal muscle tension was recorded on a computer-coupled data-acquisition system (Power-Lab, ADInstruments™, Australia) by means of an isometric force transducer. It was left for one hour for equilibration. Initially, the concentration–response curves at the concentrations of 0.5, 1, 2, 4, 8, and 16 µg/ml for the polysaccharides of *H. floresia* (Hf2s) were obtained and added cumulatively to the bath chamber (5 min for each concentration or, whenever necessary, 10 min to observe the plateau response). The difference between the peak and valley records at plateau contraction in the presence of polysaccharide was considered as the maximal effect of polysaccharide-induced contraction. The polysaccharide activity was also tested in the presence of atropine (1 µM), a muscarinic receptor antagonist, to evaluate the effect of acetylcholine on the duodenal contractile responses to the polysaccharide. In order to evaluate the possible role of extracellular Ca^{2+} effect on the

polysaccharide responses, verapamil (10^{-6} M), a voltage-gated Ca^{2+} channel blocker, or a Ca^{2+}-free medium was used. To verify tissue vitality, the duodenal spontaneous contractile were tested 30 min after the polysaccharide removal (Clemente et al., 2008).

***In vivo* gastric compliance (GC)**

Experiments were performed on the male *Wistar* rats (250–300 g, *n*=22), fasted for 24 h with access to water *ad libitum*. After urethane anesthesia (1.2 g/kg), the cervical vessels were surgically exposed, and cannulated with polyethylene catheters (PE 50) filled with saline and heparin (500 U/ml). The left jugular vein was used for polysaccharide, drugs, or vehicle administration. To evaluate the effect of Hf2s on GC, a latex balloon catheter (~4.0 ml) was introduced *per se* and then positioned in the proximal stomach of the rats. The catheter-free end was connected to a barostat liquid reservoir (DI=2.5 cm, volume=30 ml). The resulting communicant vessel system was filled with an ionic standard solution (45 mg% of NaCl and 0.3 ml% of Imbebient BBC Ornano®). The solution was pre-warmed and kept at 37°C. The barostat liquid level was set at 4.0 cm above the animal's xiphoid appendix. The gastric tonus that changed the gastric balloon volume was electronically sensored and continuously displayed by a digital plethysmometer (LE 7500-PanLab®) (Clemente et al., 2008). Following a basal period of 20 min, the animals received (i.v.) polysaccharide (20 mg/kg) or vehicle (0.2 ml of 0.9% saline). Subsequently gastric volume was monitored for a 50-min period, divided into five consecutive 10-min intervals.

Statistical analysis

The data were reported as mean±S.E.M. Analysis of variance (ANOVA) followed by the Student's *t*-test and Dunnett´s test were employed to compare the differences between the basal and experimental values. A value of $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

The *H. floresia* SPs have been recently extracted. This species is rich in three crude soluble SPs fractions (Hf1s, Hf2s and Hf3s), and they differ in molar ratios of sulfate/galactose (Amorim et al., 2011). Fraction Hf2s contained the highest sulfate content (53.08%). Besides, the anticoagulant activity of the three crude fractions measured by APTT test increased with the sulfate content. The chemical composition showed that Hf2s was composed of 6-*O*-metylgalactose and 3,6-anhydrogalactose. Here, to further evaluate other characteristics of *H. floresia* polysaccharides, Hf1s, Hf2s and Hf3s were tested by agarose gel electrophoresis procedure. These fractions were also analyzed by GPC, and only Hf2s was used on the biological assays of the gastrointestinal system.

Agarose gel electrophoresis

The electrophoretic profile is presented in Fig. 1. Agarose gel electrophoresis showed differences in resolution degree among the isolated crude SP fractions. Hf1s and Hf2s showed more homogeneous bands, with weak metachromasia and, Hf3s showed a poly-disperse polysaccharide with metachromasia intense. These differences in charge density of these algal molecules in were accordance with other studies (Assreuy et al., 2008; Rodrigues et al., 2009b). This suggested that the *H. floresia* polysaccharides occurred with distinct molecular characteristics in the algal tissue. In this way, these physico-chemical characteristics could perhaps be indicative of an important role that these macromolecules exerted on their ionic, mechanical and osmotic functions in marine algae (Kloareg and Quatrano, 1988). According to Rodrigues et al. (2009b, 2010), the technique of successive extractions could be a valuable tool for the identification of molecular characteristics among different algal species and new biological agents.

Molar mass distribution

The GPC chromatograms of Hf1s, Hf2s and Hf3s are shown in Fig 2. All the fractions presented large MM distributions. Fraction Hf1s showed four peaks of MM with elution at 6.67, 8.82, 9.95 and 10.50 ml when detected by refractive index measurements (Fig. 2A). In respect to Hf2s and Hf3s, three different peaks of MM (I, II and II) were noted, with elution volume at 7.57, 10.12 and 10.69 ml, and respectively, 6.62, 8.19 and 10.02 ml (Figs 2B and 2C). Therefore, the peaks obtained in Hf1s, Hf2s and Hf3s ranged from 8.0×10^2 to 5.0×10^6 g/mol.

These results suggested that *H. floresia* Hf2s behaved as heterogeneous system similar to other natural polysaccharides identified from *Gracilaria*

cornea (Rhodophyta) (Melo et al., 2002) and *Ulva rotundata* (Chlorophyta) (Robic et al., 2009). In fact, algae polysaccharides have MM average and with the distribution of molecular species similar in its structures, but their variations occur in the same backbone polysaccharide size (Stephen, 1995).

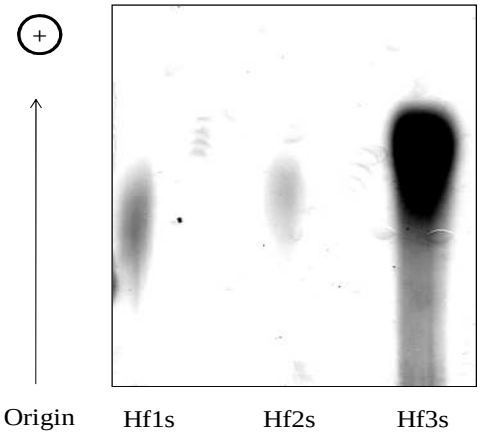

Figure 1 - Agarose gel electrophoresis of sulfated polysaccharides isolated from *H. floresia*. Fractions Hf1s, Hf2s and Hf3s present on gel were stained with 0.1% toluidine blue.

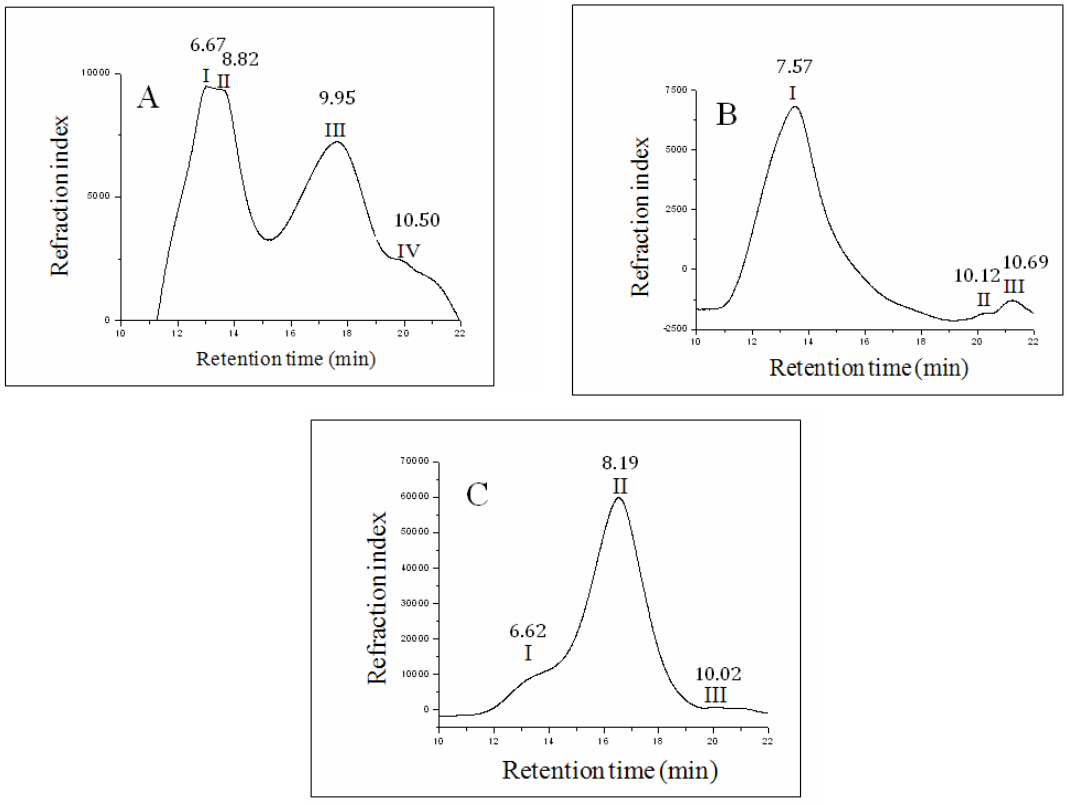

Figure 2 - GPC curves of fractions Hf1s (A), Hf2s (B) and Hf3s (C) for *H. floresia* in 0.1 M $NaNO_3$ solution.

Biological assays

In recently years, diverse biological activities have been focused for algae SPs (Nishino et al., 1991; Farias et al., 2000; Matsubara et al., 2001; Mourão, 2004; Talarico et al., 2004; Pereira et al., 2005; Pushpamali et al., 2008; Rodrigues et al., 2009b; Rodrigues et al., 2010; Rodrigues et al., 2011a). These studies suggested that was interesting to investigate this class of macromolecules in animal models (Mourão and Pereira, 1999; Farias et al., 2001; Zhang et al., 2003; Assreuy et al., 2008; Fonseca et al., 2008; Lins et al., 2009; Ananthi et al., 2010), as valuable tools on development of new pharmacological drugs. The anticoagulant activity of marine algae SPs has been widely reported. Their actions are well described (Farias et al., 2000; Pereira et al., 2005; Fonseca et al., 2008; Rodrigues et al., 2010). Although not determinant for their biological actions (Leite et al., 1998; Farias et al., 2000; Pereira et al., 2005; Fonseca et al., 2008; Rodrigues et al., 2009b), it has been accepted that the bioactivity of these compounds has positive correlation to sulfate group content (Nishino et al., 1991; Azevedo et al., 2009; Mestechkina and Shcherbukhin, 2010) and molecular weight distribution (Zhang et al., 2008; Azevedo et al., 2009; Mestechkina and Shcherbukhin, 2010).

Based on these hypotheses, in the present study, the effect of a crude SP fraction (Hf2s) isolated from *H. floresia* on *in vitro* and *in vivo* models of gastrointestinal system in rats was studied. Due to the high yield and sulfate content (Amorim et al., 2011), the Hf2s homogeneous fraction (Fig. 1) was used for these biological assays. The possible effect of Hf2s from the marine red algae *H. floresia* on intestinal contractile behavior using rat duodenal preparations *in vitro* was studied. The Hf2s demonstrated a concentration-dependent myocontractile effect on the rat duodenum with an EC_{50} value of 12.48 μg/ml (8–16 μg/ml) (Fig. 3).

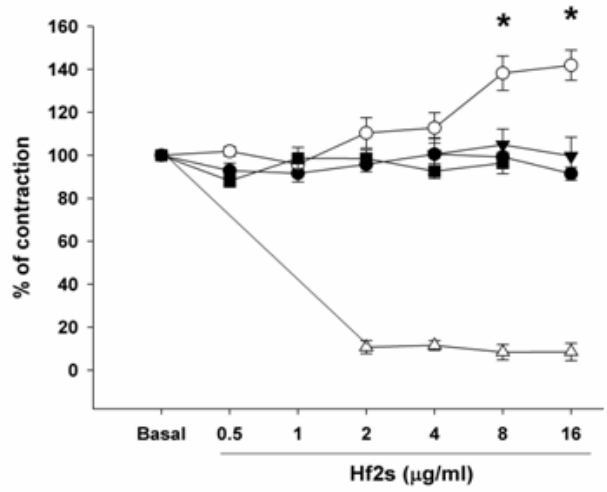

Figure 3 - Myocontractile effect of the sulfated polysaccharide from the marine red algae *Halymenia floresia* (Hf2s) on spontaneous contractions of rat-isolated duodenal *in vitro* preparations. Concentration–response curves at 0.5, 1, 2, 4, 8, and 16 μg/ml for the polysaccharides of Hf2s were cumulatively added to the bath chamber. The graph shows the mean values of the effects of vehicle Tyrode (●, *n*=6), Hf2s (○, *n*=13), Tyrode Ca^{2+}-free (▼, *n*=6), Tyrode + verapamil (△, *n*=6) or Tyrode + atropine (■, *n*=6), and SEM (vertical lines). * $P<0.05$ vs. basal interval (ANOVA and Dunnett's test).

Moreover, the inhibitory effect of Hf2s was observed to be reversible after consecutive washings. Interestingly, Hf2s, over the concentration range used in this study (0.5–16 μg/ml) did not produce spasmodic effects.

As the extracellular Ca^{2+} influx through the voltage-gated Ca^{2+} channels played an important role in gastrointestinal smooth muscle contraction (Clemente et al., 2008), the duodenal strips were pretreated with verapamil (1 μM), an inhibitor of

voltage-gated Ca2+ channels. In addition, the response of this muscle to Hf2s in a Ca^{2+}-free medium was investigated. Verapamil was observed to completely inhibit the Hf2s-elicited contractile response in the duodenum strips, and only partially diminished the phasic response, which suggested that the Hf2s-elicited contractile response depended on the release of internal Ca^{2+} entry from the extracellular space through voltage-dependent Ca^{2+} channels. Similarly, when the strips were incubated in Ca^{2+}-free medium, the Hf2s contractile response was prevented. These results suggested that the inhibition of the influx of extracellular Ca^{2+} as well as the L-type Ca^{2+} channels blocked by verapamil could contribute to elucidate the Hf2s effects on rat duodenum contractile response.

To investigate the participation of muscarinic receptors in producing Hf2s effects, the strips were pretreated with atropine (1 µM), a muscarinic receptor antagonist. Atropine pretreatment was observed to prevent the Hf2s myocontractile effect, which suggested the involvement of muscarinic acetylcholine receptors in the mediation of Hf2s contractile response in rat duodenum (Fig. 3). In this regard, it was observed that in Ca^{2+}-containing solution, acetylcholine elicited similar concentration-dependent contractile responses in the duodenum, jejunum, and ileum strips of rat intestine. However, in Ca^{2+}-free medium, acetylcholine induced phasic contractions that depended on the release of this ion from internal stores by the activation of the sarcoplasmatic-operated Ca^{2+} channels (Elorriaga et al., 1996).

Gastric emptying results from the integrated contractions of the proximal stomach, distal stomach, pylorus, and duodenum (Wingate et al., 1994). Gastric distension enhances gastric compliance (GC), which could be part of the homeostatic process to balance blood volume(Gondim et al., 2001; Graça et al., 2002).

In this study, the effects of Hf2s on GC in urethane-anesthetized rats was also investigated. Figure 4 showed that 30 min after a basal interval, Hf2s treatment decreased the GC (from 3.8±0.06 to 3.4±0.13 ml, $P<0.05$), and after over 50 min, the GC did not return to the basal levels (3.22±0.18 ml).

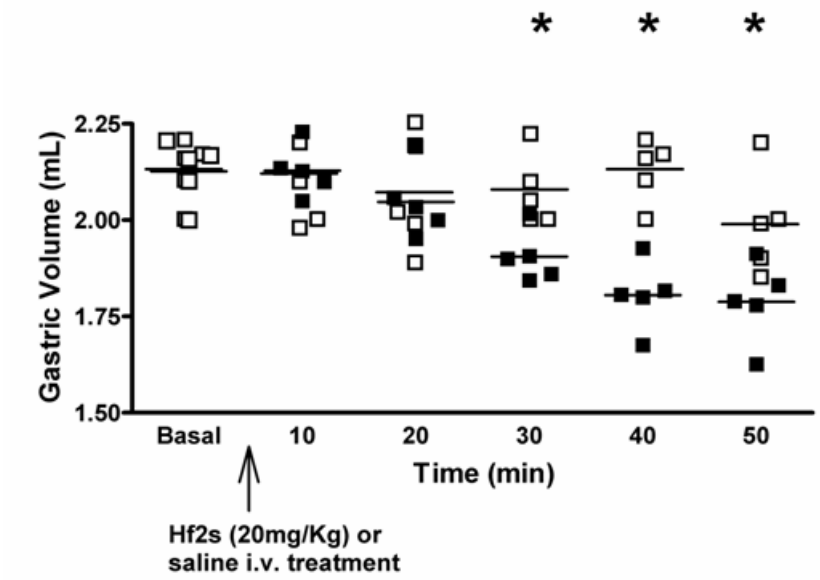

Figure 4 - Effect of the sulfated polysaccharide from the marine red algae *Halymenia floresia* (Hf2s) on the gastric volume (ml) in anesthetized rats. After 20 min (basal interval, untreated period), the animals were submitted to Hf2s (20 mg/kg, n=6, ■) or vehicle (saline 0.9%, n=6, □) to i.v. administration. Gastric volume (ml) data recorded by plethysmography were obtained before (basal interval) and 50 min after the treatments. Each value (white and black squares) pooled into consecutive 10-min intervals are presented as scatter, and a horizontal line represents the median.*$P<0.05$ *vs*. saline group (ANOVA and Dunnett's test).

In conclusion, results showed for the first time that a sulfated galactan isolated from the marine red algae *H. floresia* had promising effects on GI motor functions, which included myocontractile effect on rat duodenum and decreased GC, suggesting that the stimulatory effects of Hf2s depended on the normal activity of the voltage-gated Ca^{2+} channels and Ca^{2+} availability. Taken together, these data indicated that Hf2s could be useful when gastrointestinal contraction was necessary during motility-related disorders, and the red marine algae *H. floresia* could be a valuable and interesting natural source of bioactive compound potentially useful.

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