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CELLULAR AND MOLECULAR BIOLOGY

Preliminary report on the hemagglutinating activity of the *Scorpaena plumieri* fish venom

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Abstract: The scorpionfish *Scorpaena plumieri* is one of the most venomous fish species in the Brazilian coast. Amongst many biological activities, the *S. plumieri* fish venom (SpV) promotes hemagglutination. Although this activity appears to be associated to the presence of C-type lectins in the venom, it has not yet been chemically or functionally characterized. In the present work we sought to advance the characterization of the hemagglutinating activity associated to this venom. By fractionating SpV through saline precipitation followed by size exclusion chromatography we obtained two purified fractions - HF1 and HF3 - with Ca^{2+}-dependent agglutinating activity against rabbit erythrocytes, which remained stable upon storage at 4 and -80°C. HF1 and HF3 were bacteriostatic against Gram-positive bacteria (*Staphylococcus aureus*), displaying minimum inhibitory concentration (MIC) of 50 and 200 µg/mL, respectively. In addition, a resazurin-based viability assay revealed that both fractions, at doses up to 370 µg/mL, were cytotoxic against tumor and non-tumor cell lines. Finally, a tendency towards edema formation could be detected when the fractions - particularly HF1 - were injected into mice footpads. We believe our data contribute to a better understanding of the biological properties of the so often neglected fish venoms.

Key words: *Scorpaena plumieri*, fish venom, hemagglutination, lectins.

INTRODUCTION

A large number of aquatic venomous animals are distributed around the globe, with some of them, such as sponges, cnidarians, cone snails and fish, being associated with human envenomation (Haddad Jr. 2003, Ziegman & Alewood 2015). For that reason, the study of their venoms has gained considerable interest in the scientific community.

However, venoms from aquatic animals - particularly fish - do not usually receive as much attention as those from terrestrial animals, for the latter are encountered more often and perceived as bigger threats to humans (Church & Hodgson 2002, Ziegman & Alewood 2015). Nevertheless, phylogenetic studies reveal that there are around 3000 venomous fish species when ray-finned and cartilaginous fish are pooled together, corresponding to more than half of all venomous vertebrates (Smith et al. 2016).

Despite the wide taxonomic range of venomous fish, the venomous apparatus is similar in most species, consisting of spines usually located in their dorsal region, although pectoral, pelvic, anal and caudal spines are also common (Ziegman & Alewood 2015). The venom-secreting system is located in the spines' anterolateral cavities, and may consist of well-defined glands or more primitive structures, formed by specialized secretory cells. Envenomation occurs when the victim touches

the spines and ruptures the integumentary sheath by mechanical pressure, releasing the venom into the wound (Ziegman & Alewood 2015).

Some fish species produce venoms that may evoke severe local and systemic reactions, sometimes even leading to death (Church & Hodgson 2002, Ziegman & Alewood 2015, Reckziegel. et al. 2015, Campos et al. 2016). Among the local symptoms, excruciating pain, edema and erythema stand out. Systemic symptoms include ischemia, muscle spasms, weakness and nausea, in addition to paralysis of the affected limb, potentially including also hypotension, tachycardia and respiratory distress (Haddad Jr. et al. 2003). This highly complex pathophysiological scenario may temporarily incapacitate the victim (Haddad Jr. 2000), although the extent of the damage varies according to species, number and depth of the perforations, amount of poison released and individual reaction to venom components. Accidents caused by venomous fish can be considered a public health problem, for depending on the clinical outcome victims can take up to weeks to recover (Haddad Jr. et al. 2003).

Almost all venomous fish families and genera have representatives in Brazilian waters, including the genus *Scorpaena* (scorpionfish), considered the most dangerous in the Atlantic Ocean (Haddad Jr. et al. 2003). The black scorpionfish *Scorpaena plumieri* (Figure 1a), known in Brazil as mangangá, niquim-de-pedra or mamangava, is one of the most venomous fish in the Brazilian coast, being responsible for accidents involving mostly fishermen and unadvised tourists (Haddad Jr. et al. 2003). It usually dwells in shallow waters and reefs, disguised among rocks and plants (Humann 1994, Campos et al. 2016). Specimens reach up to 50 cm and display 12 dorsal, 2 pelvic and 3 anal short and thick mucus-covered fin spines (Figure 1b-d), which harbor primitive venom-producing glands (Moyle & Cech 1996, Haddad Jr. et al. 2003). In spite of not being lethal to humans, envenomation by *S. plumieri* can cause severe local inflammation and systemic cardio-respiratory symptoms, with intense pain having been reported by all victims (Haddad Jr. et al. 2003). Treatment usually consists of palliative measures to relieve symptoms, with the immersion of the affected area in hot water

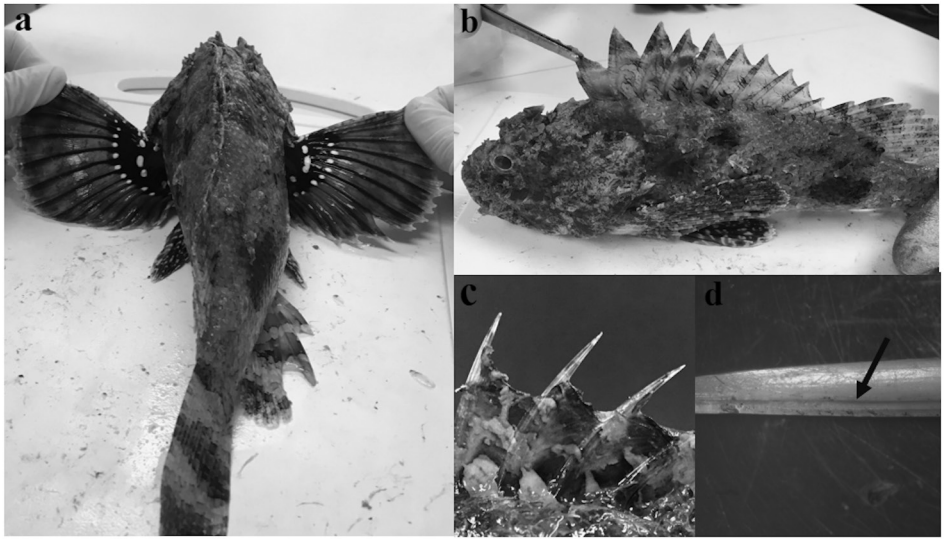

Figure 1. The scorpionfish *Scorpaena plumieri*. (a) Top view highlighting a characteristic feature of the species - white spots against a black background on the axillary part of the pectoral fins. (b) Side view of the specimen. (c) Side view of dorsal spines covered by integumentary sheath. (d) Magnified image of a spine: the black arrow indicates the anterolateral groove.

(typically about 45°C) being highly effective against the pain (Haddad Jr. et al. 2003).

Although accidents involving scorpionfish might not be as frequent as, for instance, those caused by stingrays (Haddad Jr. 2003), they can still have socioeconomic consequences. That, along with a yearning to understand the mechanisms underlying the aforementioned symptoms, drives the effort towards a thorough characterization of the activities of *S. plumieri* venom and the bioactive molecules that comprise it.

Our research group described a number of biological activities of *S. plumieri* venom. (for review see Campos et al. 2016), which was found to (i) be lethal to mice at low doses (Carrijo et al. 2005), (ii) promote dose-dependent hemolysis of rabbit erythrocytes (Carrijo et al. 2005), (iii) have proteolytic activity against gelatin, casein and fibrinogen (Carrijo et al. 2005, Borges et al. 2018), (iv) inhibit the specific binding of integrins to their ligands (Evangelista et al. 2009), (v) induce an inflammatory reaction in mice footpads, accompanied by a nociceptive response (Menezes et al. 2012), and (vi) promote - at high doses - a biphasic response in mean arterial blood pressure and isolated aortic rings, as well as a dose-dependent increase in heart rate of Wistar rats (Gomes et al. 2010, Andrich et al. 2010).

But there are still questions to be answered regarding some of the biological activities of *S. plumieri* venom. For instance, one of the protein fractions obtained through the fractionation of this venom by size exclusion chromatography displayed marked hemagglutinating activity against rabbit erythrocytes (Andrich et al. 2015, Campos et al. 2016). Ample evidence points to lectins - carbohydrate-specific binding proteins - as the molecules responsible for this effect (Kilpatrick 2002, Sharon & Lis 2004). Five isolectins (Sp-CL 1-5) were isolated from the hemagglutinating fraction of *S. plumieri* venom in a non-native, inactive form, having been classified as C-type lectins based on homology with other such molecules (Andrich et al. 2015). It stands to reason that Sp-CLs should account for the hemagglutinating activity associated to this venom, although that could not be confirmed when these proteins were first isolated (Andrich et al. 2015).

Thus, in the present work we sought to develop a robust purification strategy to obtain large quantities of active *S. plumieri* hemagglutinating fractions, in order to advance the characterization of the hemagglutinating activity associated to this venom. We believe that to be an essential step towards a better understanding of how this activity contributes to the outcome of envenomation by fish venoms.

MATERIALS AND METHODS

Animals and ethical aspects

Live specimens of *S. plumieri* were collected from seashore beaches in the city of Fundão (Espírito Santo state, Brazil) and kept in oxygenated seawater until venom extraction. Captures were authorized by the Brazilian Public Agency for Environment Affairs (IBAMA).

Male Swiss mice were provided by the animal care facility from the Federal University of Espírito Santo (UFES) and kept in temperature-controlled conditions (22-24°C) under a 12 h dark/light cycle with food and water *ad libitum*.

Rabbit blood samples (up to 2 mL) were obtained through auricular artery puncture following the application of topical anesthetic (lidocaine). Mice blood samples were kindly supplied by the Neurochemistry and Behavior Laboratory at UFES.

All procedures were conducted in accordance with the guidelines for the care and use of laboratory animals, as indicated by the

Brazilian Society of Laboratory Animal Science, having been approved by our local Ethics Committee for the Use of Animals (CEUA/UFES) under protocol number 093/2015.

Venom extraction and purification of hemagglutinating fractions

Venom samples were extracted from dorsal and anal spines in 100 mM phosphate buffer with 150 mM NaCl (pH 7.4) at 4°C (PBS), as described by Gomes et al. 2013. Hemagglutinating fractions were purified from the crude venom extract - henceforth referred to as SpV (*Scorpaena plumieri* Venom) - in the two steps briefly described below. The purification process was monitored by the hemagglutinating activity in rabbit erythrocytes as described in section 2.4.

First, agglutinant proteins were obtained from SpV by ammonium sulfate precipitation (15% w/v) at 4°C followed by centrifugation at 30.000 *g* for 30 minutes at 4°C. The precipitate, denominated P15, was resuspended in chilled PBS and loaded into a size exclusion Superose 12 column (1.0 x 30 cm, GE Healthcare) connected to a High-Performance Liquid Chromatography (HPLC system (LC-20AT, Shimadzu). The column was equilibrated and eluted with PBS at a flow rate of 0.3 mL/min and the process monitored by absorbance at 218 nm.

Protein content estimation and homogeneity evaluation

Protein content of SpV and purified hemagglutinating fractions was determined by the Lowry method (Lowry et al. 1951) with bovine serum albumin as standard. The homogeneity of active fractions obtained in the purification steps was evaluated by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE, 10%) according to Schägger & von Jagow 1987. Gels were stained with Coomassie Brilliant Blue G-250 and a protein ladder from 19 to 104 kDa (BioRad) was used as molecular weight standard.

Hemagglutinating activity assay

Hemagglutination was evaluated using washed erythrocytes suspension (2.0% v/v in PBS) according to Habermann et al. 1981, with few modifications. We began by filling a multi-well U-bottom plate with 100 µL of PBS; purified protein fractions (in 100 µL) were then added to the first column of wells and a twofold serial dilution took place. Next, we added 50 µL of the 2% erythrocytes solution and kept the plate at 4°C for 24 hours. The minimum concentration of purified fractions required for hemagglutination to be clearly visualized was defined as Minimum Hemagglutinating Concentration (MHC). For the assays described in sections 2.5 and 2.6 we used a single dose of active fractions. Rabbit erythrocytes were used for all assays, except for the one in which the specificity of the hemagglutinating activity was assessed.

Thermal stability of the hemagglutinating activity

The stability of the hemagglutinating activity was assessed after active fractions (10 µg) obtained in the chromatographic purification step were kept in different temperature conditions, namely, room temperature, 4°C, -20°C and -80°C, for 7, 30 and 60 days, as well as following lyophilization. The activity was assayed as described in section 2.4 and the results compared to those obtained immediately after the fractions had been eluted (considered 100%).

Carbohydrate-specificity and Ca^{2+}-dependency of the hemagglutinating activity

The hemagglutinating activity was assayed in the presence of monosaccharides and disaccharides (galactose, mannose, fructose, sucrose and lactose), and ethylenediaminetetraacetic acid

(EDTA). Active fractions (10 µg) obtained in the chromatographic step were previously incubated at 4°C under gentle shaking with 5, 15 or 30 mM of each carbohydrate or up to 5 mM of EDTA, in a final volume of 100 µL. The assay then proceeded as described in section 2.4, minus the serial dilution. Negative controls consisted of fraction-free trials containing only PBS, erythrocytes and carbohydrates or EDTA.

Antibacterial activity of hemagglutinating fractions

Antibacterial activity of hemagglutinating fractions was evaluated through determination of the minimum inhibitory concentration (MIC) by serial dilution, as previously described by Wiegand 2008. The assay was carried out in 96-well U-bottom sterile plates.

Gram-positive *Staphylococcus aureus* (ATCC 6538) and Gram-negative *Escherichia coli* (ATCC 25922) bacterial strains were cultured on blood agar (Fluka Analytical) and the initial inocula adjusted to match 0.5 in the McFarland scale, to ensure confluent growth. The highest concentration of active fractions employed in this assay was 200 µg/mL in 90 µL Mueller Hinton Broth 2 (Fluka Analytical), to which were added 10 µL of bacterial inocula. Positive and negative controls consisted of bacterial inocula in broth and broth only, respectively. The plates were incubated at 37°C in 5% CO_2 for 24 hours and MIC determined through the lowest concentration that inhibited visible bacterial growth. The result was confirmed by end-point absorbance reading at 620 nm in a microplate reader (Anthos, Biochrom).

To determine if the fractions were bactericidal, 10 µL of mixtures that did not show any growth after the previous step were sub-cultured on blood agar plates for 24 hours at 37°C. Minimum bactericidal concentration (MBC) was then assessed through the absence of visible bacterial colonies after the incubation period. The assay was conducted in duplicates.

Cytotoxic activity of hemagglutinating fractions

Cytotoxicity was evaluated through the resazurin-based cell viability assay using the following cell lines: human embryonic kidney (HEK293), Chinese hamster ovary (CHO) and murine macrophages (RAW264.7) as non-tumor control cells; mouse neuroblastoma (Neuro2A), rat glioma (C6), human colon carcinoma (RKO) and human cervical carcinoma (HeLa) as tumor cells. All cells were incubated with Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (Sigma Aldrich) and 1% Glutamax (Gibco) at 37°C, 5% CO_2.

Cell suspensions (135 µL, 2 x 10^5 cell/well) were incubated in 96-well flat-bottom sterile microplates with 25 or 50µg of active hemagglutinating fractions for 48 hours at 37°C, 5% CO_2. Negative controls consisted of cell suspensions with PBS only. Next, 15 µL resazurin was added to the wells at a final concentration of 100 µM and the plates further incubated for 4-6 hours at the same conditions described above. Readings were performed at 570 and 600 nm in a microplate reader (Biotek). Controls consisted of incubation medium containing the samples with resazurin only. All assays were conducted in triplicates.

Edema-inducing activity of hemagglutinating fractions

Male Swiss mice (30-40g) had their right footpads injected (intraplantar route, i.pl.) with 100 µg samples of active fractions contained in 30 µL PBS. Control animals received 30 µL of vehicle only. Edema formation was evaluated through the difference in paw thickness, measured with

a digital caliper (Zaas Precision) before and 30 minutes after the injection.

Results were expressed as percentage increase of paw thickness (mean ± SEM, N = 6). Statistical significance was evaluated trough one-way analysis of variance (ANOVA) and statistical significance set at $p < 0.05$.

RESULTS AND DISCUSSION

Fish venoms are complex mixtures of bioactive compounds, *e.g.*, high molecular weight protein lethal factors, enzymes, lectins and various non-proteinaceous molecules, such as neurotransmitters and biogenic amines (for review see Ziegman & Alewood 2015). Although cytotoxic lethal factors account for most symptoms observed upon envenomation and under experimental conditions (Khoo 2002, Church & Hodgson 2002, Ziegman & Alewood 2015, Campos et al. 2016), this richness translates into many biological actions that could contribute to the effects of these venoms.

In the present study we sought to advance the characterization of the hemagglutinating activity associated to the *S. plumieri* fish venom. In order to do so, we had first to develop a robust purification protocol that would allow us to obtain enough quantities of hemagglutinating active fractions, and, if possible, to unveil the molecules responsible for this activity.

We began the purification process by submitting 121 mg of crude venom extract (SpV) to a precipitation step with 15% (w/v) ammonium sulfate. The resuspended precipitate, referred to as P15, displayed marked hemagglutinating activity against rabbit erythrocytes, while the supernatant contained no such activity. This hemagglutinating fraction, which showed minimum hemagglutinating concentration (MHC) of 0.4 µg/mL, corresponded to = 22% of the total protein content of SpV (Table I).

The fraction P15 was then used to screen for the specificity of the hemagglutinating activity as to species. While P15 clearly agglutinated rabbit erythrocytes, its effect in mouse erythrocytes was much less pronounced (not shown). The specificity of the hemagglutinating activity associated to animal venoms appears to be rather random. For instance, BlL, a C-type lectin isolated from the venom of the snake *Bothrops leucurus*, agglutinated mainly rabbit erythrocytes, much like our own results (Nunes et al. 2011). On the other hand, Nattectin, a C-type lectin from *Thalassophryne nattereri* fish venom, agglutinated human A-type erythrocytes (Lopes-Ferreira et al. 2011) and BpLec from *Bothrops pauloensis* snake venom to dog and cat erythrocytes (Castanheira et al. 2013). This broad species-specificity is most likely due to different cells expressing different types of types of membrane glycoproteins. As the

Table I. Purification of *S. plumieri* hemagglutinating fractions.

	PROTEIN (mg)	RECOVERY (%)	MHC**
SpV	121 mg	100%	Not determined
P15	27 mg	22%	0.40 µg/mL
HF1	0.64 mg	0.52%	0.02 µg/mL
HF3	0.90 mg	0.74%	0.50 µg/mL

***MHC = Minimum Hemagglutinating Concentration.**

hemagglutinating activity of SpV was more pronounced in rabbit erythrocytes, further assays were conducted using only these cells.

For the next purification step, P15 samples (= 1 mg) were loaded into a Superose 12 column and underwent size exclusion chromatography. Although this step yielded six major protein fractions (F1 - 6) (Figure 2), only fractions 1 and 3 displayed significant hemagglutinating activity (Figure 2, insert), being henceforth referred to as Hemagglutinating Fractions 1 and 3 (HF1 and HF3). These fractions corresponded to = 0.52% and = 0.74% of the total protein content of SpV, displaying MHC values of 0.02 (20-fold higher than that of P15) and 0.5 µg/mL (same level of P15), respectively (Table I). Although this parameter cannot be established for the crude venom - for the strong hemolytic activity displayed by freshly-extracted SpV masks hemagglutination -, the yields obtained with P15, HF1 and HF3 attest to the efficiency of the purification protocol employed here (Table I).

Tricine-SDS-PAGE (10%) analysis of SpV and purified samples revealed high molecular weight (MW) protein bands (=100 kDa) in HF1, along with a faint = 45 kDa band. In HF3, intense low MW bands (= 19 and 5 kDa) were observed alongside the heavier ones (Figure 3). The same low MW bands were observed in the electrophoretic separation of F4, which did not show significant hemagglutinating activity (Figure 2). That could be interesting, as mass spectrometry analysis revealed that C-type isolectins isolated from SpV had molecular masses around 16-17 kDa (Andrich et al. 2015), values close to those observed for the low MW bands of HF3 and F4.

There is evidence pointing to C-type lectins having homo-oligomeric structures, being active when forming large supramolecular arrays (Eble 2019). Although the isolectins isolated from SpV showed sequence homology with C-type lectins from other venoms, they did not display hemagglutinating activity (Andrich et al. 2015). That could be the result of them having been obtained through reverse phase chromatography, a non-physiological method that could hinder the formation of oligomers, most likely yielding inactive monomers. This hypothesis was further supported by the mass spectrum of the fraction containing monomeric forms of the isolectins having revealed also m/z signals multiples of 16-17 kDa, *i.e.*, 34, 51 and 68 kDa (Andrich et al. 2015). Taken together, these data, along with the protein profiles of the hemagglutinating fractions obtained in the present work, point to HF1 and HF3 containing

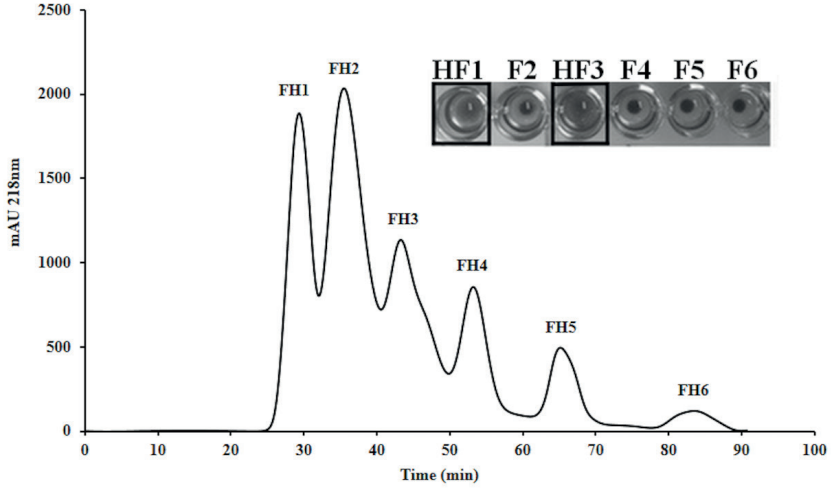

Figure 2. Elution profile of the size exclusion fractionation of P15. A sample of P15 (1 mg) was loaded into a Superose 12 column (1.0 x 30 cm) previously equilibrated and eluted with 100 mM sodium phosphate buffer with 150 mM NaCl, pH 7.4. Protein was eluted at a flow rate of 0.3 mL/min and monitored by absorbance at 218nm. Insert: representative image of three independent experiments displaying the hemagglutinating activity against rabbit erythrocytes of fractions obtained straight after elution.

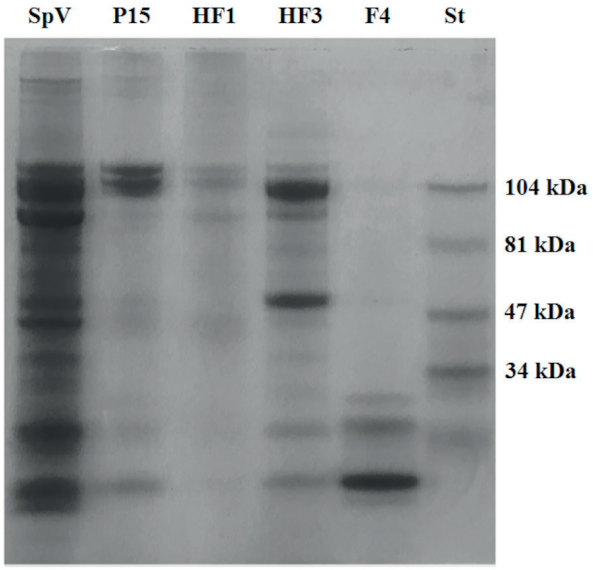

Figure 3. Electrophoretic protein profile (tricine-SDS-PAGE) of SpV and hemagglutinating fractions under non-reducing conditions. Profile obtained with a 10% gel stained with coomassie brilliant blue. Samples: µ 50 g SpV and 10-20 µg of purified fractions (P15, HF1, HF3 and F4). St: molecular weight markers.

- in addition to other proteins - oligomeric forms of the C-type lectins present in SpV. One could, therefore, suggest that in HF1 the monomers would be aggregated into hexamers and trimers, corresponding, respectively, to the = 100 and 47 kDa bands observed in this fraction's protein profile, explaining why HF1 was more active than HF3 and why F4 was not active at all

As carbohydrate-binding proteins, C-type lectins may have their activity on erythrocytes inhibited by the presence of specific sugar molecules on the agglutinating assay (Nunes et al. 2011, Castanheira et al. 2013). None of mono- and disaccharides (galactose, mannose, fructose, sucrose and lactose, at 5 to 30 mM) tested in this work inhibited the hemagglutinating activity of HF1 and HF3 (not shown). That was unexpected, as the isolectins purified from SpV had been previously shown to bind weakly to galactose (Andrich et al. 2015). The interaction between lectins and carbohydrates appears to depend on monosaccharides being arranged into more complex oligosaccharides. For instance, Plumieribetin - a B-type lectin from SpV - interacted weakly with single mannose residues, but strongly with a linear tetramannan (Evangelista et al. 2009). Thus, our results most likely reflect extremely low sugar-binding ability and specificity of HF1 and HF3 for the non-complex carbohydrates evaluated here.

C-type lectins usually depend on Ca^{2+} to perform their biological activities (for review see Eble 2019). The Ca^{2+}-chelating agent EDTA partially attenuated (50%) the hemagglutinating activity of both HF1 and HF3, showing that it depends on divalent cations (Figure 4). Similar outcomes have been reported for the hemagglutinating activity of other venoms (Carvalho et al. 1998, Castanheira et al. 2013). Although by no means conclusive, that strongly indicates that C-type lectins could indeed participate in the hemagglutinating response elicited by SpV.

One of the features that make the study of fish venoms so challenging is the lability of some of its bioactive components, and, by proxy, of their related activities (Church & Hodgson 2002, Ziegman & Alewood 2015). In fact, some of the major activities of SpV, *i.e.*, hemolytic, inflammatory, nociceptive and cardiovascular, are extremely labile (Andrich et al. 2010, Menezes et al. 2012, Gomes et al. 2016, Malacarne et al. 2018). On the other hand, the hemagglutinating activity was fully preserved when HF1 and HF3 solutions were lyophilized or kept at 4°C and -80°C for up to 30 days (not shown). These results did not come as a surprise, for the hemagglutinating activities associated to other animal venoms were reported to be quite thermostable (Nunes et al. 2011, Castanheira et al. 2013).

Animal venoms can have antimicrobial activity, which appears to be related to the presence of hemagglutinating fractions

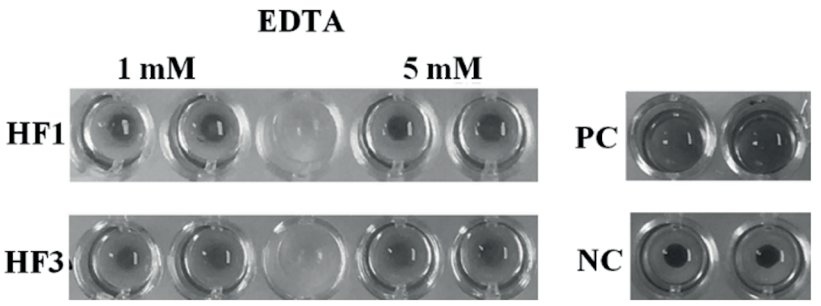

Figure 4. Evaluation of the hemagglutinating activity in the presence of EDTA. Active fractions (10 µg) were incubated at 4°C for 4h with 1 and 5 mM EDTA. Rabbit erythrocytes (2% in PBS v/v) were added after incubation and the plate kept at 4°C for 24h. Positive controls (PC): P15 samples (10 µg); negative control (NC): PBS plus erythrocytes only.

(Rádis-Baptista et al. 2006, Barbosa et al. 2010, Nunes et al. 2011, Castanheira et al. 2013). The venom of *S. plumieri* exhibited antibacterial activity against Gram-positive bacteria (*S. aureus*), while Gram-negative ones (*E. coli*) were less affected, and the proteomic analysis of *S. plumieri* venom revealed protein fragments homologous to C-type lectins (Borges et al. 2018). We then asked ourselves if the purified hemagglutinating fractions were capable of reproducing the antibacterial activity displayed by SpV. Both fractions exhibited antibacterial activity against *S. aureus*, with MIC values of 50 µg/mL and 200 µg/mL, respectively, while, at the same doses, neither was capable of visibly affecting the growth of *E. coli*. Moreover, when analyzing minimum bactericidal concentrations (MBC), we observed that the Gram-positive bacteria were able to grow upon sub-culturing regardless of HF1 and HF3, showing that these fractions are actually bacteriostatic, rather than bactericidal. That HF1 and HF3 were not able to perfectly reproduce the bactericidal effect associated to SpV can be easily explained by the crude venom containing additional compounds that could act on microorganisms.

The local inflammatory reaction associated to SpV, usually characterized by edema (Haddad et al. 2003, Menezes et al. 2012), is at least partially evoked by the *S. plumieri* multifunctional cytolysin - Sp-CTx (Malacarne et al. 2018). Other molecules from animal venoms, *e.g.*, C-type lectins, can act as pro-inflammatory agents (Saraiva et al. 2011, Ishizuka et al. 2012, Dias-Netipanyj et al. 2016), even causing paw edema in mice (Panunto et al. 2006). Thus, we conducted a preliminary assay to assess the edematogenic response of HF1 and HF3 in mice footpads. Although we did not detect a statistically significant difference between edema formation in animals receiving HF1 or HF3 (100 µg/paw) when compared to control ones (Figure 5), a clear tendency towards an increase in paw thickness could be observed, particularly following HF1 injection ($p = 0.08$). That could be simply a result of the high variability observed in the measures, hence future experiments must be conducted with a larger mice sampling, as well as different doses of the purified fractions.

From a therapeutic point of view, the ability to inhibit tumoral growth is one the most relevant biological activities of venom lectins. Snake venom lectins exhibit several antitumoral effects, *e.g.*, BlL from *B. leucurus* venom induced apoptosis in tumor cells through mitochondrial depolarization and permeability alterations (Nunes et al. 2012, Aranda-Souza et al. 2014), as did BjcuL from *B. jararacussu* (Damasio at al. 2014). In preliminary experiments, we found signs that SpV can inhibit the growth of tumor cell lines by inducing apoptosis (unpublished data). Thus, to verify if this activity could be linked to the action of hemagglutinating fractions, we conducted a resazurin-based cell viability assay with both tumor and regular cell lines. While HF3 (185 and 370 µg/mL) was cytotoxic to

all tested cell lines, except for the tumoral line Neuro2A (Figure 6b), HF1 affected only HEK293, RAW and HeLa cells in a relevant way (Figure 6a). Therefore, the cytotoxic effects evoked by the hemagglutinating fractions - particularly HF3 - appear to be broad rather than tumor-specific. That could be the result of HF3 and HF1 containing toxic molecules other than lectins, although a thorough evaluation upon the incubation of various cell lines with different doses of these fractions should be conducted in future investigations.

In conclusion, in the present study we advanced the characterization of the hemagglutinating activity associated to the *S. plumieri* venom, by developing a purification protocol that allowed us to obtain large quantities of active hemagglutinating fractions. This Ca^{2+}-dependent activity was stable when the purified fractions were kept at different temperatures. We successfully associated the hemagglutinating fractions to some of the antibacterial and cytotoxic effects exhibited by the crude venom, and, although that needs further investigation, their roles as pro-inflammatory molecules could at least be hinted at. We believe our results contribute to a better understanding of the biological properties of

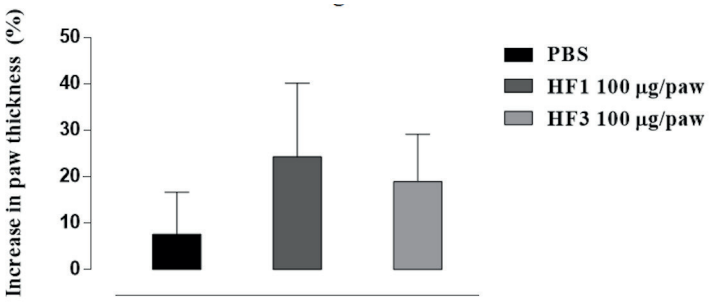

Figure 5. Edema-inducing activity of hemagglutinating fractions. HF1 and HF3 (100 µg in 30 µL) were injected (i.pl.) into the right footpads of Swiss mice. Control animals received 30 µL of PBS. Results were expressed as percentage increase of paw thickness (mean ± SEM, N = 6) and differences were considered significant when $p < 0.05$.

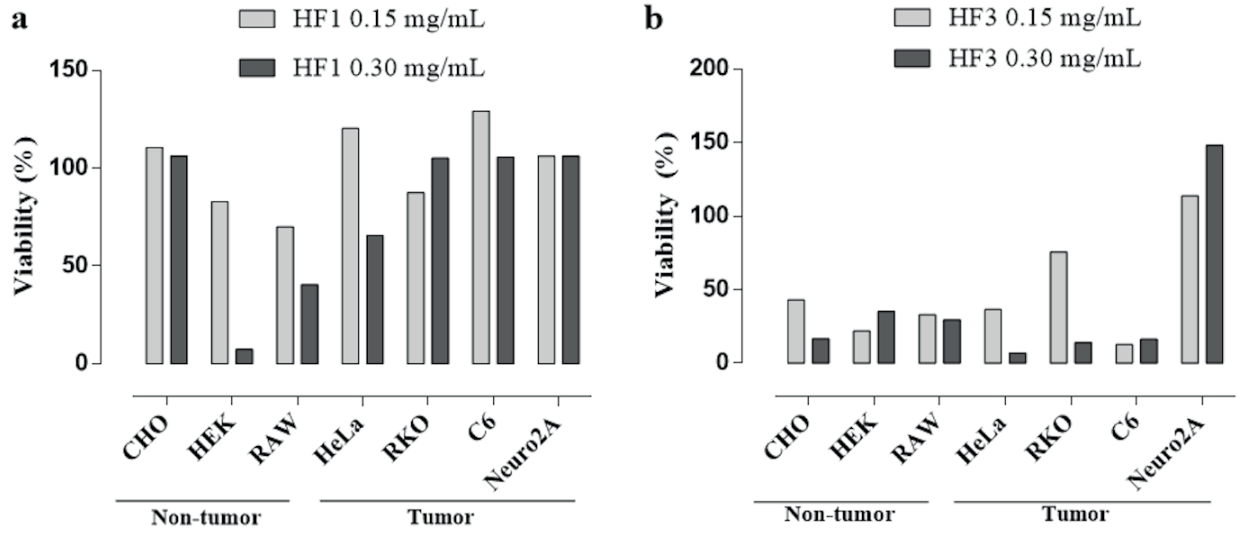

Figure 6. Cytotoxic activity of hemagglutinating fractions. Cell viability of regular (CHO, HEK293, RAW264.7) and tumor (HeLa, RKO, C6, Neuro2a) cell lines assayed through resazurin-based colorimetric assay in the presence of 25 or 50 µg of both HF1 (a) and HF3 (b). Results represent the average of triplicate readings (optical density at 570 and 600 nm) and are expressed as percentage of viability (%) compared to controls (cell suspensions in PBS), which were considered 100%.

the so often neglected fish venoms, paving the way for future studies.

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HBF conducted experiments, analyzed results and contributed to the discussion; TGS conducted experiments and analyzed results; AM designed and conducted experiments, analyzed results and contributed to the discussion; MHB designed experiments and contributed to the discussion; FVC designed experiments, contributed to the discussion and wrote the manuscript; SGF designed the study, conducted experiments and contributed to the discussion.