Anticoagulant properties of a high molecular weight polysaccharide fraction (1000RS) of the ascidian *Microcosmus exasperatus*

Diana C. Restrepo-Espinosa¹, Juliana Maria Motta², Felipe Castro O. de B. Teixeira², Yony Román³, Jhónny Colorado-Rios¹, Mauro S. G. Pavão², Alejandro Martínez³.

1 Grupo de Investigación Productos Naturales Marinos, Facultad de Ciencias Farmacéuticas y Alimentarias, Universidad de Antioquia, Calle 70 N° 52-21, Medellín, Colombia.  
2 Laboratório de Bioquímica e Biologia Celular de Glicocongejados, Instituto de Bioquímica Médica Leopoldo de Mels - Universidade Federal do Rio de Janeiro, CEP 21941-913, Rio de Janeiro, Brazil.  
3 Departamento de Bioquímica e Biologia Molecular - Universidade Federal do Paraná, CEP 81531-980, CP 19046, Curitiba, Paraná, Brazil.

ABSTRACT

Aims: The anticoagulant effect and cytotoxicity of a high molecular weight polysaccharide fraction (1000RS) obtained from the tunic of the ascidian *Microcosmus exasperatus* were evaluated.

Methods: Anticoagulant properties of 1000RS was evaluated by activated Partial Thromboplastin Time (aPTT), Thrombin Time (TT), Prothrombin Time (PT), anti-factor Xa and lupic anticoagulant (dRVVT) assays. Cytotoxicity was tested on murine hematopoietic cells using MTT assay.

Results: This galactose rich fraction showed to be a potential anticoagulant due to its inhibitory effect on the intrinsic coagulation pathway. At the same time, doses of this fraction do not affect the viability of the cells, which means that it can be used as a therapeutic agent.

Conclusion: The effect of anticoagulant in vitro of 1000RS occurs at innocuous doses, however, it still need to be tested using in vivo models and its cytotoxicity evaluated in normal human cell lines.

Keywords: anticoagulants, ascidian, *Microcosmus exasperatus*, polysaccharides.
INTRODUCTION

Sulfated polysaccharides from marine sources are currently being investigated as therapeutics in a wide range of diseases. Several reports on marine natural products have described anticoagulant and non-anticoagulant effects of novel molecules without the undesirable side effects of heparin, which interacts with distinct plasma proteins\(^2\)-\(^4\). Both positive and negative effects of heparin are related to its unique structural features such as heterogeneity of intra-chain domains and its highly negative charge and selective sulfation\(^1\). Recent studies have revealed other potential therapeutic effects of complex carbohydrates on many biological models of inflammation, cancer, metastasis, viral infection, atherosclerosis and acute tissue injury\(^4\)-\(^5\).

Diverse structural features such as sugar composition, type of linkage, molecular weight (Mw), distribution and charge density allow polysaccharides from different sources to interact with a wide variety of molecules involved in biological processes\(^6\)-\(^8\). For instance, the study of lacquer polysaccharides indicated that carboxyl and sulfate groups have a synergistic anticoagulant action; the presence of 6-O-SO\(_3\) in the side chains is a key feature for this activity while sulfation at 2-position has not such effect. Sulfation of alginate, a polymer of (1→4) linked β-D-mannuronic acid and α-L-guluronic acid that naturally contain carboxyl groups, increases its anticoagulant properties, at the same time, position of carboxyl groups may also have impact on this bioactivity\(^2\). The fucoidan obtained from Fucus vesiculosus displays procoagulant effect when it presents a minimal degree of sulfation of 0.5 and a chain of at least 70 sugar units\(^9\).

The tunic and organs of different ascidians have been studied as a source of new bioactive natural products\(^7\)-\(^10\). Among those compounds, polysaccharides like L-galactans have shown to be the main constituent of this filter feeding marine invertebrate tunic\(^11\),\(^12\). These carbohydrates present different size and structure and they likely have distinct biological effects; for instance tunic of Herdmania monus is mainly composed by a 3-O-sulfated 4-linked α-L-galactan, the galactan from Styela plicata is also substituted by sugar residues at 2-position and Ciona intestinalis possess a poorly sulfated and highly branched α-L-galactan\(^12\)-\(^13\).

Here, we aimed to evaluate anticoagulant and cytotoxic effects of a galactose-rich polysaccharide fraction obtained from the tunic of Microcosmus exasperatus. Data obtained revealed a mechanism of coagulation different from heparin-like anticoagulants at innocuous doses.

MATERIAL AND METHODS

Biological material

The ascidia Microcosmus exasperatus was collected at Guaíabara Bay, Rio de Janeiro, Brazil. Tunics were separated from the internal organs and cleaned, depigmented and freeze-dried before extraction.

Extraction of the polysaccharides from the tunic

The defatted and dried tunics (64.9 g) of M. exasperatus were extracted by three successive proteolytic digestions in a 0.1 M sodium acetate buffer containing 5 mM EDTA, 5 mM cysteine and papain (10%); each enzymatic digestion was incubated for 24 h at 60 °C\(^4\). Supernatants of the three digestions were collected and polysaccharides present in this final solution were precipitated by the exhaustive addition of ethanol and kept at 4 °C for 24 h. Finally, the crude precipitate of polysaccharides was obtained by centrifugation (4500 rpm for 10 min at 15 °C), dialyzed using a 1 kDa MWCO dialysis tube and freeze-dried.

Fractionation of the crude precipitated of polysaccharides

The isolated extract (4.1 g) was dialyzed with deionized water by using a 1000 kDa molecular weight cut-off membrane; retained and eluted fractions were collected, evaporated and freeze-dried. The retained fraction was subsequently solubilized and submitted to freezing-thawing cycles until precipitation stopped; supernatants were separated from precipitates through centrifugation for 10 min at 10 °C.

The presence of negative charges in the carbohydrates, which could be assigned to sulfate groups, was tested through the complex formed between sulfated polysaccharides and dimethylmethylene blue (DMB).

Glycosyl composition analysis

1000RS fraction (2.0 mg) was hydrolyzed with 2.0 M trifluoroacetic acid (TFA) for 8 h at 100 °C. The obtained hydrolyzate was reduced (18 h) and acetylated (12 h) by using NaBH\(_4\) (pH 9.0) and acetic anhydride:pyridine (1:1, v/v) at room temperature, respectively. Alditol acetates were separated by GC–MS (Varian Saturn 2000R-3800) using a DB-225 column (30 m × 0.25 mm i.d) and identified by comparing their retention time and mass fragmentation pattern with the alditol acetates of standard monosaccharides\(^15\).
Anticoagulant properties of a high molecular weight polysaccharide fraction (1000RS) of the ascidian Microcosmus exasperatus

Blood clotting assays

Anticoagulant activity of the fraction 1000RS at different concentrations (11.9-44.8 µg/mL) was determined by measuring the clotting time (in seconds) of unfrozen human platelet-poor plasma using an ACL ELITE PRO coagulometer at 37 °C. The activated partial thromboplastin time (aPTT), prothrombin time (PT), thrombin time (TT), anti-Xa factor and a dilute Russell’s Viper Venom time-based assay (Lupus anticoagulant-dRVVT) reagents were prepared and used according to the manufacturer’s (HemosIL®, Instrumentation Laboratory) instructions. Clot formation in PT, TT, aPTT and dRVVT assays was measured at 660 nm and calcium chloride was added to trigger coagulation reactions in aPTT assay. Unfractionated heparin (200.47 IU/mg; 6th International Standard) was used as positive control. Results were expressed as the mean of the response ± standard deviation (SD).

Chromogenic assay (anti-Xa factor) was made by incubating (30 s) 1000RS with plasma, Chromogenic substrate (anFXasub, 15 s) and Factor Xa reagent (anFxaRgt, 90 s). The concentration of residual Factor Xa was measured (60 s) by detection of paranitroaniline at 405 nm.

Cellular viability of hematopoietic cells from C57BL/6 mice (MTT assay)

Hematopoietic cells were obtained from the bone marrow of femurs and tibias of six male and female between 4 and 6 months C57BL/6 mice. After collecting bone marrow, cells were treated with ACK (Ammonium-Chloride-Potassium) solution to eliminate red cells and then incubated overnight in a flask with RPMI medium containing 10% (v/v) fetal bovine serum. Afterwards, only non-adherent cells that correspond to hematopoietic cells were collected and used subsequently. Experiments were done in 96-well plates with 5 x 10^5 cells/well (100 µL) and three different concentrations (0.5, 0.25 and 0.0078 mg/mL) of 1000RS and heparin were used for 24 h of culture. After this period, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl-2H-tetrazoliumbromide (MTT) aqueous solution (5 mg/mL, 20 µL/well) for 2 h, centrifuged at 200 g for 8 min and the supernatant solution was discarded. Formazan crystals were dissolved with a solution (200 µL) containing triton-X, HCl and isopropyl alcohol. Finally, the absorbance was measured at 560 nm in a plate reader. Cell viability was expressed as the percentage of viable cells normalized using control cells. Results are expressed as mean values and standard deviation (SD) of six different experiments in triplicate.

RESULTS

1000RS represents a yield of 5.3% of the crude extract of polysaccharide obtained from M. exasperatus. It was a fraction mainly composed by galactose (>90%), while other monomers like arabinose, xylose, mannose and glucose are present in minor proportion. A solution of this fraction formed a complex with the cationic dye DMB and the intense coloration suggests that this fraction possess negatively charged polysaccharides.

To evaluate how this fraction affects the secondary hemostasis, aPTT, TT and PT assays (Table 1) were performed in vitro.

| Sample | Amount (µg/mL) | Clotting time (s) |
|--------|----------------|------------------|
|        |                | aPTT | TT | PT  |
| Control | 0.0            | 26.7 ± 0.0 | 15.9 ± 0.0 | 13.9 ± 0.0 |
| Heparin  | 1.9            | 86.8 ± 6.4 | 169.5 ± 0.2 | 15.5 ± 0.3 |
|         | 2.8            | 163.7 ± 21.7 | 169.5 ± 0.3 | 16.9 ± 0.4 |
|         | 3.7            | 247 ± 0.0 | 169.5 ± 0.4 | 18.4 ± 0.6 |
| 1000RS   | 11.9           | 53.4 ± 1.0 | 16.3 ± 0.1 | 13.9 ± 0.2 |
|         | 23.3           | 72.3 ± 1.8 | 16.1 ± 0.2 | 15.1 ± 0.5 |
|         | 34.2           | 96.0 ± 4.0 | 19.4 ± 0.2 | 15.4 ± 0.1 |
|         | 44.8           | 129.0 ± 9.5 | 21.9 ± 0.8 | 16.4 ± 0.2 |

Clotting time (n=3) are expressed (in seconds) as the mean ± SD.

1000RS prolonged the aPTT in a dose dependent manner, whereas the TT and PT were poorly affected by the different concentrations of 1000RS (Fig.1).

This fraction did not prolong the prothrombin and thrombin, suggesting that the inhibition of the coagulation may be due to an interaction with different coagulation factors such as factor X, V and II of the common pathway. For this reason the effect of 1000RS on factor X was evaluated through the anti-Xa assay. At the same time, to better understand how this fraction interferes with the thrombus formation, the lupus anticoagulant assay was also carried out (Table 2).

All protocols used for the animal experiments were approved by the Animal Ethics committee of the Hospital Naval Marcílio Dias, Rio de Janeiro (Law n° 11.794 of 8 October 2008, Legislative Decree n° 6.899 of 15 July 2009).

Table 1. Anticoagulant activity of the 1000RS fraction (Fig.1)
The anticoagulant activity of 1000RS is not due to the inhibition of the factor X, since the amount of residual Xa at different concentrations of the fraction was lower than the control (Table 2). At the same time, the activity of 1000RS as a lupus anticoagulant was evaluated using the diluted Russell’s Viper Venom test (dRVVT); this fraction did not impair the activation of factor X by an enzyme present in the viper venom.

Furthermore, as the ascidian polysaccharide fraction showed to be a potential anticoagulant fraction with probably less adverse effects than heparin or other known anticoagulants, it is important to determine if this fraction contains carbohydrates that could be cytotoxic at the anticoagulant doses. For this purpose, in vitro cell viability was evaluated with the MTT assay using hematopoietic cells obtained from C57BL/6 mice (Fig. 2).
After treatment of cells with 1000RS and heparin, the viability observed has not significantly changed and sometimes it was even higher than the control (set as 100% of viable cells). These results indicate that polysaccharides that constitutes 1000RS fraction are potential anticoagulant agents with non-cytotoxic effect at least at doses used here.

**DISCUSSION**

Previous reports on the polysaccharide composition of ascidian tunics had demonstrated that this tissue is mainly constituted by a high molecular weight galactan, which have different sulfation pattern according to the specie of tunicate\textsuperscript{17,18}. This new fraction from *M. exasperatus* also showed the presence of polysaccharides that are mainly constituted by galactose residues, which may be negatively charged along the chain. Additionally, this fraction was retained by the 1000 kDa MWCO membrane, which supports the existence of high molecular weight polysaccharides in this ascidian.

Plasma proteases that are required for clot generation are common targets of several anticoagulants. Although those substances can effectively avoid thrombus formation and growth, their use represents a significant risk of excessive bleeding and hemostatic disorders\textsuperscript{19}. Considering that sulfated polysaccharides have frequently been shown as anticoagulant substances, we hypothesized that 1000RS could be a potential inhibitor of the coagulation process\textsuperscript{20,21}.

1000RS can significantly inhibit the intrinsic coagulation pathway, but neither the extrinsic pathway nor the thrombin-mediated fibrin formation are affected by this polysaccharide fraction\textsuperscript{22}. Thus, it does not alter the coagulation process by interacting with factor VII or factor II (prothrombin) of the extrinsic and common coagulation pathways, respectively\textsuperscript{23}. Additionally, the poor effect on thrombin and prothrombin time suggests that the extent of fibrinogen is normal and remains unaffected in the presence of this fraction, resulting in normal fibrin formation. Also, the lack of inhibitory activity on thrombin indicates that thrombin-induced activation of factor V, factor VIII and platelets, essential components of blood coagulation, remains unaltered\textsuperscript{24}.

The aPTT prolongation by 1000RS was 22.2-fold lower than heparin; the clotting time increases, in average, 2.36 s (r= 0.9619) and 59.36 s (r= 0.9830) after the treatment at different concentrations of the polysaccharide fraction and heparin, respectively. Although heparin is more potent, this result showed that 1000RS has anticoagulant activity and probably inhibits clot formation through the interaction with one or more key intrinsic pathway clotting factors. Heparin acts by blocking activated factor X (factor Xa)\textsuperscript{23,24}.

The amount of residual factor Xa after treatment with this fraction was lower than control at the different concentrations tested\textsuperscript{25}, which means that it does not bind to the active site of activated Factor X (FXa) and allows the interaction between FXa and its substrate. Therefore the thrombin generation can be normally achieved\textsuperscript{26}. Furthermore, an enzyme present in the viper venom did not activate factor X in the presence of 100RS fraction; with this activated factor in the presence of factor V and phospholipid, prothrombin is converted to thrombin\textsuperscript{27-29}. Hence, it does not decrease the availability of phospholipids to support this reaction and the clot is formed at all the concentrations tested. The fact that this fraction does not act as a lupus anticoagulant, along with the negative effect on factor X and thrombin time, confirmed that it is not an inhibitor of the common coagulation pathway. This indicates that the anticoagulant effect is mainly due to the inhibition of an intrinsic coagulation factor such as prekallikrein, high molecular weight kininogen, XII, XI, IX and VIII factors\textsuperscript{30,29}. Consequently, it can be assumed that 1000RS does not behave as a heparin-like anticoagulant, being a more specific inhibitor and possibly reducing some side effects related to the continuous use of anticoagulants such as heparin. Nevertheless, the fact that this inhibitor may acts directly against a specific coagulation factor could represent a bleeding risk factor.

This fraction did not display cytotoxic effect on hematopoietic cells obtained from C57BL/6 mice at anticoagulant doses. Cellular viability was higher than that obtained with the control at some of tested concentrations. It could directly correlate to the increase in number of metabolic active cells. Those murine hematopoietic stem cells continue viable after treatment at different concentrations of 1000RS and heparin.

**CONCLUSION**

The highest anticoagulant effect of 1000RS occurs at innocuous doses, however, the anticoagulant effect and innocuousness should be done using *in vivo* anticoagulant models and more accurate viability assays with normal human cell lines. This outcome is also promising when comparing 1000RS with other bioactive polysaccharides such cellulose, starch sulfuric acid esters and chitin disulfuric acid, which have been shown to be considerably cytotoxic\textsuperscript{30}.

Regarding the lack of activity on factor X or thrombin, it could be assumed that 1000RS does not interact with some serine protease inhibitors (serpins) such as antithrombin III (ATIII) and heparin cofactor II (HCII); regulatory plasmatic proteins which mainly inactivate these two serine proteases in plasma\textsuperscript{31}. Once again, it supports the idea that the ascidian polysaccharide fraction does not have the same mech-
anism of action shared by several sulfated polysaccharides that, like heparin, has an anticoagulant action based on the potentiation of these natural inhibitors. This opens the possibility of having a new anticoagulant substance that can catalyze the allosteric change of other serpins like kallikrein (PI4), protein C inhibitor (PCI), Protein Z-dependent protease inhibitor (ZPI), which can inhibit some homeostatic blood proteases of the intrinsic pathway like kallikrein, active protein C and FXIa, respectively. It is also possible that it can act joining the protease with its respectively serpin through a “bridge” made of its glycosidic chain.

ACKNOWLEDGEMENTS

The authors would like to thank the Colombian agency Departamento Administrativo de Ciencia, Tecnología e Innovación (COLCIENCIAS), Universidad de Antioquia and the company Humax Pharmaceutical S.A for qualifying and funding the project 463359937207. Research group Marine Natural Products acknowledges to Estrategia de Sostenibilidad 2014-2015 Codi (Universidad de Antioquia) and Programa de Doctorado Nacional (Convocatoria 567 de 2012-COLCIENCIAS) for financial support for PhD student Diana C. Restrepo-Espinosa to develop this work. We thank also the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and professors Thales R. Cipriani and Marcello Iacomini for their help in the isolation of the fraction.

REFERENCES

1. Paulo Mourão. Perspective on the Use of Sulfated Polysaccharides from Marine Organisms as a Source of New Antithrombotic Drugs. Mar Drugs 2015;13(5):2770-84.
2. Yang J, Du Y, Huang R, Wan Y, Wen Y. The structure–anticoagulant activity relationships of sulfated lacquer polysaccharide. Int J Biol Macromol 2005;36(1):9-15.
3. Cassinelli G, Naggi A. Old and new applications of non-anticoagulant heparin. Int J Cardiol 2016;212:514–521.
4. Ling L, Camilleri ET, Helledie T, Samsonraj RM, Titmash DM, Chua RJ, et al. Effect of heparin on the biological properties and molecular signature of human mesenchymal stem cells. Gene 2016;576(1 Pt 2):292.
5. Page C. Heparin and Related Drugs: Beyond Anticoagulant Activity. Int Sch Res Not 2013;2013:e910743.
6. Zhang Z, Till S, Jiang C, Knappe S, Reutterer S, Scheiflinger F, et al. Structure-activity relationship of the pro- and anticoagulant effects of Fucus vesiculosus fucoidan. Thromb Haemost 2014;111(3):429-37.
7. Kozłowski EO, Lima PC, Vicente CP, Lotufo T, Bao X, Sugahara K, et al. Dermatan sulfate in tunicate phylogeny: Order-specific sulfation pattern and the effect of [→4IdoA(2-Sulfate) β1→3GalNAc(4-Sulfate)β1→] motifs in dermatan sulfate on heparin cofactor II activity. BMC Biochem 2011;12:29.
8. Lee D-H, Hong J-H. Immune-Enhancing Effects of Polysaccharides Isolated from Ascidian (Halocynthia roretzi) Tunic. ResearchGate 2015;44(5):673-80.
9. Núñez-Pons L, Carbone M, Vázquez J, Rodriguez J, Nieto RM, Varela MM, et al. Natural Products from Antarctic Colonial Ascidians of the Genera Aplidium and Synoicum: Variability and Defensive Role. Mar Drugs 2012;10(8):1741-64.
10. Sarhadizadeh N, Alkhami M, Ehsanpour M. Evaluation of antibacterial, antifungal and cytotoxic agents of Ascidian Phallusia nigra (Savigny, 1816) from Persian Gulf. Eur J Exp Biol 2014;4(1):250-253.
11. Pereira MS, Melo FR, Mourao PAS. Is there a correlation between structure and anticoagulant action of sulfated galactans and sulfated fucans? Glycobiology 2002;12(10):573-80.
12. Santos JA, Mulloy B, Mourao PAS. Structural diversity among sulfated alpha-L-galactans from ascidians (tunicates). Studies on the species Ciona intestinalis and Herdmania mornus. Eur J Biochem 1992;204(2):669-77.
13. Pomin VH. Phylogeny, structure, function, biosynthesis and evolution of sulfated galactose-containing glycans. Int J Biol Macromol 2016;84:372-9.
14. Gomes AM, Kozłowski EO, Pomin VH, de Barros CM, Zaganeli JL, Pavão MSG. Unique extracellular matrix heparan sulfate from the bivalve Nodilpecten nodosus (Linnaeus, 1758) safely inhibits arterial thrombosis after photochemically induced endothelial lesion. J Biochem Chem 2010;285(10):7312-23.
15. do Nascimento GE, Corso CR, de Paula Werner MF, Baggio CH, Iacomini M, Cordeiro LMC. Structure of an arabinogalactan from the edible tropical fruit tamarillo (Solonum betaceum) and its antinociceptive activity. Carbohydr Polym 2015;116:300-6.
16. Stockert JC, Blázquez-Castro A, Cañete M, Horobin RW, Villanueva Á. MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. Acta Histochem 2012;114(8):785-96.
17. Riss TL, Moravec RA, Niles AL, Benink HA, Worzella TJ, Minor L. Cell Viability Assays [Internet]. Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2015.
18. Pomin VH, Mourão PA de S. Structure versus anticoagulant and antithrombotic actions of marine sulfated polysaccharides. Rev Bras Farmacogn 2012;22(4):921-8.
Anticoagulant properties of a high molecular weight polysaccharide fraction (1000RS) of the ascidian Microcosmus exasperatus

19. Gailani D, Renné T. Intrinsic Pathway of Coagulation and Arterial Thrombosis. Arterioscler Thromb Vasc Biol 2007;27(12):2507-13.

20. Björk I, Lindahl U. Mechanism of the anticoagulant action of heparin. Mol Cell Biochem 1982;48(3):161-82.

21. Faham S, Hileman R, Fromm J, Lindhardt R, Rees D. Heparin structure and interactions with basic fibroblast growth factor. Science 1996;271(5252):116-20.

22. Vasconcelos AFD, Dekker RFH, Barbosa AM, Carbonero ER, Silveira JLM, Glauser B, et al. Sulfonation and anticoagulant activity of fungal exocellular β-(1→6)-d-glucan (lasiodiplodan). Carbohydr Polym 2013;92(2):1908-14.

23. Hood JL, Eby CS. Evaluation of a Prolonged Prothrombin Time. Clin Chem 2008;54(4):765-8.

24. Hirsh J, Anand SS, Halperin JL, Fuster V. Mechanism of Action and Pharmacology of Unfractionated Heparin. Arterioscler Thromb Vasc Biol 2001;21(7):1094-6.

25. Newall F. Anti-factor Xa (anti-Xa) assay. Methods Mol Biol 2013;992:265-72.

26. Cabral K, Ansell J. The role of factor Xa inhibitors in venous thromboembolism treatment. - PubMed - NCBI. Vasc Health Risk Manag 2015;11:117-23.

27. Jacquot C, Wool GD, Kogan SC. Dilute Russell Viper Venom Time Interpretation and Clinical Correlation: A Two-Year Retrospective Institutional Review. Blood 2016 [cited 2017 mar 26];128. Available from: http://www.bloodjournal.org/content/128/22/26097?sso-checked=true

28. Moore GW, Rangarajan S, Savidge GF. The Activated Seven Lupus Anticoagulant Assay Detects Clinically Significant Antibodies. Clin Appl Thromb 2008;14(3):332-7.

29. Radhakrishnan K. The dilute Russell’s viper venom time. Methods Mol Biol 2013;992:341-8.

30. Jayakumar R, Nwe N, Tokura S, Tamura H. Sulfated chitin and chitosan as novel biomaterials. Int J Biol Macromol 2007;40(3):175-81.

31. Schoen P, Lindhout T, Hemker H. Ratios of anti-factor Xa to antithrombin activities of heparins as determined in recalcified human plasma. - PubMed - NCBI. Br J Haematol 1992;81:255-62.

32. Bhakuni T, Ali MF, Ahmad I, Baner S, Ansari S, Jairajpuri MA. Role of heparin and non-heparin binding serpins in coagulation and angiogenesis: A complex interplay. Arch Biochem Biophys 2016;604:128-42.

33. Law RH, Zhang Q, McGowan S, Buckle AM, Silverman GA, Wong W, et al. An overview of the serpin superfamily. Genome Biol 2006;7(5):216.