Journal of Natural Medicines (2019) 73:614–619
https://doi.org/10.1007/s11418-019-01289-5

NOTE

Rhamnan sulfate extracted from *Monostroma nitidum* attenuates blood coagulation and inflammation of vascular endothelial cells

Takayuki Okamoto[1] · Nobuyuki Akita[2] · Masahiro Terasawa[3] · Tatsuya Hayashi[4] · Koji Suzuki[5]

Received: 8 November 2018 / Accepted: 5 February 2019 / Published online: 22 February 2019

Abstract

Rhamnan sulfate (RS) is a polysaccharide with a rhamnose backbone isolated from *Monostroma nitidum*. Like heparin, it exerts anticoagulant activity in the presence of antithrombin. Endothelial cells facilitate the crosstalk between blood coagulation and vascular inflammation. In this study, we compared the effect of RS with that of heparin on blood coagulation and vascular endothelial cells in the presence or absence of inflammatory factors, using human umbilical vein endothelial cells. We found that RS significantly enhances inhibition of thrombin and factor Xa in the presence of antithrombin as well as heparin, and that RS inhibits tissue factor expression and von Willebrand factor release from the endothelial cells treated with or without lipopolysaccharide, tumor necrosis factor-α, or thrombin. Heparin did not show any effects on endothelial cell inflammation. Our findings suggest that RS, like heparin, is an antithrombin-dependent anticoagulant and, unlike heparin, is a potent anti-inflammatory agent acting on vascular endothelial cells.

Keywords *Monostroma nitidum* · Seaweed · Rhamnan sulfate · Anti-coagulation · Anti-inflammation · Vascular endothelium

Abbreviations

APTT	Activated partial thromboplastin time
HUVEC	Human umbilical vein endothelial cells
LPS	Lipopolysaccharide
PT	Prothrombin time
RS	Rhamnan sulfate
TF	Tissue factor
TNF	Tumor necrosis factor
VWF	Von Willebrand factor

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11418-019-01289-5) contains supplementary material, which is available to authorized users.

✉ Koji Suzuki
suzukiko@suzuka-u.ac.jp

[1] Department of Pharmacology, Faculty of Medicine, Shimane University, Izumo, Shimane, Japan

[2] Department of Clinical Engineering, Faculty of Medical Engineering, Suzuka University of Medical Science, Suzuka, Mie, Japan

[3] Konan Chemical Manufacturing Co., Ltd., Yokkaichi, Mie, Japan

[4] Department of Biochemistry, Mie Prefecture College of Nursing, Tsu, Mie, Japan

[5] Department of Molecular Pathobiology, Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science, Suzuka, Mie, Japan

Introduction

Seaweeds are traditionally consumed by people inhabiting coastal areas in East Asia. They contain abundant amounts of vitamins, minerals, soluble dietary fibers, peptides, and lipids. Recent studies have demonstrated that seaweeds and seaweed-derived products possess promising health-promoting properties suitable for enriching functional foods. *Monostroma* (*M.*) *nitidum* is a green alga that grows in shallow waters near Japan. Rhamnan sulfate (RS), a sulfated polysaccharide, is the main constituent of the intercellular space in *M. nitidum* [1]. The main repeating unit of RS consists of rhamnose with a sulfate group substituent that forms long linear chains with branched side chains [2–4]. Several in vitro and in vivo studies have reported that RS has anticoagulant [5, 6], antiviral [3, 7, 8], and anti-obesity [1] activities.

In a normal blood vessel, endothelial cells play an important role in the regulation of blood coagulation and platelet activation by synthesizing various anticoagulant factors, such as thrombomodulin, heparan sulfate proteoglycans

(antithrombin-binding polysaccharide), nitric oxide, prostaglandin I_2, and ecto-ATP/ADPase [9]. However, with respect to thrombus formation during inflammation, endothelial cells play an important role in the initiation of blood coagulation and platelet activation. Upon inflammation, activated endothelial cells express tissue factor (TF) to initiate extrinsic blood coagulation, and also secrete von Willebrand factor (VWF) to create a scaffold for platelet aggregation [10, 11].

Heparin, another sulfated polysaccharide, binds to antithrombin in the blood and changes its conformation to enhance the inhibitory activity of antithrombin toward several serine proteases of the blood coagulation system, mainly thrombin and factor Xa [12, 13].

Previous studies have shown that RS inhibits thrombin in the presence of antithrombin as well as heparin [5, 6]; however, the effect of RS on factor Xa is unknown. Moreover, the effect of RS on vascular endothelial cell activation during inflammation is not yet well understood. In this study, we investigated the effect of RS, compared with that of heparin, on blood coagulation and on vascular endothelial cells in the presence or absence of inflammatory factors using human umbilical vein endothelial cells (HUVECs).

Results and discussion

Preparation and characterization of RS

RS was extracted from *M. nitidum* by hot water and purified using anion-exchange column chromatography as described previously [3]. The purified RS eluted as a single peak with a shoulder in front of the main peak (M_w 5.6×10^5) in gel permeation chromatography.

Several studies have shown that RS has anticoagulant activity [5, 6, 14–16]. To characterize the anticoagulant activity of our RS preparation, we evaluated the effect of RS on activated partial thromboplastin time (APTT) and prothrombin time (PT) (Supplementary Fig. 1). The data show that RS prolonged PT and APTT, but the anticoagulant activity of RS was much lower than that of heparin, almost one-tenth of the activity of heparin. We confirmed that the anticoagulant activity of RS toward thrombin requires antithrombin (Fig. 1a), as previously described [5], and found that RS also inhibits factor Xa in the presence of antithrombin (Fig. 1b).

Effect of RS on endothelial cell activation under inflammation

We next examined the effect of RS on the inflammation of endothelial cells. Proinflammatory stimuli, such as lipopolysaccharide (LPS, endotoxin from Gram-negative bacteria), tumor necrosis factor (TNF)-α (a major proinflammatory cytokine), and the coagulation factor, thrombin, have been shown to significantly induce the expression of TF and VWF in activated endothelial cells [17–20]. TF expression on the cell surface contributes to the initiation of cell-based thrombus formation [21], and VWF released into the blood stimulates platelet activation in thrombotic microangiopathy [11].

We first exposed cultured HUVECs to RS or heparin. RS had no impact on cell survival according to the results of a cytotoxicity assay (Supplementary Fig. 2), and slightly decreased both TF expression and VWF release in the absence of LPS treatment (Supplementary Fig. 3a, c). Heparin weakly decreased TF expression and increased VWF release in the absence of LPS treatment (Supplementary Fig. 3b, d).

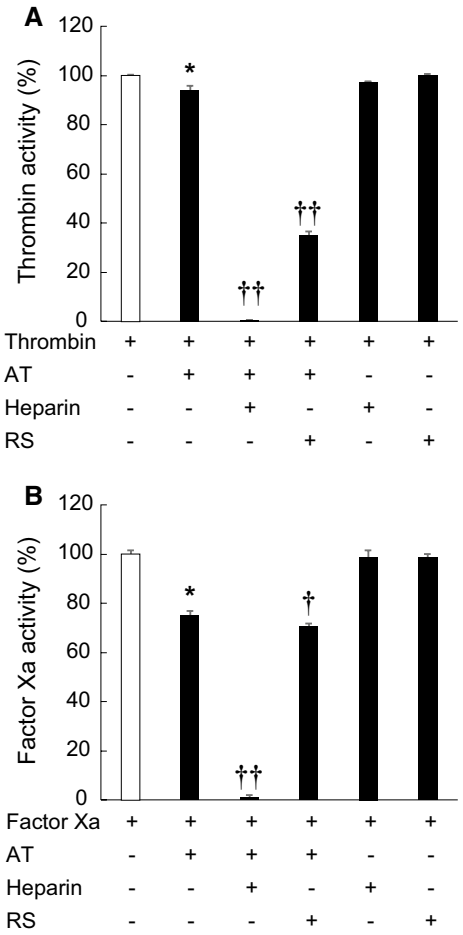

Fig. 1 Effects of RS or heparin on thrombin and factor Xa in the presence or absence of antithrombin. Residual activity of thrombin (2.5 nM) treated with 2 μg/ml heparin or RS in the presence or absence of 5 nM antithrombin (**a**). Residual activity of factor Xa (2.5 nM) treated with 2 μg/ml heparin or RS in the presence or absence of 25 nM antithrombin (**b**). Experimental conditions are described in 'Experimental'. Data are shown as the mean ± SD of three experiments. $^*P<0.05$ compared to thrombin or factor Xa without antithrombin. $^{\dagger}P<0.05$ compared to factor Xa plus antithrombin. $^{\dagger\dagger}P<0.01$ compared to thrombin or factor Xa plus antithrombin

We then exposed cultured HUVECs to LPS and evaluated TF expression and VWF release in the presence of RS or heparin. As shown in Fig. 2a, c, LPS strongly stimulated the endothelial cells, increasing TF expression and VWF release, whereas RS significantly suppressed this TF expression and VWF release in a concentration-dependent manner. In contrast, heparin did not significantly affect LPS-induced TF expression (Fig. 2b) and only slightly enhanced LPS-induced VWF release (Fig. 2d).

To determine whether RS suppresses endothelial cell activation induced by other proinflammatory stimuli, we next treated HUVECs with TNF-α and thrombin and evaluated TF expression and VWF release. RS strongly decreased TF expression and slightly suppressed VWF release induced by TNF-α (Fig. 3a, c). Heparin did not alter TNF-α-induced TF expression or VWF release (Fig. 3b, d). The increases in TF expression and VWF release in endothelial cells induced by thrombin were similar to those induced by LPS and TNF-α. RS reduced thrombin-induced TF expression by approximately 50% (Fig. 4a) and tended to suppress VWF release (Fig. 4c). Treatment with low-concentration heparin increased thrombin-induced TF expression (Fig. 4b), but heparin did not affect thrombin-induced VWF release (Fig. 4d). In these experiments, we cultured and treated HUVECs in serum-free media; thus, the present findings suggest that endothelial cell activation by proinflammatory stimuli was directly suppressed by RS without inhibition of thrombin protease activity, because antithrombin was absent in the culture medium. These findings indicate that, unlike heparin, RS has an inhibitory effect on endothelial cell activation induced by various types of inflammatory stimuli.

The mechanism of the RS-dependent anti-inflammatory effect on vascular endothelial cells is still unknown. Previous reports on the effect of the glycocalyx on vascular endothelial cells under inflammatory conditions [22] may be relevant to the anti-inflammatory effects of RS on endothelial cells. The glycocalyx is found on the surface of animal cells and is composed of glycoconjugates and proteoglycans, including sulfated polysaccharides such as heparan sulfate, chondroitin sulfate, and dermatan sulfate [23]. It has been shown that the glycocalyx in vascular endothelial cells is involved in the regulation of blood coagulation and inflammation by affecting the binding of leukocytes, platelets, and coagulation/inflammation-related factors [24]. Thus, similar to that of the glycocalyx, the sulfated polysaccharide, RS, may function to protect endothelial cells from inflammatory factors.

Recently, anticoagulant therapeutic approaches have been shown to be effective in the prevention of thrombotic disorders such as stroke, venous thromboembolism, and cardiovascular events. Based on the preventive activities of RS against blood coagulation and endothelial inflammation, RS

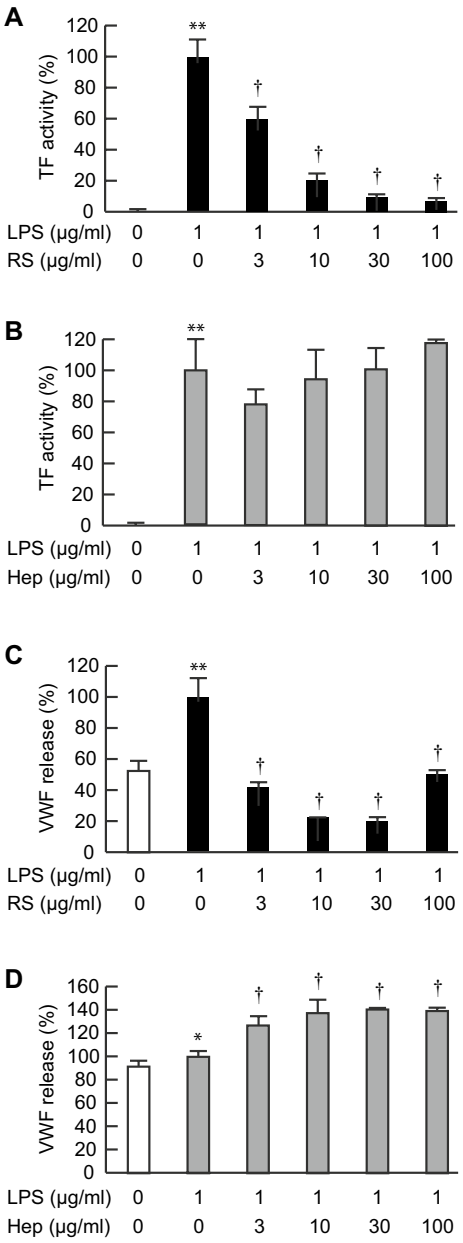

Fig. 2 Effect of RS or heparin on LPS-induced TF expression and VWF release in HUVECs. TF expression in HUVECs treated with LPS (1 μg/ml) or phosphate-buffered saline (PBS) in the presence of RS (0–100 μg/ml) (**a**) or heparin (Hep) (0–100 μg/ml) (**b**). TF activity of HUVECs treated with LPS in the absence of RS or heparin was designated to be 100%. VWF release from HUVECs treated with LPS (1 μg/ml) or PBS in the presence of RS (0–100 μg/ml) (**c**) or heparin (Hep) (0–100 μg/ml) (**d**). Concentration of VWF in culture medium of HUVECs treated with LPS in the absence of RS or heparin was designated to be 100%. Open columns indicate TF activity and VWF concentration under conditions without LPS, RS, and heparin in PBS. Black and gray columns indicate TF activity and VWF concentration in the presence of LPS. Data are shown as the mean ± SD of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to reactions without LPS treatment in the absence of RS or heparin. $^{\dagger}P < 0.01$ compared to LPS treatment in the absence of RS or heparin

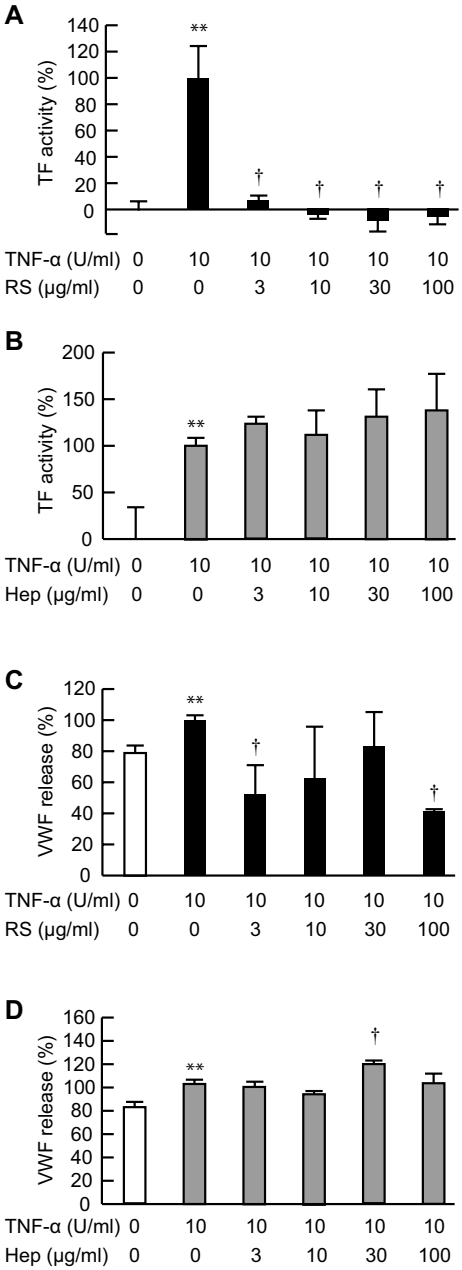

Fig. 3 Effect of RS or heparin on TNF-α-induced TF expression and VWF release in HUVECs. TF expression in HUVECs treated with TNF-α (10 U/ml) or PBS in the presence of RS (0–100 μg/ml) (**a**) or heparin (Hep) (0–100 μg/ml) (**b**). TF activity of HUVECs treated with TNF-α in the absence of RS or heparin was designated to be 100%. VWF release in HUVECs treated with TNF-α (10 U/ml) (**c**) in the presence of RS (0–100 μg/ml). VWF release from HUVECs treated with TNF-α (10 U/ml) in the presence of heparin (Hep) (0–100 μg/ml) (**d**). Concentration of VWF in culture medium treated with TNF-α in the absence of RS or heparin was designated to be 100%. Open columns indicate TF activity and VWF concentration under conditions without TNF-α, RS, and heparin in PBS. Black and gray columns indicate TF activity and VWF concentration in the presence of TNF-α. Data are shown as the mean ± SD of four independent experiments. $^{**}P < 0.01$ compared to reactions without TNF-α treatment in the absence of RS or heparin. $^{\dagger}P < 0.01$ compared to TNF-α treatment in the absence of RS or heparin

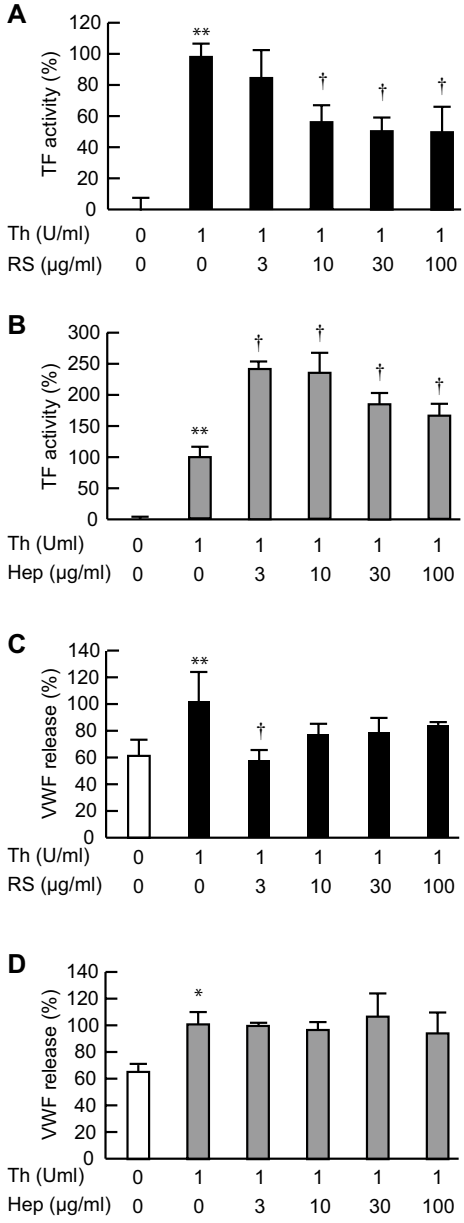

Fig. 4 Effect of RS or heparin on thrombin-induced TF expression and VWF release in HUVECs. TF expression in HUVECs treated with thrombin (Th) (1 U/ml) or PBS in the presence of RS (0–100 μg/ml) (**a**) or heparin (Hep) (0–100 μg/ml) (**b**). TF activity of HUVECs treated with thrombin in the absence of RS or heparin was designated to be 100%. VWF release from HUVECs treated with thrombin (Th) (1 U/ml) or PBS in the presence of RS (0–100 μg/ml) (**c**) or heparin (Hep) (0–100 μg/ml) (**d**). Concentration of VWF in culture medium treated with thrombin in the absence of RS or heparin was designated to be 100%. Open columns indicate TF activity and VWF concentration under conditions without thrombin, RS, and heparin in PBS. Black and gray columns indicate TF activity and VWF concentration in the presence of thrombin. Data are shown as the mean ± SD of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to reactions without thrombin treatment in the absence of RS or heparin. $^{\dagger}P < 0.01$ compared to thrombin treatment in the absence of RS or heparin

may be a candidate as a beneficial food supplement possessing antithrombotic effects.

Experimental

Preparation of RS

Crude RS was isolated from *M. nitidum* extract as described previously with slight modifications [3]. The crude RS preparation was dissolved in H_2O and treated with actinase E (Kaken Pharmaceutical, Tokyo, Japan) at 50 °C for 16 h. The treated extract was then applied to a Cellfine A-200 (JNC, Tokyo, Japan) anion-exchange chromatography column and was successively eluted with H_2O, 7 M urea in 2 M KCl. The fractions, which were monitored by the phenol-H_2SO_4 method, were collected, dialyzed, and freeze-dried.

Measurement of thrombin and factor Xa activities

Thrombin and factor Xa activities were measured according to a previously described method [25]. A thrombin solution (2.5 nM) (Wako Pure Chemical, Tokyo, Japan) was mixed with RS (2 μg/ml) or heparin (unfractionated heparin, 2 μg/ml) in the absence or presence of antithrombin (5 nM) (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C for 15 min. Residual thrombin activity was measured with a thrombin-specific substrate (200 μM, Boc-Val-Pro-Arg-MCA) (Peptide Institute, Osaka, Japan) using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA). A factor Xa solution (2.5 nM) (Sigma-Aldrich) was mixed with RS (2 μg/ml) or heparin (2 μg/ml) in the absence or presence of antithrombin (25 nM) and incubated at 37 °C for 15 min. Residual factor Xa activity was measured with a factor Xa-specific substrate (200 μM, Boc-Ser-Gly-Arg-MCA; Peptide Institute).

Measurement of TF expression

TF expression in HUVECs was measured by monitoring factor X activation, which depends on the presence of the factor VIIa/TF complex, according to the method described previously [26]. The HUVECs were stimulated with LPS (1 μg/ml) (Sigma-Aldrich), bovine thrombin (1 unit (U)/ml) (Wako Pure Chemical), or TNF-α (10 U/ml) (Sigma-Aldrich) in the presence of various concentrations of RS or heparin for 4 h. Factor Xa activity was measured using a synthetic substrate (200 μM, Boc-Ser-Gly-Arg-MCA). TF expression, as a percentage of factor Xa activity, in LPS-, thrombin-, or TNF-α-treated HUVECs in the absence of RS or heparin was designated to be 100%.

Measurement of VWF antigen

The HUVECs were treated with LPS (1 μg/ml), thrombin (1 U/ml), or TNF-α (10 U/ml) in the presence of various concentrations of RS or heparin for 4 h. The concentration of VWF in the culture medium released from the HUVECs was measured by a method described previously [26]. The concentration of VWF in the culture medium stimulated with LPS, thrombin, or TNF-α in the absence of RS or heparin was designated to be 100%.

Statistical analysis

Data were collected from four independent experiments and are presented as the mean ± SD. Statistical analyses were performed using Dunnett's multiple comparison tests. $P < 0.05$ was considered statistically significant.

Acknowledgements This work was supported in part by a Grant-in-Aid for challenging Exploratory Research (Grant number 18659280), a Grant-in-Aid for Scientific Research (B) (Grant number 21309292), and a Grant-in-Aid for Scientific Research (C) (Grant numbers 25460396 and 16K08633) from the Japan Society for the Promotion of Science.

Compliance with ethical standards

Conflict of interest This study was performed as a collaborative investigation funded by the Konan Chemical Manufacturing Co. Ltd. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

References

1. Zang L, Shimada Y, Tanaka T, Nishimura N (2015) Rhamnan sulphate from *Monostroma nitidum* attenuates hepatic steatosis by suppressing lipogenesis in a diet-induced obesity zebrafish model. J Funct Foods 17:364–370. https://doi.org/10.1016/j.jff.2015.05.041
2. Harada N, Maeda M (1998) Chemical structure of antithrombin-active Rhamnan sulfate from *Monostrom nitidum*. Biosci Biotechnol Biochem 62:1647–1652. https://doi.org/10.1271/bbb.62.1647
3. Lee JB, Koizumi S, Hayashi K, Hayashi T (2010) Structure of rhamnan sulfate from the green alga *Monostroma nitidum* and its anti-herpetic effect. Carbohydr Polym 81:572–577. https://doi.org/10.1016/j.carbpol.2010.03.014
4. Tako M, Yamashiro Y, Teruya T, Uechi S (2017) Structure-function relationship of rhamnan sulfate isolated from commercially cultured edible green seaweed, *Monostroma nitidum*. Am J Appl Chem 5:38–44. https://doi.org/10.11648/j.ajac.20170502.13
5. Li H, Mao W, Hou Y, Gao Y, Qi X, Zhao C, Chen Y, Chen Y, Li N, Wang C (2012) Preparation, structure and anticoagulant activity of a low molecular weight fraction produced by mild acid hydrolysis of sulfated rhamnan from *Monostroma latissimum*. Bioresour Technol 114:414–418. https://doi.org/10.1016/j.biortech.2012.03.025
6. Yamashiro Y, Nakamura M, Yogi T, Teruya T, Konishi T, Uechi S, Tako M (2017) Anticoagulant activity of rhamnan sulfate isolated from commercially cultured *Monostroma nitidum*. Int J Biomed Mater Res 5:37–43. https://doi.org/10.11648/j.ijbmr.20170503.12

7. Lee JB, Hayashi K, Hayashi T, Sankawa U, Maeda M (1999) Antiviral activities against HSV-1, HCMV, and HIV-1 of rhamnan sulfate from *Monostroma latissimum*. Planta Med 65:439–441. https://doi.org/10.1055/s-2006-960804
8. Lee JB, Hayashi K, Maeda M, Hayashi T (2004) Antiherpetic activities of sulfated polysaccharides from green algae. Planta Med 70:813–817. https://doi.org/10.1055/s-2004-827228
9. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM (1998) Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 91:3527–3561
10. Sadler JE (1998) Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem 67:395–424. https://doi.org/10.1146/annurev.biochem.67.1.395
11. Noone DG, Riedl M, Licht C (2018) The role of von Willebrand factor in thrombotic microangiopathy. Pediatr Nephrol 33:1297–1307. https://doi.org/10.1007/s00467-017-3744-y
12. Björk I, Lindahl U (1982) Mechanism of the anticoagulant action of heparin. Mol Cell Biochem 48:161–182. https://doi.org/10.1007/BF00421226
13. Chuang YJ, Swanson R, Raja SM, Olson ST (2001) Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence. Evidence for an exosite determinant of factor Xa specificity in heparin-activated antithrombin. J Biol Chem 276:14961–14971. https://doi.org/10.1074/jbc.M011550200
14. Li N, Liu X, He X, Wang S, Cao S, Xia Z, Xian H, Qin L, Mao W (2017) Structure and anticoagulant property of a sulfated polysaccharide isolated from the green seaweed *Monostroma angicava*. Carbohydr Polym 159:195–206. https://doi.org/10.1016/j.carbpol.2016.12.013
15. Liu X, Du P, Liu X, Cao S, Qin L, He M, He X, Mao W (2018) Anticoagulant properties of a green algal rhamnan-type sulfated polysaccharide and its low-molecular-weight fragments prepared by mild acid degradation. Mar Drugs. https://doi.org/10.3390/md16110445
16. Liu X, Wang S, Cao S, He X, Qin L, He M, Yang Y, Hao J, Mao W (2018) Structural characteristics and anticoagulant property in vitro and in vivo of a seaweed sulfated rhamnan. Mar Drugs. https://doi.org/10.3390/md16070243
17. Nemerson Y (1988) Tissue factor and hemostasis. Blood 71:1–8
18. Wada H, Wakita Y, Shiku H (1995) Tissue factor expression in endothelial cells in health and disease. Blood Coagul Fibrinolysis 6(1):S26–S31
19. Esmon CT (2003) Inflammation and thrombosis. J Thromb Haemost 1:1343–1348
20. Schneppenheim R, Budde U (2011) von Willebrand factor: the complex molecular genetics of a multidomain and multifunctional protein. J Thromb Haemost 9(Suppl 1):209–215. https://doi.org/10.1111/j.1538-7836.2011.04324.x
21. Yau JW, Teoh H, Verma S (2015) Endothelial cell control of thrombosis. BMC Cardiovasc Disord 15:130. https://doi.org/10.1186/s12872-015-0124-z
22. Kolarova H, Ambruzova B, Svihalkova Sindlerova L, Klinke A, Kubala L (2014) Modulation of endothelial glycocalyx structure under inflammatory conditions. Mediators Inflamm 2014:694312. https://doi.org/10.1155/2014/694312
23. Reitsma S, Slaaf DW, Vink H, van Zandvoort MA, Oude Egbrink MG (2007) The endothelial glycocalyx: composition, functions, and visualization. Pflugers Arch 454:345–359. https://doi.org/10.1007/s00424-007-0212-8
24. van den Berg BM, Vink H, Spaan JA (2003) The endothelial glycocalyx protects against myocardial edema. Circ Res 92:592–594. https://doi.org/10.1161/01.Res.0000065917.53950.75
25. Morita T, Kato H, Iwanaga S, Takada K, Kimura T (1977) New fluorogenic substrates for alpha-thrombin, factor Xa, kallikreins, and urokinase. J Biochem 82:1495–1498
26. Okamoto T, Akita N, Nagai M, Hayashi T, Suzuki K (2014) 6-Methylsulfinylhexyl isothiocyanate modulates endothelial cell function and suppresses leukocyte adhesion. J Nat Med 68:144–153. https://doi.org/10.1007/s11418-013-0784-x