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Anticoagulant and antithrombotic activity of a sulfate obtained from a glucan component of the lichen *Parmotrema mantiqueirense* Hale

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Abstract

A β-(1→6)-linked glucan (β-G) was isolated from the lichen *Parmotrema mantiqueirense* Hale and was sulfated, to give a product (β-G-SO_4) with 76.8% sulfate, corresponding to a degree of substitution of 1.95. Both β-G and β-G-SO_4 were evaluated by its activated partial thromboplastin (APTT) and thrombin time (TT), using pooled normal human plasma, and compared with that having 140 USP units/mg (porcine intestinal mucosa heparin). Anticoagulant activity was detected for β-G-SO_4, but not β-G. The in vivo antithrombotic properties of β-G-SO_4 were determined using a stasis thrombosis model in Wistar rats, with an intravenous administration of 0.5 mg/kg body weight totally inhibiting thrombus formation. It caused dose-dependent increases in tail transection bleeding time. The results obtained show that this sulfated polysaccharide is a promising anticoagulant and antithrombotic agent.

Keywords: Anticoagulant activity; Sulfated polysaccharide; Antithrombotic

1. Introduction

Anticoagulants have been used widely for blood treatment during dialysis and surgery, as medication of disseminated intravascular coagulation and thrombosis in various diseases, and for blood testing in vitro (Tamada, 2004). Heparin, a heterogeneous sulfated polysaccharide from the growing family of known glycosaminoglycans, is widely used as a therapeutic anticoagulant and antithrombotic (Jouault et al., 2001). Heparin is a linear polysaccharide with a disaccharide repeated unit containing preponderantly of α-D-glucosamine alternating with α-L-iduronic acid. The structure of heparin is however made more complex by variation in its pattern of substitution with *N*- and *O*-sulfate, and *N*-acetyl groups (Mourão & Pereira, 1999).

The anticoagulant effect of heparin is expressed through binding of the polysaccharide to antithrombin (AT), which leads to acceleration of the rate at which this proteinase inhibitor complexes with and thereby inactives the enzymes involved in blood coagulation (Razi et al., 1995). Heparin has a specific pentasaccharide sequence that mediates the interaction with AT (Atha, Lormeau, Petitou, Rosemberg, & Choay, 1985; Lindahl, Balckstroem, Thumberg, & Leder, 1980). Heparin suffer from number of major disadvantages, these include extreme structural diversity, problems with animal pathogen contamination due to its animal origin, poor bioavailability, the problem of heparin-induced thrombocytopenia developing in some heparin recipients, and the risk of bleeding (Beijering, ten Cate, & ten Cate, 1996; Hirsh, 1991).

Attempts have been made to develop new anticoagulant and antithrombotic drugs. Various studies have concerned new sulfated polysaccharides that show anticoagulant/antithrombotic properties. Sulfated polysaccharides that are either of natural, semisynthetic, or synthetic origin, among them dextran sulfate, chitin and chitosan sulfate, carrageenan, and others, have multiple biological activities including

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anticoagulant and antithrombotic activities (Huang, Du, Yang, & Fan, 2003). These polysaccharides show no structural homology with heparin, except high sulfate contents (Jouault et al., 2001) It is generally accepted that the anticoagulant activity of the polysaccharides partly results from the strong interaction between the negatively charged sulfated groups and some positively charged peptidic sequences (Bourin & Lindahl, 1993).

We also describe the preparation of a sulfated glucan (β-G-SO_4) by esterefication of a β-(1→6)-linked glucan (β-G), extracted from the lichenized fungus *Parmotrema mantiqueirense* Hale. β-G-SO_4 was then examined for its anticoagulant and antithrombotic properties.

2. Experimental

2.1. Lichenized fungus

The lichenized fungus *P. mantiqueirense* Hale (Parmeliaceae family) was collected in 1996, in Lapa, State of Paraná, Brazil. It was identified by Prof. Dra. Sionara Eliasaro (Department of Botanic of the Federal University of Paraná-UFPR) and has its voucher (No. 33355) deposited in the Herbarium UPCB of UFPR.

2.2. Materials

Heparin from porcine intestinal mucosa (140 USP units/mg) was obtained from Sigma (St Louis, MO) and rabbit brain thromboplastin from Instrumentation Laboratory (Lexington, MA). Normal human plasma was obtained by centrifugation (2000×*g* for 15 min at 22 °C) of citrated normal human plasma (1/10 vol. of 3.8% trisodium citrate) from a pool of healthy volunteer donors, and frozen at −20 °C in aliquots of 0.5 ml until further use. Activated partial thromboplastin (APTT) and PT were determined with reagents from Instrumentation Laboratory (Lexington, MA) and thrombin time (TT) with those from Behring (Marburg, Germany). Pyridine, formamide, chlorosulfonic acid, methanol, ethanol, and trifluoroacetic acid were from Merck (Darmstadt, Germany). All other chemicals and reagents used were of analytical grade.

2.3. Isolation of glucan and its sulfation

A lichen sample (100 g) was successively refluxed in $CHCl_3$–MeOH (500 ml, 2:1 v/v) and 80% aqueous MeOH (500 ml), in order to extract low molecular components. The residual material was then extracted successively with 2 and 10% aq. KOH (800 ml) containing a trace of $NaBH_4$ at 100 °C for 3 h. The extracts obtained with 10% aq. KOH were combined, neutralized with HOAc, dialyzed against tap water for 72 h, and the retained solution was freeze thawed to give a precipitate and supernatant. The latter was then treated with Fehling solution (Jones & Stoodley, 1965) and resulting precipitate of Cu^{++} complexes removed. These were neutralized with HOAc, dialyzed against tap water and deionized with mixed ion exchange resins, giving rise to a glucan (β-G, 2.1 g).

β-G was sulfated according to the method described by O'Neill (1995) with slight modifications as follows. The native polysaccharide (2.0 g) was solubilized in formamide (100 ml) and pyridine (100 ml) by vigorous stirring for 24 h, followed by dropwise addition of chlorosulfonic acid (25 ml) over 1 h at 0 °C, the mixture being maintained at 4 °C for 12 h. Ice-water was added, followed by 10% (w/v) aqueous $NaHCO_3$ until effervescence ceased. The solution was then dialyzed against water to remove pyridine, salts, and potential degradation products and then freeze dried, providing the sodium salt of the sulfated glucan. Sulfated glucan was stored until use in a desiccator. The degree of substitution (DS) of sulfated derivative was determinated by hydrolysis with 1 M HCl for 5 h at 100 °C, the resulting $BaSO_4$ being measured turbidimetrically (Dodgson & Price, 1962).

2.4. Monosaccharide composition

Hydrolysis was carried out with 1 M TFA at 100 °C for 8 h and the hydrolyzates then evaporated to dryness, followed by successive reduction with $NaBH_4$ and acetylation with Ac_2O-pyridine (1:1, v/v; 2 ml) at room temperature for 12 h (Wolfrom & Thompson, 1963a,b). The resulting alditol acetates obtained were analyzed by GC-MS using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer, with a DB-225 capillary column (30 m–0.25 mm i.d.), and He as carrier gas. The analysis was carried out from 50 to 220 °C at 40 °C/min maintaining the temperature constant at the end of the gradient (18 min).

2.5. Methylation analysis

Fractions β-G and β-G-SO_4 (5 mg) were per-*O*-methylated according to the method of Ciucanu and Kerek (1984), using powdered NaOH in Me_2SO–MeI. The per-*O*-methylated derivatives were treated with refluxing 3% HCl–MeOH for 2 h at 80 °C, and then with 0.5 M H_2SO_4 at 100 °C for 14 h, followed by neutralization with $BaCO_3$. The resulting mixtures of *O*-methylaldoses were reduced with $NaBH_4$ and acetylated as cited above to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC-MS. The analysis was carried out from 50 to 215 °C at 40 °C/min maintaining the temperature constant at the end of the gradient (31 min). The resulting partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976).

2.6. Determination of homogeneity and molar mass

The elution profile of fraction β-G was determined by high performance size-exclusion chromatography (HPSEC), using a WATERS 510 HPLC pump at 0.6 ml/min with four gel permeation columns in series with exclusion sizes of 1×10^6, 4×10^5, 8×10^4, and 5×10^3 Da, using a refractive index (RI) detector. The eluent was 0.1 mol/l aq. $NaNO_3$ containing 200 ppm aq. NaN_3. Samples, previously filtered through a membrane (0.22 μm; Millipore), were injected (250 μl loop) at a 2 mg/ml concentration.

The specific RI increment (d*n*/d*c*) was determined for fraction β-G. The sample was dissolved in 50 mM $NaNO_3$ and five increasing concentrations, ranging from 0.2 to 0.7 mg/ml, were used to determine the slope of the increment. Results were processed in software provided by the manufacturer (Wyatt Technologies).

2.7. Nuclear magnetic resonance spectroscopy

NMR spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer with a 5 mm inverse probe. ^{13}C-NMR (100.6 MHz) and ^{1}H-NMR (400.13 MHz) analyses were performed at 50 or 30 °C on sample dissolved in D_2O. Chemical shifts of samples are expressed in ppm (δ) relative to acetone at δ 30.20 and 2.22 for ^{13}C and ^{1}H signals, respectively.

2.8. Clotting assay

The assay was carried out using heparin, β-G and β-G-SO_4, dissolved in saline at various concentrations. Normal human plasma (90 μl) was mixed with 10 μl of a solution of β-G-SO_4 (0–20 μg), β-G (0–2 mg) or heparin (0–20 μg). For controls, saline was added to plasma in a ratio of 1:10. APTT measurements were performed using a kit obtained from Instrumentation Laboratory (IL test™, Lexington, MA). Plasma (100 μl), containing various concentrations of β-G-SO_4, β-G or heparin, was incubated at 37 °C for 1 min. One hundred microliter of bovine cephalin was then added and incubated at 37 °C. After 3 min incubation, 100 μl of pre-warmed 0.25 M $CaCl_2$ solution were added to the mixtures and the clotting time was measured in quadruplicate using a COAG-A-MATE® XM coagulometer (Organon Teknika Corporation, Durhan, NC) up to 300 s. For determination of TT, normal human plasma (100 μl) containing various concentrations of heparin, β-G and β-G-SO_4, was incubated at 37 °C for 2 min, at which the thrombin time reagent, 200 μl, (Behring, Marburg, Germany) was added. The time for the appearance of a fibrin clot (s) was measured up to 300 s using a COAG-A-MATE® XM coagulometer (Organon Teknika Corporation, Durhan, NC). For determination of PT, normal human plasma (100 μl) containing various concentrations of heparin, β-G and β-G-SO_4 was incubated at 37 °C for 2 min, then, 200 μl of rabbit brain calcium tromboplastin was added and the clotting time recorded using a COAG-A-MATE® XM coagulometer. All assays were performed in duplicate and repeated at least three times on different days ($n=6$).

2.9. Animals

Experiments were conducted on male Wistar rats weighing 250–300 g, which were anesthetized with a mixture of ketamine (Fort Dodge; 100 mg/kg intramuscularly) and xylasine (Virbac; 16 mg/kg intramuscularly).

All recommendations of the Brazilian National Law (No. 6.638, 05 November 1979) for scientific management of animals were respected.

2.10. Ex vivo determination of APTT

Animals were anesthetized as described above and the carotid artery was carefully exposed and dissected free from surrounding tissue. Heparin (0–500 μg/kg), vehicle (PBS) or β-G-SO_4 (0–1 mg/kg) was administered into the carotid artery. After 5, 15, 30 and 60 min samples of blood was collected (0.5 ml in 3.8% trisodium citrate, 9:1, v/v). Each blood sample was centrifuged ($2000\times g$, 10 min) and plasma was stored at −20 °C until use. APTT was determined using a kit obtained from Instrumentation Laboratory (IL test™, Lexington, MA). Plasma (100 μl), was incubated at 37 °C for 1 min. One hundred microliter of bovine cephalin was then added and incubated at 37 °C. After 3 min of incubation, 100 μl of pre-warmed 0.25 M $CaCl_2$ solution were added to the mixtures and the clotting time was measured using a COAG-A-MATE® XM coagulometer (Organon Teknika Corporation, Durhan, NC) up to 300 s. For each treatment group ($n=6$) the mean ex vivo APTT ± SD was determined.

2.11. Tail transection bleeding time

For evaluation on the bleeding effect, rats were anesthetized as described above. The right carotid artery of animals was cannulated for administration of β-G-SO_4 (0.1–1 mg/kg), vehicle (PBS), or heparin (25–100 μg/kg). Bleeding was induced by section of the tail extremity 3 mm from the tip. The tails were blotted with tissue paper every 30 s and the time up to cessation of bleeding were noted. The compounds were administered 5 min before tail transection. For each treatment group ($n=6$) the mean bleeding time ± SD was determined.

2.12. Stasis-induced venous thrombosis model

Thrombus formation was induced by promoting a combination of stasis and hypercoagulability (Berry, Girardi, Lochot, & Lecoffre, 1994). Rats were anesthetized and their right carotid artery was cannulated for injection of

β-G-SO_4, vehicle (PBS), heparin, and thromboplastin. The abdomen of each animal was opened surgically and, after careful dissection, the abdominal vena cava was exposed and dissected free from the surrounding tissue, all its side-branches being ligated between the left renal and femoral veins. β-G-SO_4 (0.25–2 mg/kg), heparin (25–100 μg/kg), or vehicle, were administered as a single bolus injection. After 5 min, thrombus formation was induced by the injection by thromboplastin (10 mg/kg) followed 10 s later by stasis of a 1 cm segment of the abdominal vein cava, which was maintained for 20 min. The formed thrombus was removed, immediately blotted twice on paper and weighed. For each treatment group ($n=6$) the mean thrombus weight ± SD was determined.

2.13. Statistics

All results are expressed as the mean ± standard deviation (SD). To analyze the data statistically, we performed one-way analysis of variance (ANOVA) for repeated measurements of the same variable, and used Tukey test to determine which means were significantly different from that of the control. A value of $p<0.05$ was considered significant.

3. Results and discussion

3.1. Chemical characterization of β-G and β-G-SO_4

The precipitate obtained using Fehling solution (fraction β-G; 2.1% yield), contained glucose as the main component. HPSEC analysis showed a homogeneous profile with $M_r = 4.2\times10^5$ kDa ($dn/dc = 0.141$). Their ^{13}C NMR spectra containing six signals of equal area, identical to those of a (1→6)-linked β-glucan (pustulan) (Sassaki et al., 2002). The β-configuration was confirmed by virtue of a high-field H-1 signal at δ 4.51 and a low field C-1 at δ 103.5. The C-6 signal at δ 69.3 shows 6-*O*-substitution of Glc*p* units, confirmed by a corresponding inverted CH_2 signal in the DEPT spectrum. The other signals observed at δ 73.6, 76.1, 70.0, and 75.4 corresponding to C-2, C-3, C-4, and C-5, respectively. These data are in agreement with those of a methylation analysis, which showed only the derivatives 2,3,4,6-Me_4-Glc*p* (0.6%) and 2,3,4-Me_3-Glc*p* (99.4%).

Comparison of methylation analysis data for β-G and β-G-SO_4 demonstrated that sulfate groups were inserted principally in the OH-groups at C-2 and C-4. It was observed unsulfated internal chain units (13%), unsulfated non-reducing end units (0.4%), 4-*O*-sulfated units (20.3%), 2-*O*-sulfated units (25.6%), 3-*O*-sulfated units (5.1%), 2,4-di-*O*-sulfated unit (30.0%), and 2,3,4-tri-*O*-sulfated units (5.6%).

3.2. In vitro anticoagulant activity of β-G-SO_4

The anticoagulant activity of β-G-SO_4 was investigated by the classical coagulation assays APTT, TT and PT, using heparin (140 USP units/mg) as a reference. APTT is related to the intrinsic coagulation phase in plasma, PT with the extrinsic phase, and TT with the third coagulation phase in plasma. These tests are often referred to as functional tests because they monitor clot formation. (Leadley, Chi, Rebello, & Gagnon, 2000). Table 1 illustrates the anticoagulant activity of β-G-SO_4, β-G and heparin as measured by APTT, TT and PT. β-G-SO_4 was able to prolong APTT and TT in a concentration-dependent manner. β-G-SO_4 prolonged APTT and TT beyond 300 s at concentrations greater than 14 μg/ml, 2.4-fold more β-G-SO_4 than heparin being required to prolong APTT and TT to 300 s. At 8, 10 and 12 μg/ml of plasma, the anticoagulant activity was ~4.4 (119.8 ± 1.9 s), 7.4 (202.6 ± 1.0 s) and, 9.5 (261.1 ± 1.6 s) times greater than that of the control (27.4 s), in respect to APTT. The anticoagulant effect of the β-G-SO_4 was also evaluated by the TT test, the anticoagulant activity being about 8.6 (160.7 ± 1.1 s), 11.5 (215.9 ± 0.7 s), and 14.0 (262.5 ± 0.8 s) times greater than that of the control (18.7 s) at the same concentrations. The prolongation of APTT suggested inhibition of the intrinsic coagulation pathway, whereas prolongation of TT indicated inhibition of thrombin-mediated fibrin formation. Similar to heparin, the weakest effect was observed in the PT assay for the sulfated polysaccharide. Since the anticoagulant effect of heparin is not mainly mediated by a modulation of the extrinsic system, it appears that β-G-SO_4 is a poor inhibitor of the extrinsic pathway. The relative lack of a β-G-SO_4 effect on the PT is consistent with the observation that this test is also not sensitive to heparin and several other sulfated polysaccharides (Yoon et al., 2002). β-G did not inhibit APTT, TT and PT assays, because the presence of sulfated groups is an essential requirement for anticoagulant activity (Huang et al., 2003). β-G-SO_4 showed an important in vitro anticoagulation action, evidenced by a increased dose-dependence of APTT and TT. This action results from its high level of negative charge density produced by the sulfate groups (DS 1.95). The anticoagulant activity increased with the sulfate ester content, indicating the sulfate esters played a major role in its biological activity.

3.3. Stasis-induced venous thrombosis model

The antithrombotic efficacy of β-G-SO_4 was evaluated in rats by the stasis-induced venous thrombosis model, being observed on thrombus formation (Fig. 1). In vehicle-treated rats, the average thrombus weight was 23.4 ± 1.7 mg. The inhibition of thrombus formation increased in a dose-dependent manner. The percentage of thrombosis inhibition was 24.3, 64.5 and 83.5% at 0.05, 0.1 and 0.3 mg/kg body weight, respectively.

Table 1
Anticoagulant activity measured by activated partial thromboplastin time (APTT), thrombin time (TT), and prothrombin time (PT) of normal human plasma containing β-G, β-G-SO_4 and heparin

Derivative	APTT		TT		PT	
	Plasma (μg/ml)	Time (s)	Plasma (μg/ml)	Time (s)	Plasma (μg/ml)	Time (s)
Native glucan (β-G)	0	27.7±0.8[a]	0	18.7±0.5[a]	0	15.4±0.5[a]
	10	27.7±0.8[a]	10	18.7±0.5[a]	10	15.4±0.5[a]
	50	27.7±0.8[a]	50	18.7±0.5[a]	50	15.4±0.5[a]
	500	27.7±0.8[a]	500	18.7±0.5[a]	500	15.4±0.5[a]
	1000	27.7±0.8[a]	1000	18.7±0.5[a]	1000	15.4±0.5[a]
Sulfated glucan (β-G-SO_4)	0	27.7±0.8[a]	0	18.7±0.5[a]	0	15.4±0.5[a]
	2	45.0±1.2*	2	21.5±1.2*	2	16.9±1.1
	4	64.7±1.6*	4	56.1±0.6*	4	17.0±1.4
	6	95.8±0.9*	6	100.3±0.9*	6	18.8±1.1*
	8	119.8±1.9*	8	160.7±1.1*	8	20.3±1.1*
	10	202.6±1.0*	10	215.9±0.7*	10	21.3±1.0*
	12	261.1±1.6*	12	262.5±0.8*	12	22.3±1.2*
	14	>300[b]	14	>300[b]	14	26±1.1*
Heparin[c]	0	27.7±0.8[a]	0	18.7±0.5[a]	0	15.4±0.5[a]
	2	85.2±1.3*	2	59.6±1.1*	2	17.0±0.6*
	4	169.2±1.1*	4	105.3±0.9*	4	17.5±0.4*
	6	>300[b]	6	>300[b]	6	18.0±0.5*

Results are expressed as means time±SD ($n=6$/group). Statistical significance compared to control values is denoted by asterisks where *$p<0.05$ versus controls, Tukey's tests.
[a] Coagulation time similar to that of pooled human plasma. Standard values (26–37, 14–21, and 11–16 s for APTT, TT, and PT, respectively).
[b] No statistical tests were performed with times longer than 300 s.
[c] Heparin from porcine intestinal mucosa at a concentration of 140 USP units/mg.

β-G-SO_4 was an effective antithrombotic agent, and prevented thrombosis in a dose-dependent manner and a dose of 0.5 mg/kg body weight completely prevented thrombosis after 20 min stasis. Unfractionated heparin was more effective than β-G-SO_4 and completely prevented thrombosis in the same experimental model, at a dose of 0.1 mg/kg body weight. We evaluated the antithrombotic properties of β-G-SO_4 using a venous stasis model in rats (Berry et al., 1994). Many models of thrombosis have been developed for rodents, for antitrombotic efficacy on the venous side, several compounds were evaluated using methods that cause stasis of blood in inferior vena cava. In this model, a section of the vena cava is isolated and ligated so that the stasis in this region promotes thrombus generation. The lack of antithrombotic activity of the native glucan, demonstrated the importance of the sulfate groups for antithrombotic activity, as was observed for its anticoagulant action. When other sulfated polysaccharides were compared with β-G-SO_4, we observed that a chemically sulfated (1→3)-linked β-glucan completely inhibited thrombus formation, but only at a concentration 20 times greater than that of unfractionated heparin (Alban, Jeske, Welzel, Franz, & Fareed, 1995) and a fucosylated chondroitin sulfate completely inhibited thrombus formation at a dose of 1.5 mg/kg body weight, 25 times greater than unfractionated heparin (Mourão, Guimarães, Mulloy, Thomas, & Gray, 1998). Only a concentration 4 times greater of β-G-SO_4 was necessary to obtain the same effect as heparin.

3.4. Ex vivo determination of APTT

In order to determine whether β-G-SO_4 could exert its anticoagulant action in vivo, we tested for APTT ex vivo

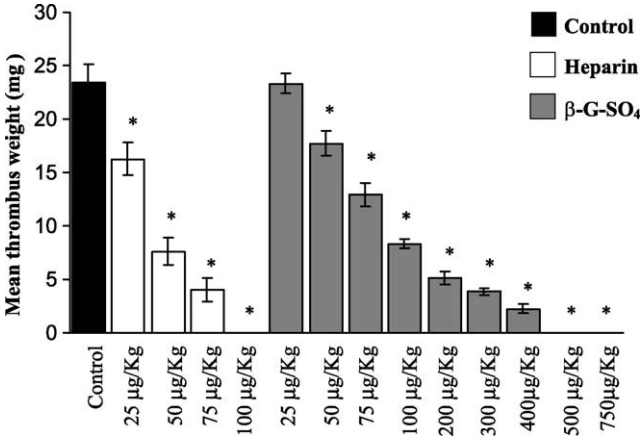

Fig. 1. Effects of β-G-SO_4 on the stasis-induced venous thrombosis model. After being cannulated, the abdomen of each animal was opened surgically and the abdominal vena cava was exposed, all its side-branches being ligated between the left renal and femoral veins. The β-G-SO_4 (25–750 μg/kg) (hatched columns), heparin (25–100 μg/kg) (open columns) or vehicle (PBS) (filled columns) was administered. After 5 min, thrombus formation was induced by the injection by thromboplastin (10 mg/kg) followed 1 min later by stasis of a 1 cm segment of the abdominal vein cava, which was maintained for 20 min. The formed thrombus was removed, immediately blotted twice on paper and weighed. For each treatment group ($n=6$) the mean thrombus weight±SD was determined. Statistical significance compared to control values is denoted by asterisks where *$p<0.05$ versus controls, Tukey's tests.

Table 2
Ex vivo anticoagulant activity of β-G-SO_4

Polysaccharide	APTT ex vivo[a]		Polysaccharide	APTT ex vivo[a]	
	Plasma (μg/kg)	Time (s)		Plasma (μg/kg)	Time (s)
Sulfated glucan	0	19.7±1.1[b]	Heparin[c]	0	19.7±1.1[b]
(β-G-SO_4)	200	53.2±0.8*		50	19,7±1.2
	300	82.2±0.8*		75	21.1±1.4
	500	150.7±1.2*		100	41.3±0.9*
	600	174.8±1.2*		200	57.3±0.8*
	800	246.3±0.9*		300	108.9±1.0*
	900	273.1±0.9*		400	161.1±1.5*
	1000	>300[d]		500	>300[d]

Results are expressed as means time±SD ($n=6$/group). Statistical significance compared to control values is denoted by asterisks where *$p<0.05$ versus controls, Tukey's tests.

[a] β-G-SO_4 and heparin at the indicated doses were administered intravenously 5 min before collection of blood samples (0.5 ml) into 3.8% trisodium citrate (9:1, v/v). APTT was determined on ex vivo rat plasma as described in Section 2.2.

[b] Coagulation time similar to that of pooled rat plasma.

[c] Heparin from porcine intestinal mucosa at a concentration of 140 USP units/mg.

[d] No statistical tests were performed with times longer than 300 s.

using a rat model. APTT determined for β-G-SO_4 doses of 200, 300, 500, 600, 800, 900 and 1000 μg/kg, after 5 min of drug administration, was increased in a dose-dependent manner by 2.7, 4.2, 7.6, 8.9, 12.5, 13.8 and >15.3-fold, respectively (Table 2). Twofold more β-G-SO_4 than heparin was required to prolong APTT to 300 s. This result indicates that the β-G-SO_4 has an anticoagulant action in vivo.

3.5. Tail transection bleeding time

The tail rat transection bleeding time was measured to determine the antihemostatic effects of β-G-SO_4. The effect of β-G-SO_4 was assessed based on blood loss from a cut rat tail, after intravascular administration of the polysaccharide.

Table 3
Effects of β-G-SO_4 on the tail transection bleeding time in rat

Compound	Dose (μg/kg)	Bleeding time (s)[a]	Increase (%)
Vehicle	–	960±50.2	–
β-G-SO_4	100	920±77.4	–
	250	960±82.7	–
	500	1425±125.5*	48.4
	1000	2000±164*	108.3
Heparin[b]	25	945±56.1	–
	50	1380±65.7*	43.7
	75	2075±44.1*	116.1
	100	2685±64.8*	179.7

Effects of β-G-SO_4 on the tail transection bleeding time in rat. Statistical significance compared to control values is denoted by asterisks where *$p<0.05$ versus controls, Tukey's tests.

[a] The left jugular vein of animals was cannulated for intravenous injection of (0.1–1 mg/kg), vehicle (PBS) or heparin (25–100 μg/kg). After 5 min, bleeding was induced by section of the extremity of the tail 3 mm from the tip. The tails were blotted with tissue paper every 30 s the time to cessation of bleeding was noted. For each treatment group the mean bleeding time±SD was determined for $n=6$/group.

[b] Heparin from porcine intestinal mucosa at a concentration of 140 USP units/mg.

Both heparin and β-G-SO_4 caused dose-dependent increases in the tail transection bleeding time (Table 3). In the vehicle-treated control group, the bleeding time averaged at 960±50.2 s ($n=6$). β-G-SO_4 caused an increase in bleeding time of 48.4 and 108.3% at 500 and 1000 μg/kg body weight, respectively. Heparin produced a strong hemorrhagic effect at 100 μg/kg: the increased in bleeding times was 179.7%. The ideal clinical anticoagulant would reliably and predictably inhibit thrombin without substantially increasing the risk of bleeding (Guglielmone, Agnese, Montoya, & Cabrera, 2002). According to our results, β-G-SO_4 produced a short prolongation of the rat tail transaction bleeding time, when compared with heparin.

4. Conclusion

Our study shows that β-G-SO_4 is effective in vitro and in vivo as an anticoagulant and antithrombotic agent for thrombosis in rats. These results suggest that β-G-SO_4 may be a promising antithrombotic agent for the treatment of various thrombotic diseases.

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