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A sulfated polysaccharide, fucans, isolated from brown algae *Sargassum vulgare* with anticoagulant, antithrombotic, antioxidant and anti-inflammatory effects

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ABSTRACT

Fucan (SV1) sulfated polysaccharides from the brown algae *Sargassum vulgare* were extracted, fractionated in acetone and examined with respect to chemical composition, anticoagulant, anti-inflammatory, antithrombotic effects and cellular proliferation. These polysaccharides contain low levels of protein, high level of carbohydrate and sulfate. Monosaccharides analysis revealed that SV1 was composed of fucose, galactose, xylose, glucuronic acid and mannose. SV1 polysaccharide prolonged activated partial thromboplastin time (aPTT) and exhibited high antithrombotic action *in vivo*, with a concentration ten times higher than heparin activity. PSV1, a purified form in gel filtration showed very low biological activities. SV1 stimulated the enzymatic activity of FXa. Its action on DPPH radical scavenging activity was 22%. This polymer has no cytotoxic action (hemolytic) on ABO and Rh blood types in different erythrocyte groups. It displays strong anti-inflammatory action at all concentrations tested in the carrageenan-induced paw edema model, demonstrated by reduced edema and cellular infiltration.

1. Introduction

Seaweeds have been the focus of growing interest in the biomedical area, mainly due to their bioactive substances, which show great potential for anti-inflammatory, antimicrobial, antiviral, and anti-tumoral drugs (Bluden, 1993). Sulfated fucans, polysaccharides that contain neutral sugars, substantial percentages of L-fucose and sulfated ester groups, are constituents of brown algae and some marine invertebrates (Berteau & Mulloy, 2003). Their structure varies according to algal species and extraction procedure. Although several studies have attempted to determine the fine structure of fucans, only a few examples of regularity were found. The linkages, branching, sulfate positions and composition of monosaccharides differ significantly, and the relationship between structure and biological activity has yet to be established (Holtkamp, Kelly, Ulber, & Lang, 2009).

Sargassum (Phaeophyceae) is an algal genus with an extensive geographical range (Duarte, Cardoso, Noseda, & Cerezo, 2001). Early studies of fucans from the *Sargassum* genus indicate they are generally composed of glucuronic acid, mannose, and galactose residues, with partially sulfated side-chains consisting of galactose, xylose, and fucose. Fucans from *Sargassum* are widely studied owing to their broad therapeutic applications (Zhang, Hu, Liu, & Shuai, 2011).

Most thromboembolic processes require anticoagulant therapy. This explains current efforts to develop specific and potent anticoagulant and antithrombotic agents (Cumashi et al., 2007). Since the 1940s, heparin, sulfated polysaccharides, has been the predominant drug for treatment and prevention of venous thrombosis and thromboembolism. However, an obvious side effect of heparin administration is hemorrhagic. It is well-documented that marine brown algae are an abundant source of anticoagulant polysaccharides containing a variety of sulfated L-fucans with anticoagulant activity (Olson & Björk, 1993; Zhu et al., 2009). The proposed mechanism of anticoagulant action for fucoidan was predominantly related to *in vitro* potential ion of natural inhibitors of activated factor II (thrombin) and activated factor X (Ananthi et al., 2010).

Oxidative stress has been defined as a disturbance in the equilibrium between pro-oxidant and antioxidant systems in favor of pro-oxidation, due to intracellular signaling and defense against microorganisms (Ananthi et al., 2010). Other symptoms may result from ROS production associated with activation of the immune system. Cell membrane lipids and proteins are also sites of free radical reactions. Several investigations have been conducted to verify and to demonstrate the antioxidant properties in algae (Yuan et al., 2005).

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We previously demonstrated that *Sargassum vulgare* synthesizes alginic acid and heterofucans (Dietrich et al., 1995). In the present study, a fucan (SV1) was obtained from *S. vulgare* and its anticoagulant, antithrombotic, anti-hemolytic, antioxidant and anti-inflammatory properties were assessed using several *in vitro* and *in vivo* tests.

2. Experimental

2.1. Materials

Coomassie Brilliant Blue; KBr, agarose gel 1,3-diaminopropane; bovine thrombin; bovine serum albumin, toluidine blue, Heparin, *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, *N*-benzoyl-Phe-Val-Arg-γ-nitroanilide hydrochloride, D_2O, *N*-cetyl-*N,N,N*-trimethylammonium bromide,5-diphenyltetrazolium bromide, Folin Ciocalteau, glucose, galactose, arabinose, fucose, mannose, glucuronic acid, xylose, and rhamnose were purchased from Sigma (St. Louis, EUA) alkaline protease from *Esporobacillus* (BioBrás, Montes Claros, MG, Brazil), aPTT and PT commercial kits were purchased from Labtest (São Paulo, SP).

2.2. Animals

Experiments were conducted using male Wistar rats weighing 250–300 g, which were anesthetized with a mixture of ketamine (100 mg/kg intramuscularly) and xylasine (16 mg/kg intramuscularly). Experiments were in accordance with Brazilian National Law for the scientific management of animals.

2.3. Polysaccharide extraction

The brown seaweed *S. vulgare* was collected at Búzios beach on the south coast of Rio Grande do Norte state, Brazil. Seaweeds were stored in our laboratory and dried at 50 °C under ventilation in an oven, ground in a blender and incubated with acetone to eliminate lipids and pigments. About 50 g of powdered algae was suspended with five volumes of 0.25 M NaCl and pH was adjusted to 8.0 with NaOH. Ten milligrams of maxataze, an alkaline protease from *Esporobacillus* (BioBrás, Montes Claros, MG, Brazil), was then added to the mixture for proteolytic digestion. After incubation for 24 h at 60 °C under agitation, and periodic pH adjustments, the mixture was filtered through cheesecloth and precipitated with increasing amounts of ice-cold acetone (0.3, 0.5, 1.0 and 1.5 v) under gentle agitation at 4 °C (Silva et al., 2005). Precipitates formed were collected by centrifugation at 10,000 × *g* for 20 min and dried under vacuum. The volume fraction obtained with 1.0 v, denominated SV1, was chosen for analysis because it exhibits a higher yield.

2.4. Chemical composition, monosaccharide composition analysis by HPLC and characterization by electrophoresis

Total sugars were determined using a phenol–H_2SO_4 reaction, with D-fucose as standard, as previously described (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Sulfate content was measured after acid hydrolysis (HCl 6 N, 6 h, 100 °C) using the turbidimetric method (Dodgson & Price, 1962). Protein content was quantified with Coomassie Brilliant Blue reagent and bovine serum albumin as standard (Bradford, 1976). Phenolic compounds were measured by the Folin Ciocalteau method (Swain & Hills, 1959), with some modifications.

2.5. Monosaccharide composition

After hydrolysis (2 M HCl, 100 °C, 2 h), the fraction of total monosaccharide composition was determined by high performance liquid chromatography (HPLC), with a refractive index detector and LiChroCART® 250-4 column. Glucose, galactose, arabinose, fucose, mannose, glucuronic acid, xylose, and rhamnose were used as the standard for analysis.

2.6. Characterization of polysaccharides fractionated in acetone by electrophoresis and purified by gel filtration

Several fractions (0.3, 0.5, 1.0 and 1.5 v) of sulfated polysaccharides from *S. vulgare* (∼15 μg) was applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05 M 1,3-diaminopropane/acetate (pH 9.0). These sulfated fucans were fixed in the gel with 0.1% *N*-cetyl-*N,N,N*-trimethylammonium bromide solution (Leite et al., 1998). After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v). The polysaccharides (SV1) had high yielding and was subjected to gel-permeation chromatography on Sepharose CL-4B (140 cm × 1.8 cm) using 0.2 M acetic acid as eluent. The elution was monitored for total sugar (Dubois et al., 1956). To estimate the MW of the polysaccharide, we used dextrans of different sizes as standards (Pharmacia). The eluted polysaccharide was dialyzed against water, freeze-dried and used in the assays.

2.7. Infrared and NMR spectroscopies of polysaccharides

For infrared analysis a sample of 5 mg of each polysaccharides SV1 and PSV1 were mixed thoroughly with dry potassium bromide (100 mg). Pellets was prepared and scanned on a Nicolet 5PC Fourier transform infrared spectrophotometer.

NMR spectroscopy of ^1H and ^{13}C spectra spectra was recorded at 500 MHz at using a Varian Unity 500 spectrometer. The sulfated fucan sample (PSV1) (∼10 mg) was converted to sodium salt by passage through a 10 cm × 1 cm column of Dowex 50-X8 Na^+ form, and all samples were dissolved in approximately 0.7 mL of 99.8% D_2O.

2.8. Anticoagulant activity

Blood was collected by venous punction and mixed carefully with 3.2% sodium citrate at a proportion of 9:1. Next, the blood was centrifuged at 1000 × *g* for 10 min at ambient temperature. After centrifugation, the supernatant was removed and stored in plastic siliconized tubes, representing the citrated pool of plasma. Activated partial thromboplastin time (aPTT) and prothrombin time (PT) of the plasma pool mixed with SV1 in different concentrations were determined using commercial kits from Labtest (São Paulo, SP). The aPTT test evaluates the influence of compounds under intrinsic and common coagulation pathways, while the PT test assesses their influence under extrinsic and common coagulation pathways (Silva et al., 2005).

2.9. Enzymatic activity of coagulation factors (chromogenic substrate assay)

2.9.1. Assay of thrombin activity

The chromogenic substrate assay of thrombin was carried out in a hemolysis tube with a final volume of 1 mL, according to a modified method developed by Gaspar, Crause, and Neitz (1995), using 8 NIH/mL of bovine thrombin in 50 mM Tris–HCl + 0.1 M NaCl buffer (pH 8.0). The substrate *N*-benzoyl-Phe-Val-Arg-γ-nitroanilide hydrochloride was dissolved in buffer and methanol at a final concentration of 3 mM. In order to perform the assay, 30 μL of 8 NIH/mL thrombin, 815 μL of buffer, and 10 μL of SV1 (12.5, 25 and 50 μg) were incubated for 10 min at 37 °C, and 25 μL of thrombin chromogenic substrate at 3 mM concentration was then added. The mixture was incubated for 20 min at 37 °C. After incubation, 120 μL

of 30% acetic acid was added to stop the reaction. Absorbance was measured by a microplate reader at 405 nm. The same reagents were used for the blank sample; however, the substrate was only added after the reaction had been stopped with 30% acetic acid. In order to assess total enzyme activity (100% of enzymatic activity), the sample was not added to the reaction mixture.

2.9.2. FXa activity assay

The chromogenic substrate assay of thrombin was conducted in a 96-well microtiter plate, with final volume of 150 μL, according to a modified method developed by Gaspar et al. (1995). FXa was dissolved in 150 mM pH 7.4 PBS buffer at a concentration of 0.2 U/mL and the chromogenic substrate *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide was dissolved in PBS buffer at a final concentration of 1 mM/mL. To initiate the reaction, 75 μL of PBS buffer, 20 μL of FXa and 10 μL of SV1 (12.5, 25 and 50 μg/10 μL) were incubated for 10 min at 37 °C. Next, 25 μL of chromogenic substrate of FXa (1 mM/mL) was added and the mixture was incubated for an additional 30 min at 37 °C. Following incubation, 25 μL of 30% acetic acid was added to stop the reaction. Absorbance was measured by a microplate reader at 405 nm. The same reagents were used for the blank sample, although the substrate was only added after the reaction was stopped with 30% acetic acid. In order to analyze total enzyme activity (100% of enzymatic activity), the sample was not added to the reaction mixture.

2.10. In vivo antithrombotic activity

Antithrombotic activity was determined according to Reyers, Mussoni, Donati, and Gaetano (1980). Polysaccharides were dissolved in saline. The abdominal cavity was opened and the inferior vena cava exposed. Samples were applied in the caudal vein at a final volume of 100 μL in saline. After 5 min, a ligation was made in the inferior vena cava. After 1 h, the cavity was reopened and a new ligature was made 1 cm below the first. The fragment of the vena cava between the two ligatures was removed and placed in a Petri dish. Any thrombus formed was removed, placed on previously dried filter paper and weighed. The difference in paper weight before and after thrombus addition was the weight of the thrombus formed.

2.11. Antioxidant action

Scavenging activity of 1,1-diphenyl-2-picrylhidrazyl (DPPH) radical was determined according to Ye, Wang, Chunhong, Liu, and Zeng (2008), with slight modifications. We added 0.1 mL of different SV1 concentrations (0.3–2.5 mg/mL) to 1.5 mL 0.1 mM ethanol solution of DPPH. After 30 min at ambient temperature, absorbance was measured at 517 nm. The scavenging activity of DPPH radicals was calculated using the following equation: scavenging activity (%) = $(1 - A_{sample}/A_{control}) \times 100$ where $A_{control}$ is absorbance of the ethanol solution of DPPH without the sample (which was replaced by ethyl alcohol) and A_{sample} represents absorbance of the ethanol solution of DPPH with tested samples.

2.12. Cytotoxic action of SV1 on ABO and different RH group erythrocytes

The direct hemolytic assay was performed as described by Belokoneva, Villegas, Corzo, Dai, and Nakajima (2003) with slight modifications. Blood samples were collected in EDTA tubes. Red blood cells were rinsed with PBS buffer (0.15 M; pH 7.4) until the supernatant reached a transparent state. A 10% red blood cell suspension in PBS buffer was incubated with different amounts of SV1 (50, 100 and 150 μg) at ambient temperature for 1 and 6 h. After incubation, the red blood cell suspension was centrifuged at 1000 × *g* for 5 min and supernatants were read at 540 nm. As positive and negative controls, the red blood cell suspension was incubated under the same conditions with 1% Triton X-100 and PBS buffer, respectively. Results were expressed as hemolysis percentage (%) = $(A_{sample} - A_{PBS})/(A_{1\%\ Triton\ X\text{-}100} - A_{PBS})$ where A_{sample}, A_{PBS}, and $A_{1\%\ Triton\ X\text{-}100}$ refer to tested samples, negative control (PBS), and positive control (1% Triton X-100), respectively. Incubation was carried out in triplicate using different groups of red blood cells (A, B, AB and O groups), as well as different Rh groups (A positive and A negative red blood cells).

2.13. Cell viability assay (MTT test)

The MTT assay was performed using RAW 264.7 (mouse leukaemic monocyte macrophage cell line) as described by Mosmann (1983). The culture was exposed to 25, 50, and 100 μg of SV1 (triplicate) and incubated for 24 h at 37 °C. After incubation, 100 μL of DMEM medium containing MTT (final concentration of 5 mg/mL) was added to each well and the microplate was incubated for 4 h at 37 °C. The supernatant was removed and 100 μL of ethanol was added to each well to solubilize the formazan crystals. After homogenization of the mixture, absorbance was measured by a microplate reader at 570 nm. To control the reaction (100% of proliferation), SV1 was replaced by a DMEM medium. Each concentration of the respective sulfated polysaccharide was assayed sevenfold. The cell proliferation inhibition percentage was calculated according to the following equation:

$$\%\text{Inhibition} = \frac{\text{Abs. 570 nm control} - \text{Abs. 570 nm sample}}{\text{Abs. 570 nm control}} \times 100$$

2.14. Action of SV1 in carrageenan-induced paw edema

Animals received intraperitoneal pre-treatment with SV1 at different concentrations (10, 30 and 50 mg/kg) and the control group was treated with saline. After 30 min, individuals were lightly anesthetized with ethyl ether and injected intradermally with 100 μL of carrageenan in the right hind paw. The left paw received 100 μL of saline and was used as a control. Carrageenan was dissolved in 0.1% saline solution. Paw edema was measured immediately after and 4 h after carrageenan injection. The difference in volume between the right and left hind paws, measured with a pachymeter, was considered paw edema (Silva et al., 2010). Six rats per group were used in the experiments. After 4 h, the plantar region was excised for histological analysis.

2.15. Statistical analysis

Values were expressed as mean ± SEM. Analysis of variance (ANOVA) and the Tukey–Kramer test were used to assess biological activity data, with $p < 0.05$ accepted as statistically significant. Differences in the assay and between treatment and control were compared using an unpaired Student's *t*-test.

3. Results and discussion

3.1. Chemical characterization, IR and NMR spectroscopies

This study applies methodology combining proteolysis with non-specific proteolytic enzymes from sporobacillus such as maxatase, and acetone precipitation in several volumes (Dietrich et al., 1995). This solvent in varying concentrations gradually decreased the dielectric constant of water, promoting different populations of

Table 1
Percentage of total sugars, proteins, sulfate and monosaccharides of fucans extrated from *S. vulgare* by HPLC.

Fractions	Total sugars[a] (%)	Protein[b] (%)	Fucose (%)	Mannose (%)	Galactose (%)	Xylose (%)	Glucuronic Acid (%)	Sulfate[c] (%)
0.3 v	16.6	0.7	2.0	1.3	nd	3.4	89.4	3.1
0.5 v	27.8	1.5	4.0	3.5	5.3	14.8	62.8	10.4
1.0 v	63. 1	0.8	36.8	12.4	17.1	8.1	11.1	22.6
1.5 v	57.8	nd	53.1	5.7	3.1	nd	15.6	17.3

nd: not detected.
[a] Dubois method (1956).
[b] Bradford (1976).
[c] Dodgson and Price (1962).

sulfated polysaccharide precipitation rates. Four heteropolysaccharide fractions (0.3, 0.5, 1.0 and 1.5) were obtained from the brown seaweed *S. vulgare* (Table 1). The fraction denominated SV1 was eluted with 1.0 v of acetone and chosen according to its high yield.

Chemical analysis indicates SV1 (Table 1) is composed primarily of carbohydrates (63.1%) and displays high sulfate content (22.6%), with low protein contamination (0.8%) and phenolic compound content (2.9%). HPLC analysis of the monosaccharide composition from SV1 showed it is a heteropolysaccharide composed of fucose, galactose, xylose, glucuronic acid and mannose, thereby confirming it as a fucan or heterofucan (Table 1). Several investigations have demonstrated that chemical compositions and structures of fucans from brown algae are very complex, varying from species to species (Dietrich et al., 1995; Leite et al., 1998). Fractionated polysaccharides from *S. vulgare* were submitted to agarose gel electrophoresis (0.3, 0.5, 1.0 and 1.5) to determine the efficiency of the fractioning process applied. The electrophoretic profile in agarose gel, using a buffer system of 1,3-diaminopropane and toluidine blue staining, confirmed SV1 as a polydisperse and highly metachromatic fucan, thereby proving that SV1 is a sulfated polysaccharide (Fig. 1). The electrophoretic profiles showed the presence of a polydisperse component in all fractions, while the other fractions, SV0.3 and SV1.5 (lines 1 and 4) showed a single band each (Fig. 1A).

3.2. IR and NMR spectroscopies

The spectra of the SV1 and purified SV1 in Sepharose CL-4B (PSV1) samples (Fig. 1B) were scanned between wavenumbers 4000 and 500 cm^{-1} both exhibited major absorption bands at around 3423 and 3028 cm^{-1} that were interpreted as being due to O—H stretching. Infrared spectroscopy of the SV1 and PSV1 fractions demonstrated absorption at 2143 and 1641 cm^{-1}, corresponding to C—H of the methyl group of fucose and carboxyl groups of uronic acid, respectively. Absorption at 840 cm^{-1} is a region of particular importance, indicating the presence of a sulfate in the axial position of the C4 of fucose (Fig. 2, red line), whereas an equatorial position would provoke a low band near 820 cm^{-1} (Duarte et al., 2001; Olson et al., 1993). An absortion band was found in all samples at approximately 1255 cm^{-1}, attributed to S=O stretching vibration and suggesting the presence of ester sulfate. This was confirmed by examining the infrared spectra of several sulfated polysaccharides. It is inferred that most of the sulfate groups in fucan are on the C-4 of fucose (the only C atom in the molecule with an axial hydroxyl), in agreement with chemical data. Infrared spectroscopy of SV1 showed signals corroborating those found in other studies of the brown algae *Sargassum stenophilum* (Duarte et al., 2001).

We observed in the infra red spectrum that the fraction purified in Sepharose CL-4B, (PSV1) not showed absorptions at 1255 and 840 cm^{-1}. This demonstrates loss of sulfate/polymers of sugars during purification. Thus, is evident the importance of sulfate group on biological activities. SVP1 fraction was used only in the infrared and NMR spectroscopies.

NMR spectroscopy is a convenient method to obtain structural information on polysaccharides. NMR analysis was used to determine the characteristics of a purified SV1 fraction (SVP1). The fraction no purified showed a spectrum very bad. Moreover, studies on this polysaccharide have indicated a highly complex structure. The ^{1}H NMR spectrum of PSV1 were similar to the ^{1}H from *Fucus vesiculosus* and *Laminaria brasiliensis*, with pooly resolved signals (Ale, Maruyama, Tamauchi, & Mikkelsen, 2011). The ^{1}H NMR spectrum of fraction of polysaccharide contained an intense broad signal (5.0–5.5 ppm) and several minor signals in the α-anomeric region, as well as two intense signals in the high field region assigned to the protons of methyl groups (Fig. 2A). The signal at 4.61 ppm (Fig. 2A) was attributed to a 3-linked D-galactopyranosyl residue when compared to data obtained by Ale et al. (2011). Thus, the complexity of fraction polysaccharides (SV1) had poor quality spectra (^{1}H and ^{13}C) and not was showed.

The ^{13}C NMR spectrum from PSV1 exhibited considerable complexity, showing three signals in the α-anomeric region. Signals between 105 and 74 ppm correspond to sulfated or glycosylated carbons in the fucopyranose ring, while the group of signals at 71–66 ppm may arise from unsubstituted carbons. Moreover, in ^{13}C, signals were recorded at 103.1 and 61.3 for galactoses and at 100.8 for xylose (Fig. 2B). We also observed a very low signal at 178 ppm related to glucuronic acid (not shown). These values are consistent with those reported by Pereira, Mulloy, and Mourão (1999) for fucans isolated from echinoderms, for fucans from *Lessonia vadosa* (Chandia, Ortiz, & Mansilla, 2005; Croci et al., 2011), suggesting that even a sulfated polysaccharide isolated from a given species of brown algae may be a mixture of structurally different sugars in polymers. Thus, despite increasing efforts, the structure–activity relationship of fucoidans is still an unresolved issue.

3.3. Anticoagulant activity

The PT and the aPTT tests are used to distinguish the effects on extrinsic and intrinsic coagulation pathways, respectively. Anticoagulant activity data are shown in Fig. 3. SV1 dramatically increases clotting time at different concentrations ($p < 0.001$) in relation to the control. This was measured with the aPTT test, which evaluates action of the compound in the intrinsic and common pathway of blood coagulation. The results suggested that SV1 promoted a maximum increase in clotting time, detected by aPTT at a concentration of 50 and 100 μg/mL. None of the fractions (SV1 and PSV1) had an anti-clotting effect when examined by the prothrombin time (PT) test. PSV1 fraction not showed anticoagulant activity. Literature reports indicated molecular weight, chain length, charge density and the three-dimensional structure of the sulfated polysaccharide influence its interactions with the coagulation proteins (Olson, Bjork, & Bock, 2002).

3.4. Assay activity of thrombin and Factor Xa

Sulfated polysaccharides from algae may display direct inhibitory action under coagulation factors, in addition to indirect

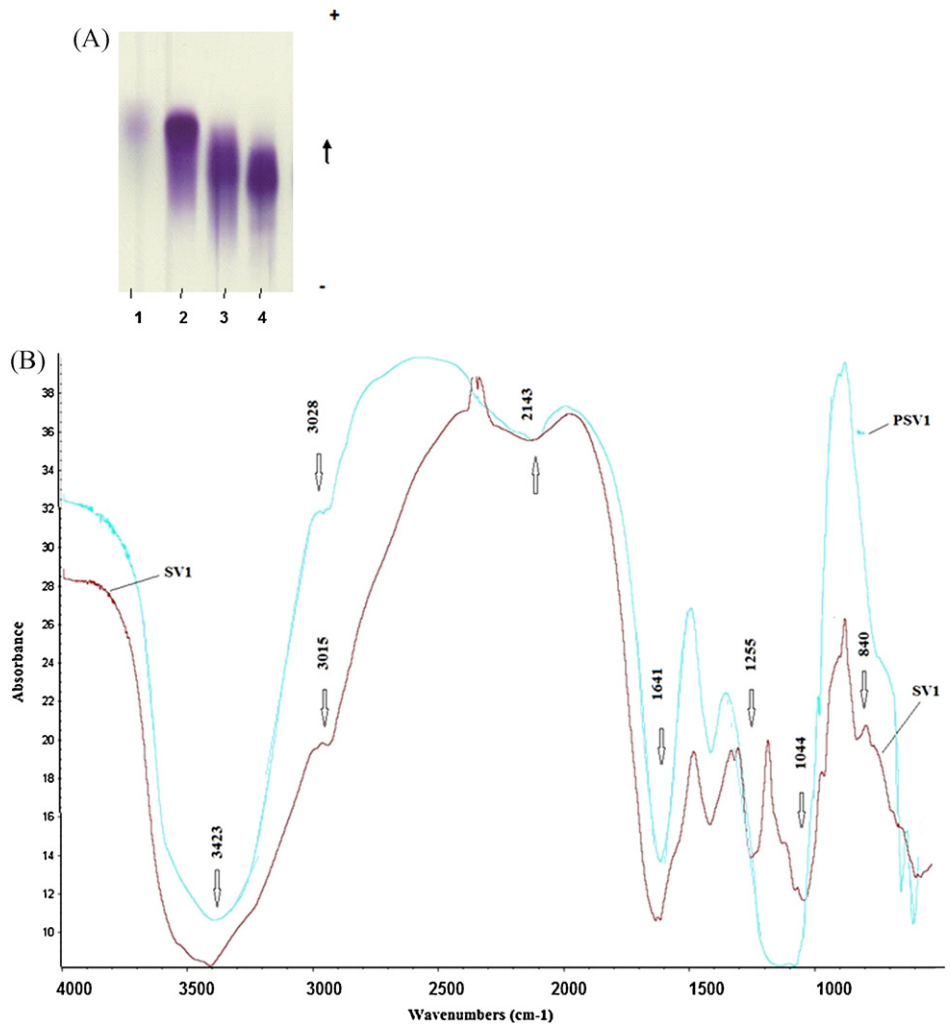

Fig. 1. Electrophoretic profile in agarose gel and infrared analysis of sulfated polysaccharides from *S. vulgare*. (A) The sulfated fucans obtained by precipitation in acetone (0.3–1.5 v) were applied to the 0.5% agarose gel in 0.05 M 1,3-diaminepropane acetate buffer, at pH 8.5. Fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v). Each fraction contained 50 μg of polysaccharides in 5 μL. 0.3 Fraction (1 line); 0.5 Fraction (2 line); 1.0 Fraction (SV1) (3 line); 1.5 Fraction (4 line). (B) Infrared analysis of sulfated polysaccharides from *Sargassum vulgare* scanned between 4000 and 400 cm^{-1}. (A – red) fractionated with 1.0 v acetone (SV1) and (B – blue) SV1 purified by Sepharose CL-4B (PSV1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

inhibitory action mediated by antithrombin and heparin II cofactor (Pereira & Mulloy, 2008). Evaluation of SV1 action on thrombin and factor Xa reaction was performed at different concentrations (3.1, 6.2, 12.5, 25 and 50 μg/10 μL), using chromogenic substrates for these proteins (Fig. 4A). SV1 demonstrated optimal inhibitory activity of thrombin at 12.5/10 μL (55.3 ± 1.13%) and 25 μg/10 μL (54.2 ± 2.18%). There was no statistically significant difference between concentrations of 12.5 and 25 μg/10 μL ($p < 0.05$). Different behavior was observed for Factor Xa (FXa), where SV1 stimulated activity at 12.5 μg/10 μL (131.7 ± 3.4%) and 25 μg/μL (108.9 ± 5.58%). Data showed that SV1 anticoagulant activity results from its direct action on the enzymatic activity of thrombin and stimulation of FXa enzymatic activity (Fig. 4B).

3.5. Antithrombotic activity in vivo

SV1 fraction displays anticoagulant activity and as such, we opted to perform an antithrombotic assay *in vivo* at two different concentrations. In order to compare the antithrombotic activity of SV1 with heparin, an antithrombotic trial was conducted on rats. The results (see Fig. 5) demonstrated that SV1 has a strong antithrombotic effect similar to that of heparin when used at a concentration ten times higher (10 μg/g by body weight), exhibiting a significant difference in relation to the control ($p < 0.001$). However, when 5 μg/g by body weight was used, this activity decreased considerably in relation to the control ($p < 0.01$).

3.6. Antioxidant activity

The DPPH free radical is a stable free radical, widely accepted as a tool for estimating free radical-scavenging activities of antioxidants (Jiménez-Escrig, Jiménez-Jimenez, Pulido, & Saura-Calixto, 2001). The present study tested SV1 at different concentrations (0.15, 0.3, 0.6, 1.2 and 2.5 mg/mL) in a control system of scavenging radicals. As shown in Table 2, SV1 displays DPPH scavenging activity of 22.2% at 2.5 mg/mL. There are some reports in the literature regarding antioxidant activity of algae, assessing alcoholic and aqueous extracts of seaweed. Recent studies with a heterofucan from the brown seaweed *Sargassum filipendula* indicate these polysaccharides exhibit significant antioxidant action (Costa et al., 2011). The antioxidant activity of fraction purified PSV1 was low (date not shown). Polysaccharides extracted from *Sargassum*

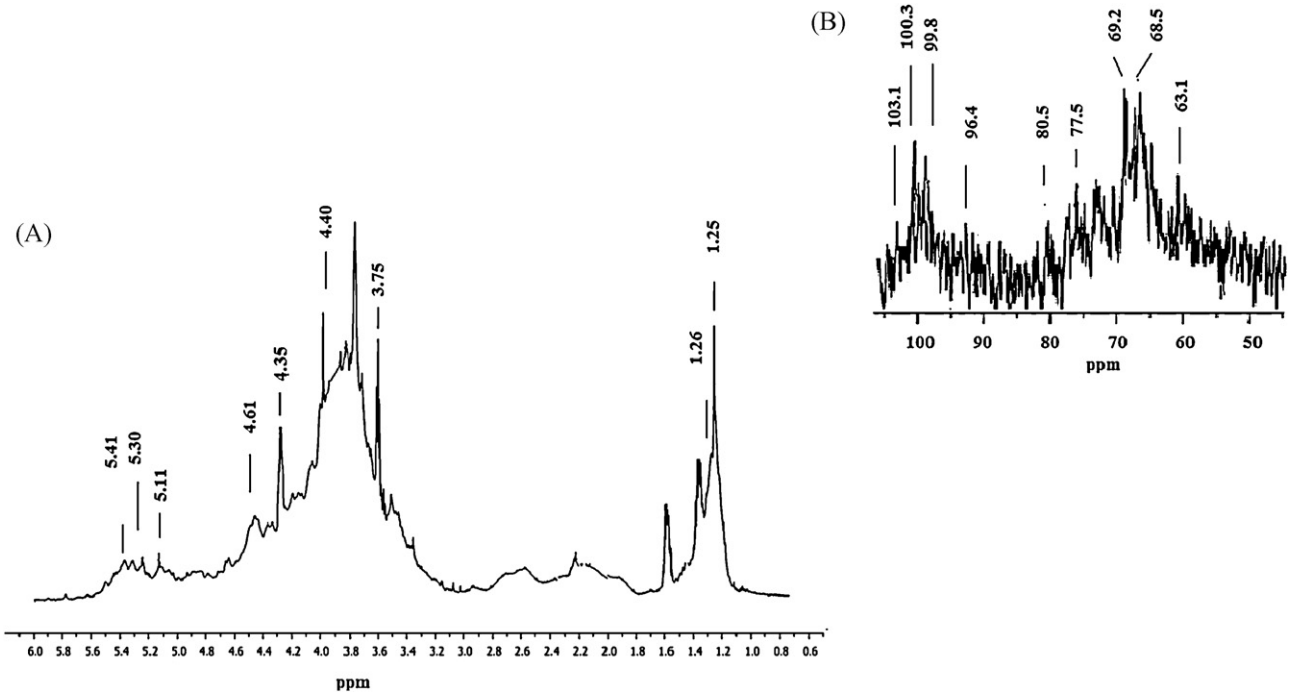

Fig. 2. NMR spectrum of sulfated polysaccharides from *S. vulgare* at 60 °C in D_2O solution. (A) ^1H NMR of SV1 and (B) ^{13}C of PSV1 at 55–105 ppm.

pallidum at test concentrations of 3.8 mg/mL showed a 19.1% scavenging capacity for DPPH radicals (Ale et al., 2008). The manifested antioxidant activity of some algal species investigated increases their importance as a potential new source of natural additives, primarily when considering the inverse relationship between dietary intake of antioxidant-rich foods and incidences of human diseases (Lu & Foo, 2000).

3.7. Cytotoxic action of SV1 under ABO and Rh blood types in different erythrocyte groups

Occurrence of erythrocyte hemolysis in the blood is consistent with oxidative damage to the plasma membrane (Chukhlovin, 1996). According to the methodology applied, SV1 showed no cytotoxic activity on erythrocytes, demonstrated by the lack of significant hemolysis in tested concentrations. There was no statistically significant difference between erythrocytes of the Rh positive and negative groups (Fig. 6A), although a significant

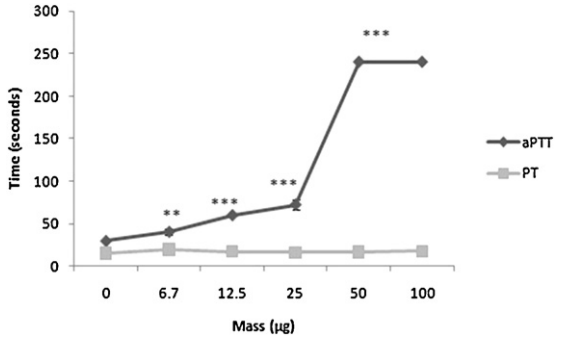

Fig. 3. Anticoagulant activities (aPTT and PT) of sulfated polysaccharides from SV1. Activated partial thromboplastin time (aPTT) and prothrombin time (PT) of the plasma pool treated with different amounts of SV1 (0, 6.7, 12.5, 25, 50, 100 µg). Significance level related to negative control $p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Values are expressed as mean ± SEM ($n = 3$).

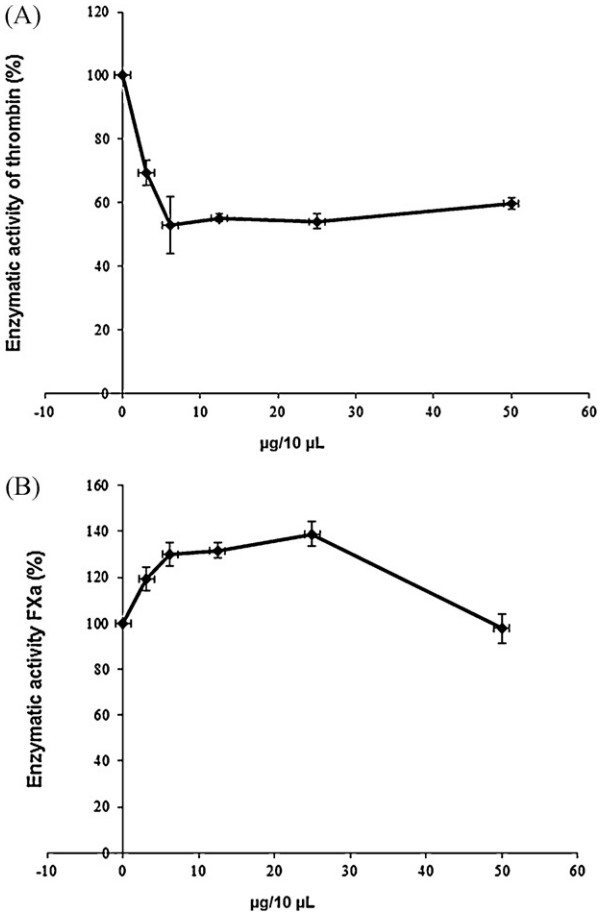

Fig. 4. SV1 action on thrombin and FXa. (A) Effect of SV1 on thrombin activity. (B) Effect of SV1 on Factor Xa activity. Each value depicts the mean ± SD of three determinations.

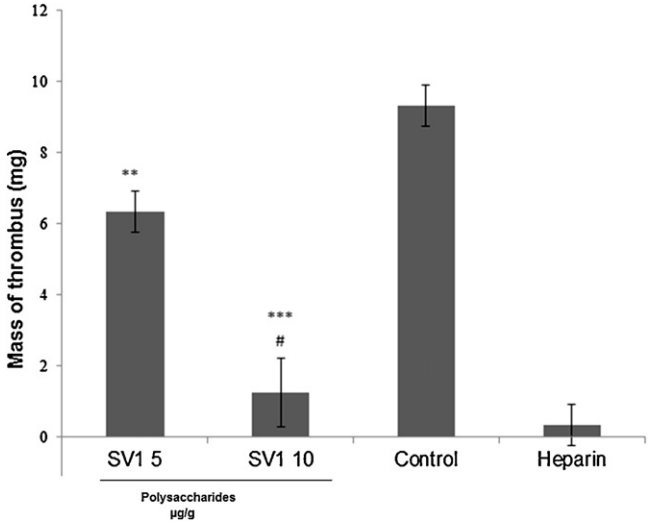

Fig. 5. Antithrombotic activity *in vivo* of sulfated polysaccharides from SV1. Significance level related to negative control $^{**}p < 0.01$ and $^{***}p < 0.001$. Significance level between heparin and SV1 $^{\#}p > 0.05$. Values are expressed as mean ± SEM ($n = 3$).

difference was recorded among erythrocytes of A and B, AB, and O groups (Fig. 6B). Thus, PSV1 does not cause erythrocyte membrane damage in any of the blood groups tested. Li, Li, and Zhou (2007) found that a polysaccharide extract from *Lycium barbarum*

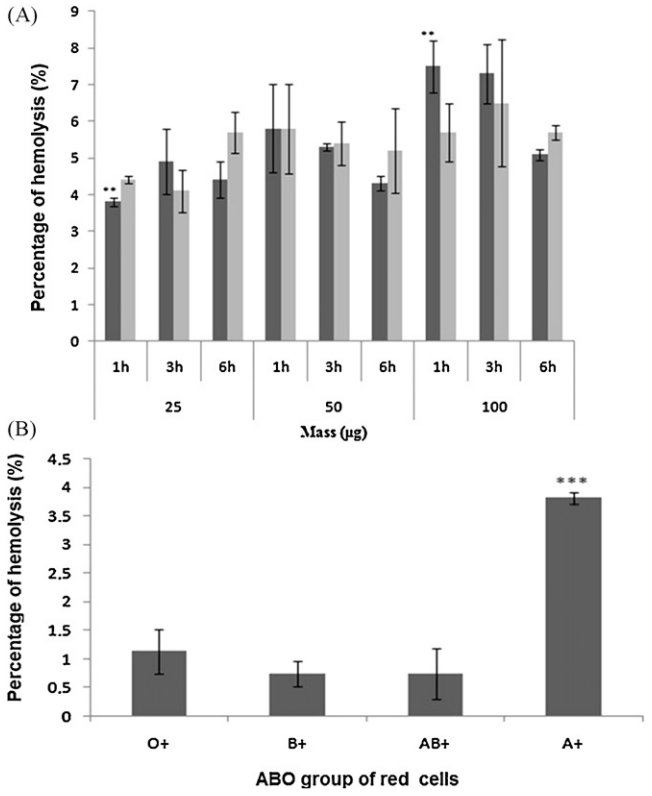

Fig. 6. Cytotoxic action of SV1 on ABO and Rh blood types in different erythrocyte groups. (A) Direct hemolysis on A positive and A negative erythrocytes. Erythrocytes were incubated for 1 and 6 h using different amounts of SV1. There was no statistically significant difference between Rh groups tested ($p > 0.05$). Significance level between A+ and A− in the first time period ($^{**}p < 0.01$). (B) Direct hemolysis on A, B, AB and O positive blood group erythrocytes. Erythrocytes were incubated for 1 h with 50 µg of SV1. No statistically significant difference was recorded ($p > 0.05$) between erythrocytes of B, AB and O blood groups. $^{*}p < 0.05$. Values are mean ± SEM ($n = 3$).

Table 2
Percentage of DPPH radical scavenging ability of sulfated polysaccharides from SV1.

Sulfated polysaccharides (mg/mL)	DPPH radical scavenging (%)
	10.0 ± 0.7
0.3	11.2 ± 0.4
0.6	11.9 ± 0.2
1.2	12.3 ± 1.8
2.5	22.2 ± 0.4
3.0	22.0 ± 0.6

fruits exhibited strong anti-hemolytic activity in rat erythrocytes (Li et al., 2007).

3.8. Cell viability

Cell viability was verified using RAW 264.7 (mouse leukaemic monocyte macrophage cell line) by MTT assay. SV1 displayed proliferative action on RAW 264.7 cells. When this lineage was treated with different amounts (25, 50, and 100 µg) of SV1 for 24, 48 and 72 h, the cell proliferation observed was 127.67 ± 3.08% when cells were incubated with 25 µg of SV1 for 72 h (Fig. 7). No statistically significant difference was found between the tested concentrations at the different times ($p > 0.05$).

Cell proliferation was observed in the MTT assay when macrophages from RAW 264.7 cells were incubated with SV1. This fraction promoted cell division, increasing the mitochondrial function for MTT conversion to formazan, the compound detected in the assay. The present study found that SV1 stimulates the proliferation of macrophages, cells of the innate immune system, and is a potential target for new studies related to the role of this compound in the fight against cancer *via* stimulation of the innate immune system.

3.9. Action of SV1 in carrageenan-induced paw edema

The role of SV1 in carrageenan-induced acute inflammation was evaluated at concentrations of 10, 30 and 50 mg/kg. Edema was reduced for all concentrations tested, with a significant difference in relation to the control ($p\square 0.001$) and no statistically significant difference when compared to saline ($p > 0.05$) (Fig. 8A). Histological analysis showed a significant decrease in cell infiltration in the plantar region of animals treated with different doses of SV1 (Fig. 8B). Similar results were recorded by Ananthi et al. (2010) when testing a polysaccharide from the marine brown alga *Turbinaria ornata* in carrageenan-induced paw edema, with measurements of 0.9 mm ± 0.09 mm and 0.51 mm ± 0.06 mm in the fifth hour after treatment, using 20 mg/kg of the polysaccharide. Thus, the present

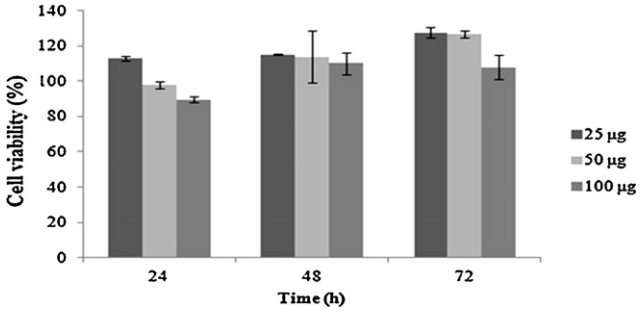

Fig. 7. Cell viability of polysaccharides (SV1) using RAW 264.7. The culture was exposed to 25, 50, and 100 µg of SV1 (triplicate) and incubated for 24 h at 37 °C and 5% CO_2. Values are expressed as mean ± SEM ($n = 3$).

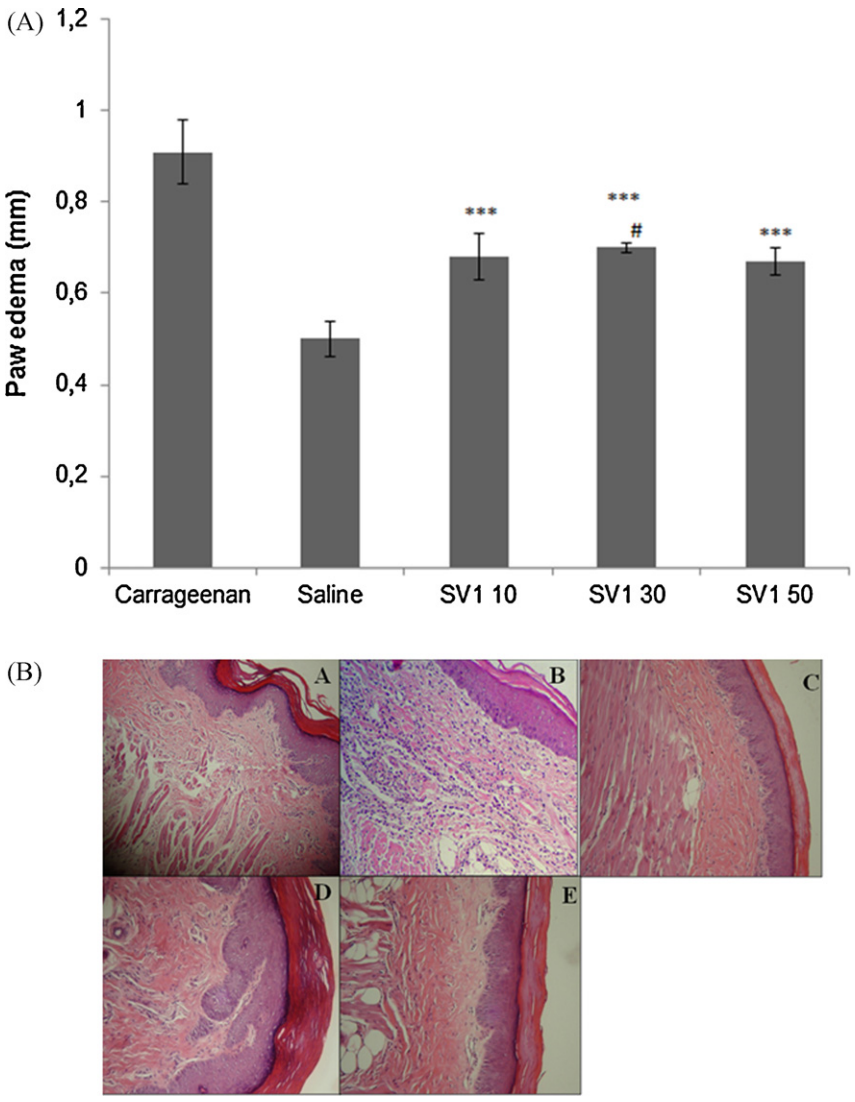

Fig. 8. Anti-inflammatory action of SV1 in a carrageenan-induced paw edema model and histological analysis. (A) Carrageenan (positive control), saline and fraction SV1 (10, 30 and 50 mg/kg). Significance level related to negative control $^{***}p < 0.001$. Significance level between SV1 30 and saline $^{\#}p > 0.05$. Values are expressed as mean ± SEM ($n = 6$). (B) Histological analysis of carrageenan-induced paw edema treated with different concentrations of SV1. HE (10×) (A) positive control (carrageenan); (B) negative control (saline); (C) animals treated with SV1 at 10 mg/kg; (D) animals treated with SV1 at 30 mg/kg; (E) animals treated with SV1 at 50 mg/kg.

investigation demonstrated that SV1 showed significant reduction in the biphasic response of carrageenan-induced edema.

4. Conclusions

This study demonstrated that a fucan SV1 soluble in water was isolated from *S. vulgare*. This fractions was extracted by differential precipitation in acetone (1.0 v) and selected due to its high yield and significant anticoagulant activity. We suggest that the strongly antithrombotic and anticoagulant effects of SV1 may be a result of its direct inhibition of thrombin. SV1 stimulates the proliferation of macrophages, but prevents arrival of these cells at the injury site, possibly owing to their ability to bind to cell membrane receptors. SV1 demonstrated strong anti-inflammatory activity, which may be related to its antioxidant and antihemolytic actions. PSV1 purified fraction of SV1 was used infrared and NMR spectroscopies and showed very low biological activities. It is possible that the loss of sulfate inserted in the polymer decreased biological effects. SV1 have anti inflammatory effect in carrageenan-induced paw edema.

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