Invitro anticoagulant activity of marine algae species *Acanthaphora spicifera* (Rhodophyceae)

P.Pandian
Department of Pharmacy,
Annamalai Univeristy,
Chidambaram, India.

Abstract

Hemostasis is the process of formation of clots within the walls of damaged blood vessels. To prevent abnormal bleeding and to maintain intravascular blood in a fluid state, in this study we aimed to evaluate the possible anticoagulant effect of marine algae extracts of *Acanthaphora spicifera.* The algae was collected from different locations of Mandapam area, Tamilnadu, extracted with chloroform, methanol solvent the standard kit was purchased for the anticoagulant assay the APTT, PT, TT assay were performed and different concentration was used from 2mg/ml to 20mg/ml the result was tabulated and the methanol solvent possess more anticoagulant than chloroform as APTT value for 20mg/ml is 175sec, PT values is 48sec and TT values is 82 respectively.

Introduction

The coagulation system can be divided into the extrinsic and intrinsic pathway. Activation of the extrinsic pathway is generally considered to initiate both haemostasis and thrombosis. Haemostasis is initiated when blood is exposed to tissue factor located in the adventitia of blood vessels, and thrombosis is initiated when blood is exposed to tissue factor in the necrotic core of the ruptured atherosclerotic plaques, in the subendothelium of injured vessels and on the surface of activated leucocytes attracted to the damaged vessel (**Davie et al, 1991**) The prothrombin time test (also known as the pro test or PT test) is a useful screening procedure for the extrinsic coagulation mechanism including the common pathway. It detects deficiencies in factor II, V, VII, and X. The prothrombin time test is frequently used to follow oral anticoagulant therapy that inhibit factors II, VII, IX and X. Thromboplastin activates the extrinsic coagulation system in plasma in the presence of calcium ions. The subsequent clotting time is dependent on the concentration of factors II, V, VII and X. Thus prolongation indicates a deficiency in one or more of these factors (**Quick, 1966, 1970**). Sulfated polysaccharides from algae possess important pharmacological activities such as anticoagulant, antioxidant, antiproliferative, antitumoral, anticomplementary, anti-inflammatory, antiviral, antipeptic and antiadhesive activities (**Cumashi A, et al 2007, Azevedo TCG et al 2009**). The relationship between structure and biological activities of algal sulfated polysaccharides are not yet clearly established (**Bilan MI, & Usov A,2008**). However, it is most likely than some structural features are required for biological activities, especially sulfate clusters to ensure interactions with cationic proteins (**Mulloy B ,2005**). Indeed, the importance of the molecular size has been reported (**Silva TM et al 2005, Albuquerque IR, et al 2004**). Anticoagulant activity is among the most widely studied properties of sulfated polysaccharides. Unfractionated heparins and low molecular weight heparins are the only sulfated polysaccharides currently used as anticoagulant drugs. However, these compounds have several side effects such as bleeding and thrombocytopenia, which increasing the necessity to look for alternative sources of anticoagulant agents (**Nader HB,et al 2004**). Algal sulfated polysaccharides have been described to possess anticoagulant activity similar to heparin. Various sulfated polysaccharides have been extracted from species from order Bryopsidales (Chlorophyta) and Dictyotales and Fucales (Phaeophyta) (**Fonseca RJC,et al 2008, Shanmugam,et al 2001, Medeiros VP et al,2008**) The proposed mechanisms of action of these compounds are predominantly related to the ‘‘in vitro’’ inhibition of factors Xa and IIa mediated by antithrombin and heparin cofactor II (**Li B et al,2008**). There has been increasing interest in the systematic screening of bioactive compounds from natural resources such as marine organisms. Sulphated polysaccharides are a class of compounds having hemi-ester sulphate groups in their sugar units. In marine algae, they occur as sulphated fructose and sulphated galactans (**Painter, 1983**)

The present study was designed to check invitro anticoagulant activity of the marine red algae *Acanthaphora spicifera* with various solvents.

Material and method

Collection of marine algae:

Fresh algae of *Acanthaphora spicifera* (Rhodophyceae) were collected from different locations of Mandapam area, Tamilnadu, South East Coast of India. In the earlymorning 5 am to 11.30am during which the tidal height was from 0.77 meter to 0.08 meter(lat 9° 15' N; long 79°E). Then the algae were washed thoroughly with sea water to remove extraneous materials and brought to the laboratory in plastic bag containing water toprevent evaporation. Samples were then shade dried until constant weight obtained and ground in an electric mixer. The powdered samples subsequently stored in refrigerator (**Sellappa Sudha et al, 2014**)

Preparation of Extracts:

The shade dried material was extracted with analytical grade petroleum ether, chloroform

and methanol for 8 hours by continuous hot percolation in Soxhlet apparatus **(Harborne, 1984)**.. The Extracts were dried under vacuum. The dry drug extract were dissolved in dimethyl formamide (DMF) the chloroform and methanol extract are used for further studies

Anticoagulation assay

Blood was drawn from healthy people without history of bleeding disease (**Wenjun Mao et al 2006, A.K Siddhanta et al, 2002**). Nine parts of blood was collected by vein puncture and mixed with one part of 3.8% Tri Sodium Citrate. Blood was centrifuged at 5000rpm at 10mins. Supernatant was collected designated as plasma. Test samples were prepared at different concentrations as 2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml, 10mg/ml,12mg/ml,14mg/ml, 16mg/m, 18mg/ml, 20mg/ml.

APTT assay

APTT assay was performed by using the standard APTT assay Kit purchased from Diagnostic Enterprises, Himachal Pradesh. 100µl of plasma was mixed with 10µl of solution of different concentrations of sulphated polysaccharides and then 100µl of prewarmed APTT assay reagent was added and incubated for 3 minutes at 37°C and to this 100µl of calcium chloride is added and the time taken for formation of clot was recorded.

PT assay

Assay was performed by using the standard PT assay Kit purchased from Diagnostic Enterprises, Himachal Pradesh. Prothrombin time (PT) assay was carried out using normal human plasma (100µl) mixed with solution of algal extract (10µl) then after 3 min incubation, clotting was induced by the addition of thrombo reagent (200µl) and clotting time was recorded.

Thrombin time (TT) assay

Thrombin time (TT) assay was performed with citrated normal human plasma (90µl) was mixed with 10µl of a solution of algal extract and incubated for 1min at 37^{0}c. Then TT assay reagent (200µl), prewarmed for 10min at 37^{0}c, was added and clotting time was recorded.(**H.J.Zhang et al,2008**)

Result and Discussion

Anticoagulation activity

Crude extracts of different concentrations were taken and the anticoagulation activity profile was established by using Activated Partial Thromboplastin Time (APTT) Prothrombin time (PT) and Thrombin time assay. APTT assay of chloroform extract was found to have APTT activity of 156seconds at 20 mg/ml and 175 seconds at 20mg/ml for methanol extract.The results have been tabulated in table 1and 2

Prothrombin(PT) assay

In PT various concentration was performed the chloroform extract of *Acanthaphora spicifera* was 40 seconds at 20mg/ml and the methanol extract was 48 seconds at 20mg/ml The results have been tabulated in table 1and 2

Thrombin time (TT) assay

In TT various concentration was performed the chloroform extract of *Acanthaphora spicifera* was 63 seconds at 20mg/ml and the methanol extract was 82 seconds at 20mg/ml The results have been tabulated in table 1and 2

Anticoagulant activity associated with polysaccharides in marine algae was first reported in *Iridae laminariodes* by (chargaff et al 1936) due to the presence of galactan sulphuric acid. after this many seaweed species were reported to possess anticoagulant activity and they were *Grateloupia filicina* (**Muruganantham,2001**) *Grateloupia indica* and etc

Heparin has been widely used in anticoagulant therapy for more than 50 years. The major mechanism by which unfractionated heparin exerts its anticoagulant effect is by accelerating serine proteinase inhibitor plasma factor such as thrombin (factor IIa) and factor Xa. But being from animal origin, heparin can induce diseases in mammals, such as avian influenza and bovine spongiform encephalopathy (**Mendes et al. 2009**). These reasons reinforce the need to find a new anticoagulant and antithrombotic agent replacing heparin. The anticoagulant activity of the sulphated polysaccharides depends on their degree of substitution, their molecular weights and the position of the sulfate group (**Jiraporn et al., 2009).**Thus in the clotting cascade blocks the intrinsic pathway by inhibiting factors XII, XI, X, IX, VIII, prothrombin which identified by the results of APTT assay. Anticoagulant activity is largely dependent on the sugar composition, sulfate content, sulfate position and molecular weight of the compound. (**Shanmugam and Mody,2000**) This anticoagulation activity may be due to the presence of uronic acids. The present results are in concomitant with (**Toshihiko et al ,2003 and El-Baroty et al, 2005**). who explained that, the polysaccharides containing uronic acids, carrying a negative charge, have the ability for binding calcium ions and therefore prevent the formation of clot. In addition, variation in anti-coagulating activity of the different solvents is probably due to the quantity of uronic acids in polysaccharides and some conformational differences in the molecules of these polysaccharides While, (**Mao et al,2006**) declared that the anticoagulant activities of the sulfated polysaccharides from the red algae *Acanthaphora spicifera.* It also blocks extrinsic pathway by inhibiting factors X, V, prothrombin which was identified through PT and also by TT.

Conclusion

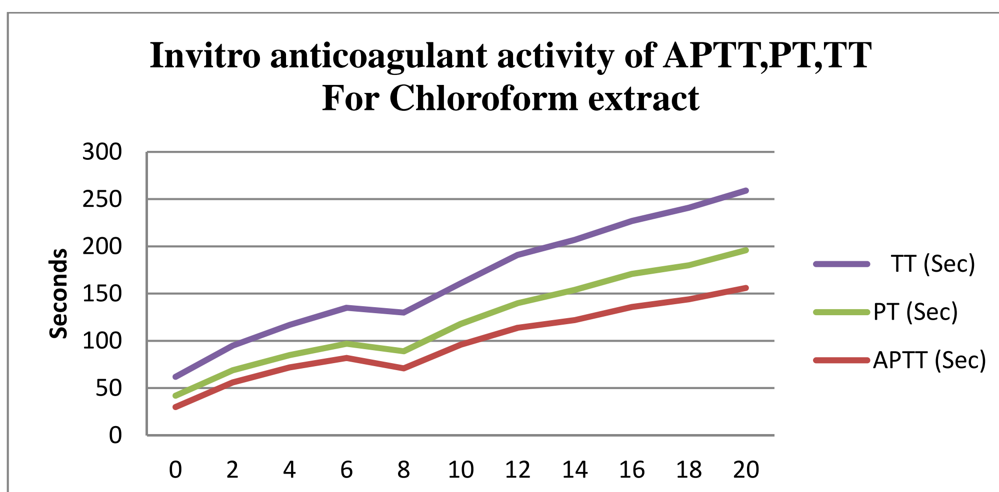
In this research work, Based on that different concentration of samples are prepared, tested for anticoagulant activity the methanol extract possess more anticoagulant activity than chloroform extract and it would studied only invitro of anticoagulant assay in future in vivo study is possessed and the compound responsible for the anticoagulant is evaluated.

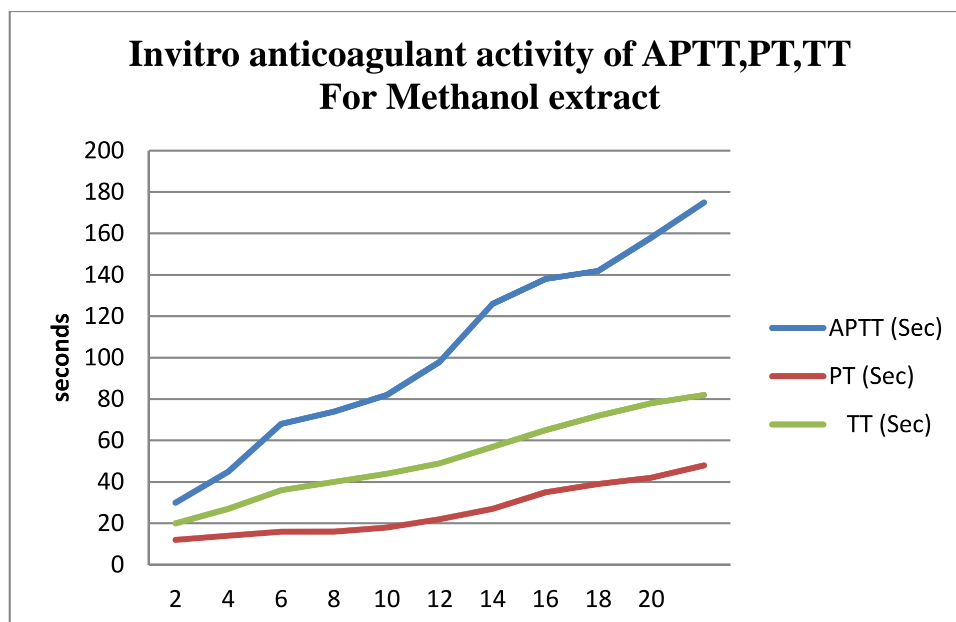
Table-1 Invitro anticoagulant activity of APTT, PT, TT for Chloroform extract

Sample (mg/ml)	APTT (Sec)	Sample (mg/ml)	PT (Sec)	Sample (mg/ml)	TT (Sec)
control	30	control	12	control	20
2	56	2	13	2	26
4	72	4	13	4	32
6	82	6	15	6	38
8	71	8	18	8	41
10	96	10	22	10	43
12	114	12	26	12	51
14	122	14	32	14	53
16	136	16	35	16	56
18	144	18	36	18	61
20	156	20	40	20	63

Table-2 Invitro anticoagulant activity of APTT, PT, TT for Methanol extract

Sample (mg/ml)	APTT (Sec)	Sample (mg/ml)	PT (Sec)	Sample (mg/ml)	TT (Sec)
control	30	control	12	control	20
2	45	2	14	2	27
4	68	4	16	4	36
6	74	6	16	6	40
8	82	8	18	8	44
10	98	10	22	10	49
12	126	12	27	12	57
14	138	14	35	14	65
16	142	16	39	16	72
18	158	18	42	18	78
20	175	20	48	20	82





Reference

[1] A.K Siddhanta, A.M Goswami, M.Shanmugam, K. H. Mody, B.K.Ramavat & O.P Mairh. Sulphated galactans of marine red algae Laurencia sp. from the west coast of India. Indian J of Marine Science 2002; 31.4: 305-309

[2] Albuquerque IR, Queiroz KC, Alves LG, Santos EA, Leite EL, Rocha HA. Heterofucans from Dictyota menstrualis have anticoagulant activity. Braz J Med Biol Res 2004;37:167–71.

[3] Azevedo TCG, Bezerra ME, Santos MD, Souza LA, Marques CT, Benevides NM, et al. Heparinoids algal and their anticoagulant, hemorrhagic activities and platelet aggregation. Biomed Pharmacother 2009;63:477–83.

[4] Bilan MI, Usov AI. Structural analysis of fucoidans. Nat Prod Commun 2008;3:1639–48

[5] Chargaff, E F.W. Bancroft and M,Stanley-Brown, studies on the chemistry of coagulation II. On the inhibition of blood clotting by substance on high molecular weight. J.Biol.Chem, 1936 115,155-161

[6] Cumashi A, Ushakova NA, Preobrazhenskaya ME, D'Incecco A, Piccoli A, Totani L, et al. A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic and antiadhesive activities of nine different fucoidans from brown seaweeds. Glycobiology 2007;17:541–52.

[7] Davie E. W., Fujikawa K., Kisiel W. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry*. (1991);30:10363–10370.

[8] El-Baroty G., Shallan M., Ali M.A., Abd El-Baky H.H., El-Baz F.K., Shalaby E.A. Antiviral activity of different extracts from Spirulina maxima. 3rd Conference on Recent Technologies in Agriculture,2005, November 8-15:1-7.

[9] Fonseca RJC, Oliveira SNMCG, Melo FR, Pereira MG, Benevides NMB, Mourao PAS. Slight differences in sulfation of algal galactans account for differences in their anticoagulant and venous antithrombotic activities. Thromb Haemost 2008;99:539–45.

[10] Harborne JB. Phytochemical methods 11 Edn.In Chapman &, Hall. New York, 1984; 4-5.

[11] H-J. Zhang, W-J. Mao, F. Fang, H-Y. Li, H-H. Sun, Y. Chen,.Chemical characteristics and anticoagulant activities of a sulfated polysaccharide and its fragments from *Monostroma latissimum* Carbohydrate Polymers, 71 (2008), pp. 428-434

[12] Hui-Juan Zhang,Wen-JunMao, Fang Fang, Hong-Yan Li, Hai-Hong Sun, Yin Chen, Xiao-Hui Qi.chemical characteristics and anticoagulant activities of a sulphated polysaccharide and its fragements from *Monostromalatissimum*.Carbohydrate polymer 71(2008) 428-434.

[13] Jiraporn S., Zhenqing Z., Boyangzi L., Preeyanat V., Puttinan M., Fuming Z.,. Sulfonation of papain-treated chitosan and its mechanism for anticoagulant activity, Carbohydrate Research. 2009; 344:1190–1196

[14] Li B, Lu F, Wei X, Zhao R. Fucoidan: structure and bioactivity. Molecules 2008;13:1671–95

[15] Mao W., Zang X., Li Y., Zhang H. Sulfated polysaccharides from marine green algae Ulva conglobata and their anticoagulant activity. Journal of Applied Phycology,2006 18: 9-14.

[16] Medeiros VP, Queiroz KC, Cardoso ML, Monteiro GR, Oliveira FW, Chavante SF, et al. Sulfated galactofucan from Lobophora variegata: anticoagulant and antiinflammatory properties. Biochemistry (Mosc) 2008;73:1018–24

[17] Mendes S.F. O., dos Santos Jr A.M., Barbosa A.F.D. Vasconcelos and G. Aranda-Selverio. Sulfonation and anticoagulant activity of botryosphaeran from Botryosphaeria rhodinaMAMB-05 grown on fructose. Int. J. Biol. Macromol. 2009; 45: 305-309

[18] Mulloy B. The specificity of interactions between proteins and sulfated polysaccharides. An Acad Bras Cienc 2005;77:651–64.

[19] Muruganantham, A . Heparin from seaweeds. A search in *Grateloupia filicina* J.Ag P 1-4

[20] Nader HB, Lopes CC, Rocha HA, Santos EA, Dietrich CP. Heparins and heparinoids: occurrence, structure and mechanism of antithrombotic and hemorrhagic activities. Curr Pharm Des 2004;10:951–66.

[21] Painter, T.J. Algal polysaccharides. The Polysaccharide. Academic Press, New York, 1983 2: 195-285

[22] Quick, A.J., Bleeding problems in clinical medicine, Hemorrhagic Diseases and Thrombosis, W.B. Saunders Co., Philadelphia, 1970, 225.

[23] Quick, A.J., Coagulation, Hemorrhagic Diseases and Thrombosis, Lea and Febiger, Philadelphia, 1966, 460.

[24] Sellappa Sudha, Palanisamy Senthilkumar, and Subramanian Prakash. Antidiabetic activity of aqueous extract of *Padina boergesenii* in Streptozotocin-induced diabetic rats. International Journal of Pharmacy and Pharmaceutical Sciences 2014 6(5), P 418-422

[25] Shanmugam M, Mody K.H. Heparinoid–active sulphated polysaccharides from marine algae as potential anticoagulant agents. Current Science, 79:1672-1682, (2000).

[26] Shanmugam M, Mody KH, Siddhanta AK. Blood anticoagulant sulphated polysaccharides of the marine green algae Codium dwarkense (Boergs.) and C. tomentosum (Huds.) Stackh. Indian J Exp Biol 2001;39:365–70.
[27] Silva TM, Alves LG, de Queiroz KC, Santos MG, Marques CT, Chavante SF, et al. Partial characterization and anticoagulant activity of a heterofucan from the brown seaweed Padina gymnospora. Braz J Med Biol Res 2005;38:523–33.
[28] Toshihiko T., Amornut C., Robert L.J. Structure and bioactivity of sulfated polysaccharides. Trend in Glycosci and Glycotechnol, 2003,5:29-46,
[29] Wenjun Mao, Hongyan Li, Yi Li, Huijuan Zhamg, Xiaohui Qi, Haiong Sun, Yin Chem, Shoudong GuoA. Chemical characteristics and anticoagulant activity of the sulphated polysaccharide isolated from Monostroma latissimum. International J of Biological Macromolecules 2006; 44: 70-74.