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Inhibitory effect of a Brazilian marine brown alga *Spatoglossum schröederi* on biological activities of *Lachesis muta* snake venom

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Abstract: The ability of crude extracts of the brown seaweed *Spatoglossum schröederi* to counteract some of the biological activities of *Lachesis muta* snake venom was evaluated. *In vitro* assays showed that only the extract of *S. schröederi* prepared in ethyl acetate was able to inhibit the clotting of fibrinogen induced by *L. muta* venom. On the other hand, all extracts were able to inhibit partially the hemolysis caused by venom and those prepared in dichloromethane or ethyl acetate fully neutralized the proteolysis and hemorrhage produced by the venom. Moreover, the dichloromethane or ethyl acetate extracts inhibited the hemolysis induced by an isolated phospholipase A_2 from *L. muta* venom, called LM-PLA_2-I. In contrast, the hexane extract failed to protect mice from hemorrhage or to inhibit proteolysis and clotting. These results show that the polarity of the solvent used to prepare the extracts of *S. schröederi* algae influenced the potency of the inhibitory effect of the biological activities induced by *L. muta* venom. Thus, the seaweed *S. schröederi* may be a promising source of natural inhibitors of the enzymes involved in biological activities of *L. muta* venom.

Article

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Introduction

Marine organisms represent a valuable source of new compounds. The biodiversity of the marine environment, as well as its chemical diversity, constitute an unlimited source of substances that may lead to the development of bioactive products (Aneiros & Garateix, 2004). Through their metabolism, marine organisms produce molecules, called secondary metabolites, with ecological functions such as defense against predators, competitors and herbivores and for prevention of biofouling (Clavico et al., 2006; Teixeira, 2009). Moreover, some pharmacological properties have been reported for such metabolites, including antimicrobial (González et al., 2001), antiviral (Cirne-Santos et al., 2008), anticancer (Rocha et al., 2007), antimalarial and antituberculosis (Jongaramruong & Kongkam, 2007), antilonomic (Domingos et al., 2009) and antiophidic (Moura et al., 2010) activities. The brown algae (Phaeophyceae) are a class of almost exclusively marine organisms that have been extensively explored because of their biological potential, especially those of the family Dictyotaceae. The algae that belong to this family are the most studied and they produce a large amount of biologically-active substances, including terpenoids of different origins (De Paula et al., 2011). More than 300 diterpenes have been isolated from at least 35 species collected all over the world (Vallim et al., 2005). The genus *Spatoglossum* Kützing has twenty species but, despite this, only the diterpenoids spatane and secospatane were isolated from *S. howellii* Setchell & N.L. Gardner and *S. schmittii* W. R. Taylor, respectively (Gerwick & Fenical, 1983; Gerwick et al., 1983, De Paula et al., 2011).

Snakebite envenomation represents a serious health problem in tropical countries because of the high incidence and the sequels derived from accidents. Snake venoms are a complex mixture that induce a wide range of biological activities. Envenomation by the snake *Lachesis muta* (Linnaeus, 1766) results in systemic (hemostatic, renal, hemorrhage and neurotoxic disturbances) and local (edema, necrosis, inflammation, hemorrhage) signs and symptoms (Jorge et al., 1997; Fuly et al., 2002), which

leads to high lethality and morbidity indexes (Ministério da Saúde, 2001).

The conventional treatment for snakebites is the parenteral administration of serum (antivenom), obtained from hyperimmunized equines, that is able to neutralize the toxic systemic effects. However, antivenom has several disadvantages such as: poor availability in distant regions, the potential for inducing allergic reactions and the lack of efficient neutralization of the local tissue damage induced by venoms (da Silva, 2007; Gutiérrez et al., 2009). Therefore, the search for venom inhibitors from either natural or synthetic sources that are able to act in concert with antivenom is relevant.

The present work reports the ability of extracts from the brown seaweed *Spatoglossum schröederi* (C. Agardh) Kützing to counteract the *in vivo* and *in vitro* activities of *L. muta* snake venom. Moreover, the inhibitory effect of seaweed was also evaluated against a phospholipase A_2 enzyme (denoted LM-PLA2-I) previously isolated from this venom (Fuly et al., 1997).

Materials and methods

Algae material

Specimens of *S. schröederi* (Dictyotaceae, Phaeophyceae) were collected and identified by Dr. Roberto Villaça through snorkeling at depths ranging from 0 to 5 m at Atol das Rocas reef, Rio Grande do Norte State (03°51'03''S and 33°40'29''W), Brazil, in July, 2009. Atol das Rocas reef is a marine biological reserve in northeast Brazil and is the only atoll in the South Atlantic. Seaweeds were washed with local sea water and separated from sediments, epiphytes or other associated organisms. The specimens are deposited in the herbarium of Dr. Roberto Villaça at the Department of Marine Biology, Universidade Federal Fluminense (UFF).

Preparation of crude extract

The air-dried algal material (109.3 g) was triturated and the extracts were obtained through an exhaustive and sequential extraction with solvents of increasing polarity: n-hexane, dichloromethane and ethyl acetate. For each extraction, the solvent was evaporated under reduce pressure and the chromatographic profiles of the extracts were observed on TLC plates (SiO_2 60, F254) manufactured by Merck. The TLC plate was eluted with ethyl acetate: dichloromethane (6:4, v/v) as the solvent system. The eluted plate was revealed by spraying with ceric sulfate, followed by heating at 120 °C.

Snake venom and animals

Lyophilized *L. muta* snake venom was provided by the Fundação Ezequiel Dias, Belo Horizonte-MG, Brazil, and LM-PLA_2-I was isolated according to Fuly and collaborators (1997; 2002). Balb/c mice (18-20 g) were obtained from the Núcleo de Animais de Laboratório of the UFF. They were housed under controlled conditions of temperature (24±1 °C) and light and all of the experiments performed were approved by the UFF Institutional Committee for Ethics in Animal Experimentation (CEPA: 200/10) and were in accordance with the guidelines of the Brazilian Committee for Animal Experimentation.

Biological assays

Antihemolytic activity

The degree of hemolysis induced by *L. muta* venom or LM-PLA_2-I was determined by the indirect hemolytic test using human erythrocytes and hen's egg yolk emulsion as substrate (Fuly et al., 2002). The amount of *L. muta* venom (µg/mL) that produced 80% hemolysis was denoted as the Minimum Indirect Hemolytic Dose (MIHD). Inhibitory experiments were performed by incubating *S. schröederi* extracts with one MIHD for 30 min at room temperature and then the hemolytic activity was evaluated. Control experiments were performed by incubating venom with DMSO or saline instead of *S. schröederi* extracts.

Anticlotting assays

The clotting activity of *L. muta* venom was determined using an Amelung Model KC4A coagulometer (Labcon, Germany). Different concentrations of *L. muta* venom were mixed with bovine fibrinogen solution (2 mg/mL) or with normal citrated human plasma (donated by the blood bank of Hospital Universitário Antônio Pedro, UFF). The amount of venom that clotted fibrinogen or plasma in 60 s was denoted as the Minimum Coagulant Dose (MCD). To evaluate the inhibitory effect, *S. schröederi* extracts were incubated for 30 min at room temperature with one MCD of venom. The mixture was then added to fibrinogen or plasma and the clotting time recorded. Control experiments were performed by incubating venom with DMSO or saline instead of *S. schröederi* extracts.

Hydrolytic activity upon chromogenic substrate

The hydrolysis of the chromogenic synthetic substrate S-2238 (used for monitoring thrombin-like enzymes) by *L. muta* venom was measured in a Thermomax Microplate reader (Molecular Devices, Menlo Park, CA, USA). *L. muta* venom was incubated for 30 min at room temperature with increasing concentrations of *S. schröederi* extracts. Then, reaction was triggered

by adding S-2238 (0.1 mM, final concentration) and the reaction was monitored at 405 nm at 37 °C. The reading obtained after 20 min of reaction in the absence of extract was taken as 100% activity and compared with the values in the presence of the extracts. Control experiments were performed by incubating *L. muta* venom with DMSO (1% v/v, final concentration) or saline.

Antihemorrhagic activity

Hemorrhagic lesions produced by *L. muta* venom were quantified by using a procedure described by Kondo et al. (1960) with minor modifications (Moura et al., 2010). Briefly, samples were injected intradermally (*i.d.*) into the abdominal skin of mice. Two hours later, the animals were euthanized and the abdominal skin removed, stretched and inspected for visual changes in the internal aspect in order to localize hemorrhagic spots. Hemorrhage was quantified as the Minimum Hemorrhagic Dose (MHD), defined as the amount of venom (μg/g) able to produce a hemorrhagic halo of 10 mm (Nikai et al., 1984). The inhibitory effect of *S. schröederi* extracts was investigated by incubating extracts with one MHD for 30 min at room temperature and the mixture was then injected into mice and hemorrhage measured. Hemorrhagic activity was expressed as the mean diameter (in millimeters) of the hemorrhagic halo induced by *L. muta* venom in the presence or in the absence of *S. schröederi* extracts. Negative control experiments were performed by injecting DMSO or saline.

Antiproteolytic activity

The proteolytic activity of *L. muta* venom was determined using azocasein as substrate (0.2% w/v, in 20 mM Tris-HCl, 8 mM $CaCl_2$, pH 8.8), with minor modification (Garcia et al., 1978; Moura et al., 2010). An effective concentration (EC) was defined as the amount of *L. muta* venom (μg/mL) able to produce a variation of about 0.2 OD units at 420 nm. *S. schröederi* extract or DMSO alone were incubated with two EC of venom for 30 min at room temperature and then proteolysis was measured.

Statistical analysis

Results are expressed as the mean±SEM obtained with the indicated number of animals or experiments performed. The statistical significance of differences among experimental groups was evaluated using the Student's *t* test and *p* values of ≤0.05 were considered statistically significant.

Results and Discussion

Bioprospection studies with marine organisms for the identification of active molecules are growing and different types of substances with high biotechnological and pharmacological potential have been isolated from them (Pereira et al., 2005; Mayer et al., 2011; Cirne-Santos et al., 2008; Abrantes et al., 2010; Bianco et al., 2009a, b). Previous studies showed the ability of dolastane and secodolastane diterpenes isolated from *C. cervicornis* marine brown alga to inhibit hemolytic, proteolytic, hemorrhagic and clotting activities of *L. muta* venom (Moura et al., 2010; Domingos et al., 2011). We have now evaluated the ability of *S. schröederi* algal extracts prepared with solvents of increasing polarities (*n*-Hexane, dichloromethane, ethyl acetate) to neutralize the hemolytic, proteolytic, hemorrhagic and clotting activities of crude *L. muta* venom.

The *S. schröederi* extracts (90-900 μg/mL) inhibited the hemolysis induced by *L. muta* venom (3.6 μg/mL) or LM-PLA_2-I (9.0 μg/mL) in a concentration-dependent manner, but with potencies that varied according to the extraction solvent (Table 1). The extracts of alga prepared in dichloromethane at a 1:25 venom:alga ratio (w/w) inhibited 8.5% and 100% of the hemolysis induced by *L. muta* venom or LM-PLA_2-I, respectively (Table 1). A 100% inhibition of hemolysis was also observed for extracts of alga prepared in ethyl acetate, but not for the hexane ones. When compared to LM-PLA_2-I, extracts of alga inhibited the hemolysis induced by *L. muta* venom less well (Table 1). Phospholipase A_2 (PLA_2) enzymes are considered to be the most active pharmacological component in snake venoms (De Paula et al., 2009). PLA_2 enzymes are involved in prey digestion and also produce a wide range of pharmacological and toxic effects such as hemolysis, neurotoxicity, cardiotoxicity, effects on platelet aggregation, myotoxicity, and edema, that often contribute to the envenomation symptoms (Gutiérrez & Ownby, 2003; Otero et al., 2000). *L. muta* venom contains PLA_2 enzyme isoforms (Damico et al., 2008), one of which, denoted LM-PLA_2-I, has been isolated (Fuly et al., 2002). As shown in Table 1, at a 1:25 venom:alga ratio, the ethyl acetate extract inhibited 30% and 60% of the hemolysis induced by *L. muta* venom or LM-PLA_2-I, respectively. Curiously, at higher ratios (1:50 or 1:100), no inhibitory effect was achieved for *L. muta* venom. In contrast, at such ratios, the hemolysis induced by LM-PLA_2-I was totally inhibited (Table 1). At higher extract concentrations, less inhibition was observed for *L. muta* venom. At any concentration, *S. schröederi* extracts or DMSO did not induce hemolysis and DMSO did not interfere with the degree of hemolysis of *L. muta* venom or LM-PLA_2-I (data not shown).

As shown in Figure 1A, proteolysis caused by *L. muta* venom (0.75 μg/mL) was inhibited by different concentrations of *S. schröederi* extracts (18.75, 37.5 and 75 μg/mL). The dichloromethane and ethyl acetate

extracts presented the highest inhibitory effect, from 90 to 100% at the three venom:alga ratios (1:25, 1:50 and 1:100). The *n*-hexane extract inhibited proteolysis from 10 to 20% (Figure 1A, group 1). *In vivo* assays showed that intradermal injection of one MHD of *L. muta* venom (1.2 µg/g) produced a hemorrhage halo of about 10 mm in mice (data not shown). Figure 1B shows that only the extract prepared in ethyl acetate fully protected mice from hemorrhage caused by *L. muta* venom, while the *n*-hexane and dichloromethane extracts inhibited 4% and 40%, respectively (Figure 1B). The animals that received saline or *S. schröederi* extracts showed no hemorrhagic halo (data not shown). We suggest that the inhibitory mechanism of action of *S. schröederi* extracts on hemorrhagic activity could occur through an interaction between compounds present in the alga and the catalytic sites of the metalloproteases of venom or, alternatively, such algal compounds might chelate metal ions (Zn^{2+}) that are essential for the enzymatic activity of the metalloproteases. Envenomation by *L. muta* venom usually produces hemorrhage due to the degradation of blood vessels or the consumption of fibrinogen or other blood clotting factors, thus preventing clot formation (Markland, 1998). Moreover, *L. muta* venom is a procoagulant. Hemorrhage or clotting effects are associated with specific protease groups, the metalloprotease and serine protease. A large number of bioactive molecules with anticoagulant activity have been described in marine organisms (Jurd et al., 1995; Lee et al., 1998; Mayer et al., 2011). These molecules are regularly produced via their primary or secondary metabolism, leading to the formation of polysaccharides (Camara et al., 2011) or diterpenes (Moura et al., 2010, 2011), respectively. Rocha et al. (2005) isolated polysaccharides from *S. schröederi* with potent *in vivo* antithrombotic effect upon venous thrombosis.

As observed in Figure 2A, even at the highest concentration (62 µg/mL) the three extracts of *S. schröederi* did not inhibit plasma clotting induced by *L. muta* venom (0.62 µg/mL), but at the same concentration the ethyl acetate extract inhibited clotting of commercial fibrinogen (Figure 2B). Hence, we infer that *S. schröederi* compounds somehow interfere with the serine protease enzymes of venom. DMSO did not affect the clotting induced by *L. muta* venom (Figure 2A, B). Thrombin, a serine protease enzyme, is a pivotal enzyme in the human clotting system. It is responsible for generating thrombus through the cleavage of fibrinogen, leading to formation of a fibrin net. Thrombin also induces platelet aggregation and may active other blood clotting factors. These effects are mediated by two distinct sites, a catalytic and a pharmacological one. To test the catalytic activity of thrombin or thrombin-like enzymes, a chromogenic substrate, S-2238, is often used. We therefore evaluated the ability of *S. schröederi* extracts to inhibit the hydrolysis of S-2238. Figure 3 shows that all extracts prevented hydrolysis by *L. muta* venom, suggesting that the anticlotting effect of *S. schröederi* extracts upon fibrinogen might be associated with thrombin-like enzymes present in *L. muta* venom. Again, the ethyl acetate extract proved to be the most powerful, since at 1:50 or 1:100 venom:alga ratios a 100%-inhibition of the hydrolysis of S-2238 (Figure 3) or fibrinogen clotting (Figure 2) was seen.

Table 1. Antihemolytic effect of extracts of *S. schröederi* on *L. muta* venom or LM-PLA_2-I.

Venom source (µg/mL)	Solvent	Algal concentration (µg/mL)	% Inhibition of hemolysis
L. muta	*n*-hexane	90	42±0.7
		180	33±3.5
		360	3.5±0.7
	dichloromethane	90	8.5±2.8
		180	39±0.1
		360	0
	ethyl acetate	90	30±0.1
		180	0
		360	0
LM-PLA2-I	*n*-hexane	90	0
		180	0
		360	0
	dichloromethane	90	100
		180	100
		360	100
	ethyl acetate	90	62±5
		180	100
		360	100

L. muta venom (3.6 µg/mL) or LM-PLA_2-I (9.0 µg/mL) was incubated with *S. schröederi* extracts at 1:25, 1:50 or 1:100 ratio (w/w) for 30 min at room temperature. Then, the hemolytic test was evaluated and percentage of inhibition of hemolysis analyzed. Data are expressed as mean ± SEM of individuals experiments (n=3).

Based on our results, the polarity of the solvents (*n*-hexane, dichloromethane and ethyl acetate) influenced on the inhibition profile for the biological activities evaluated in this work. The greater the polarity of the extraction solvent, the higher was the inhibitory percentage. This could be clearly observed since the dichloromethane and ethyl acetate extracts neutralized more efficiently all of the biological activities tested. As shown in Figure 4, extracts of *S. schröederi* subjected to TLC revealed heterogeneity in each extract composition. The lanes corresponding to the dichloromethane and ethyl acetate extracts showed the presence of more polar compounds than that of the hexane extract. Such differences in extract composition could explain the different inhibitory profiles

of the extracts; and suggest that polar molecules are responsible for the observed inhibitory effects on *L. muta* biological activities.

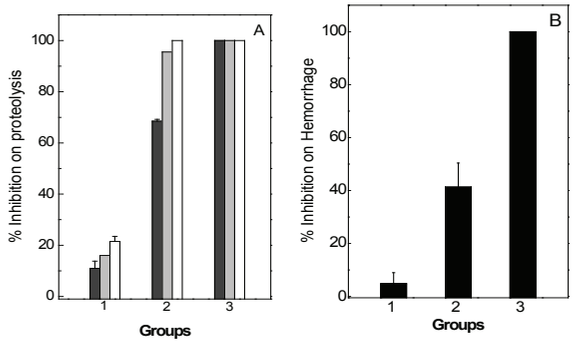

Figure 1. Antiproteolytic and antihemorrhagic effect of *S. schröederi* extracts. A. Inhibitory effect of *S. schröederi* extract at 1:25 (dark gray columns), 1:50 (gray columns) or 1:100 (white columns) venom: alga ratio (w/w) prepared in *n*-hexane (Group 1), dichloromethane (Group 2) or ethyl acetate (Group 3) upon proteolysis induced by *L. muta* venom (0.75 µg/mL). B. Inhibition of hemorrhage induced by *L. muta* venom (1.2 µg/g) in the presence of 18 µg/g of *S. schröederi* extracts prepared in *n*-hexane (Column 1), dichloromethane (Column 2) and ethyl acetate (Column 3). Data are expressed as mean±SEM of three individual experiments (n = 3).

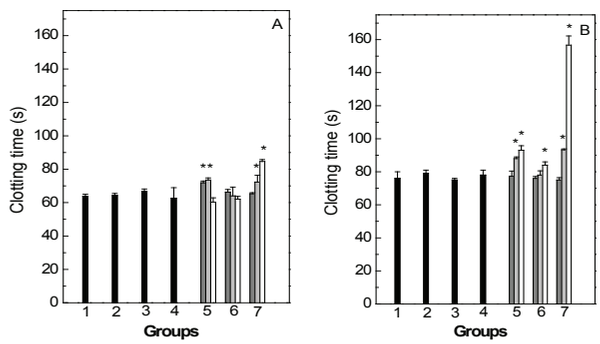

Figure 2. Anticlotting effects of *S. schröederi* crude extracts. Inhibitory effect of *S. schröederi* extracts prepared in *n*-hexane (Group 5), dichloromethane (Group 6) and ethyl acetate (Group 7) on clotting activity of human plasma (Panel A) and fibrinogen (2 mg/mL) (Panel B) caused by *L. muta* venom (0.62 µg/mL) at 1:25 (dark gray columns), 1:50 (gray columns) or 1:100 (white columns) venom:alga ratio (w/w). Columns 1, 2, 3 and 4 represent coagulation induced by *L. muta* venom (0.62 µg/mL) in the presence of 150 mM NaCl or 0.5, 1 and 2% DMSO (v/v, final concentration), respectively. Data are expressed as mean±SEM of three individual experiments (n=3). *Significance level (p<0.05) when compared to column 1 of each panel.

In conclusion, *S. schröederi* extracts may be a promising source of molecules to improve the treatment against *L. muta* snakebites and may be useful for the development of new antiophidian molecules.

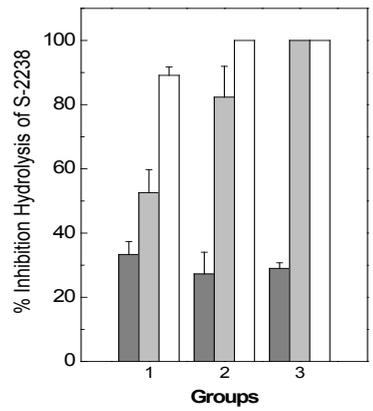

Figure 3. Effects of *S. schröederi* extracts on hydrolysis of S-2238. Inhibitory effect of *S. schröederi* extracts prepared in *n*-hexane (Group 1), dichloromethane (Group 2) and ethyl acetate (Group 3) on S-2238 hydrolysis caused by *L. muta* venom (0.3 µg/mL) at 1:25 (dark gray columns), 1:50 (gray columns) or 1:100 (white columns) venom:alga ratio (w/w). Data are expressed as mean±SEM of two individual experiments (n=2).

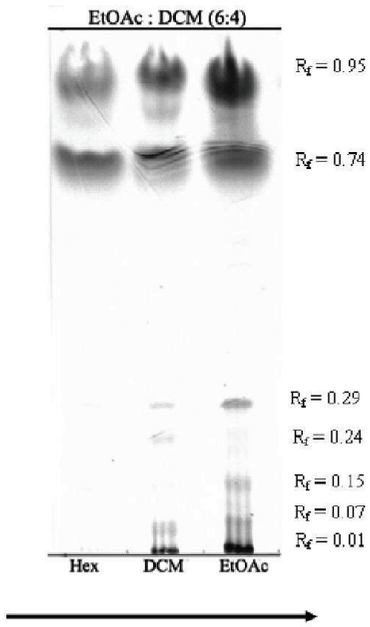

Figure 4. TLC profile of *S. schröederi* extracts on Silica 60 F254 plates eluted with ethyl acetate:dichloromethane (6:4, v/v) and revealed with ceric sulphate. Lanes are: Hex, extract in *n*-hexane; DCM, extract in dichloromethane; EtOAc, extract in ethyl acetate.

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