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Research Article

ANTICOAGULANT ACTIVITY OF MARINE GASTROPODS *BABYLONIA SPIRATA* LIN, 1758 AND *PHALIUM GLAUCUM* LIN, 1758 COLLECTED FROM CUDDALORE, SOUTHEAST COST OF INDIA

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ABSTRACT

Objectives: Molluscs are highly delicious seafood and they are also very good source for biomedically imported products. Among the molluscs some have pronounced pharmacological activities or other properties which are useful in biomedical area.

Methods: In the present study glycosaminoglycans was isolated from two marine gastropods such as *Babylonia spirata* and *Phalium glaucum*.

Results: The isolated glycosaminoglycans were quantified in crude samples and they were estimated as 8.7 gm/kg & 5.3 gm/kg crude in Babylonia spirata and Phalium glaucum respectively. Both the gastropods showed the anticoagulant activity of the crude samples 134 USP units/mg and 78 USP units/mg correspondingly in B. spirata and P. glaucum. In the agarose gel electrophoresis using acetate buffer, the band of the crude samples studied showed the band with similar mobility to standard heparin sulfate. FTIR analysis reveals the presence of anticoagulant substance signals at different ranges.

Conclusion: Among the two gastropods *B. spirata* showed more anticoagulant activity than that of *P. glaucum*.

Keywords: *B. spirata, P. glaucum* Anticoagulant activity, Agarose gel electrophoresis, FTIR

INTRODUCTION

Commonly glycosaminoglycans are classified based on their sulphate position and glycosaminoglycans include heparin, dermatan sulphate, hyaluronan, chondroitan sulphate, keratan sulphate and heparin sulphate. In this group, heparin is a sulphated mucoploysaccharide composed of α-L-iduronic, β-D-glucuronic acid and α-D- glycosamine unit, joined by 1→bonds. The structure of heparin is highly complex and heterogeneous. This compound is involved in a variety of physiological and pathological infections[1].

Anticoagulants have been widely used both clinically and *in-vitro* medical treatments. In clinical practice, they are drugs of choice for the prevention and treatment of thromboembolic disorders and prophylaxis of thrombotic events both pre and post-surgery. It is estimated that 2–4 patients out of 1,000 receive anticoagulant therapy [23]. In the materials field, anticoagulants are used to improve the hemo-compatibility of medical device and tissue engineering materials [4]. Recently, the concept of "vascular beautifying" has been promoted by the cosmetics industry [5]. Substances with anticoagulant activity are among the first choices as functional components which are being used to open up new areas of application for anticoagulants.

Heparin and heparin like substances appear to be rather ubiquitous natural products which are found in most mammalian tissues including viscera, lung, skin, kidney, liver, muscle, mast cells and basophils [6]. Their strategic location and highly charged nature make them important biological players in cell to cell and cell matrix interactions that take place during normal and pathological events, related to the cell recognition, adhesion, migration and growth [7,8,9,10] Suggested that GAGs and other important molecules involved in the calcification of carbohydrates in antler of mule deer (*Odocoileus hemionus*). However heparin and heparin like substances are not only found in higher life forms, but also in lower invertebrates [11][12],lobster[13], [14] ascidiansand tunicates[15]. A large number of animal species contain GAGs and the marine molluscs are particularly rich of those polysaccharides [16][17]. A compound named mactin had been isolated from *Mactrus pussula* and *Cyprina islandica* [18]. The chemical analyses and anticoagulant activities of mactin (glycosaminoglycan) were distinguishable from those of heparin. Heparin-like GAGs were isolated from *Chicoreus ramosus, C. virgineus* and *Hemifusus pugilinus* [19]. The sulphated glycosaminoglycans were also isolated from the bivalves and gastropods [20]. Among several invertebrates, the presences of heparin-like compounds in molluscs were observed by [21-25] studied in-vitro anticoagulant activities of alginate sulphate and its quaterized derivates. Their structure was characterized by elemental analysis such as FTIR, C-NMR and gel permeation chromatography.

[26] Have developed a method for the recovery of heparin and heparin like substances from various marine organisms like flounder, crab, mussel and clams. Since molluscs heparin contains antihrombin-dependant anticoagulant associated with the presence of unique 3-O- sulfated glycosamine residues in all anticoagulant heparin[12]. Many works have been done on anticoagulant potential of different classes of molluscs whereas fewer attempts has made on the class of gastropods. Hence, the present study deals with the isolation of GAGs, anticoagulant activity and characterization of the gastropods, *B. spirata* and *P. glaucum* collected from the cuddalore, southeast coast of India.

MATERIALS AND METHOD

Isolation of Glycosaminoglycans

The molluscs *B. spirata* and *P. glaucum* was collected from the Cuddalore (Lat. $11^0 24'$; Long.$79^0 49'$E). Shells were opened and body tissues were collected. They were blended in 0.4 M sodium sulfate solution (Na_2SO_4; 3.5 1/kg of the tissue) and kept at 55^0c for 1 h 30 min. the pH was adjusted to 11.5 by adding 10% sodium hydroxide (NaOH) solution. Aluminium sulfate ($Al_2 (SO_4)_2$) crystals (80 mg/kg tissues) were added to this solution, and the suspension was heated to 95^oc for 1 h. Cetyl pyridinum chloride (CPC) solution (3 g/100 ml of 0.8 M Nacl) was used to precipitate the crude white heparin complex. The precipitate was redissolved in 150 ml of sodium chloride solution (2.0 M) and was incubated at 300c for 30 min. the precipitate was washed, with ethanol and methanol through centrifugation, and vaccum dried.

Anticoagulant activity

The anticoagulant activities of crude GAG samples were determined by comparing with the concentration necessary to prevent the clotting of sheep plasma using USP (United State Pharmacopoeia) method.

Agarose gel electrophoresis

Electrophoresis was carried out in agarose gel plates. The gel plate was prepared with 0.6% agarose (2 to 4 mm thick) containing acetate buffer at pH 3.6 by the following method of [27].

FTIR - (Fourier Transform- Infra Red spectrum analysis)

The lyophilized GAGs samples of *B. spirata* and *P. glaucum* (10mg) was mixed with 100mg of dried potassium bromide (Kbr) and compressed to prepare as a salt disc. The disc was then read spectro photometerically (Bio- Rad FTIR-40- model, USA). The frequencies of different components present in each sample were analyzed.

RESULTS

The amount of the crude GAGs sample of *B. spirata* and *P. glaucum* was recorded as 8.7 & 5.3 gm/kg respectively. The anticoagulant activity of the *B. spirata* and *P. glaucum* crude samples was reported to be 134 USP units/mg and 78 USP units/mg (Table. 1).

Agarose gel electrophoresis

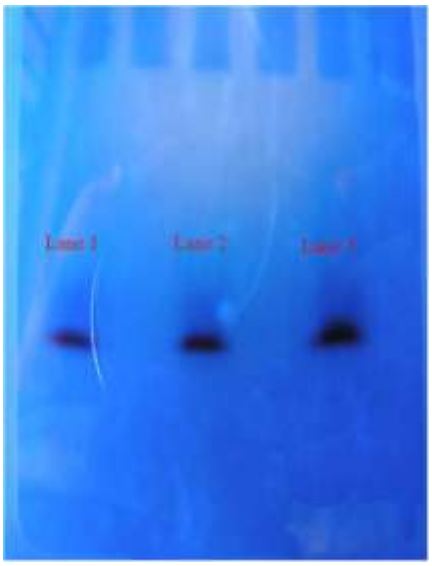
Agarose gel electrophoresis of extracted crude glycosaminoglycans from *B. spirata* and *P. glaucum* are presented in Fig.1. As it can be observed that the agarose gel electrophoresis using acetate buffer, the band of the heparin like glycosaminoglycans in the crude sample studied showed the band similar mobility to heparin sulfate. The result of agarose gel electrophoresis showed the migration of crude sample as that of standard heparin sulfate. From this it could be inferred that the crude GAGs sample contains complex mixture of anticoagulant compounds.

Table 1: Yield and anticoagulant activity of crude samples in *B. spirata* and *P. glaucum*

S. No.	Animal	Yield crude GAGs gm/kg	Anticoagulant activity USP units/mg crude sample
1	*Babylonia spirata*	8.7	134
2	*Phalium glaucum*	5.3	78

FTIR spectral analysis

Fourier Transform Infra Red spectrum of the lyophilized sample 8 peaks at 1411.89, 1292.31, 1213.23, 1157.29, 1041.56, 960.55, 840.96 and 758.02 cm^{1}.whereas the spectrum of the crude sample of *B. spirata* showed four peaks at 1215.15, 933.55, 767.67 and 678.94 cm^{-1}. *P. glaucum* showed the two peaks at 1215.15 and 763.81cm^{1}.



Lane.1. Standard heparin sulfate **Lane.2.** *B. spirata* GAGs **Lane.3** *P. glaucum* GAGs

Fig. 1: A agarose gel Image of GAGs from two gastropods

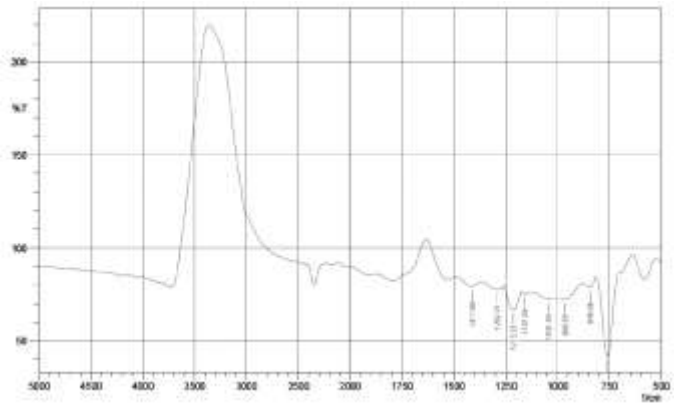


Fig. 2: FTIR spectrum of Standard heparin sulfate

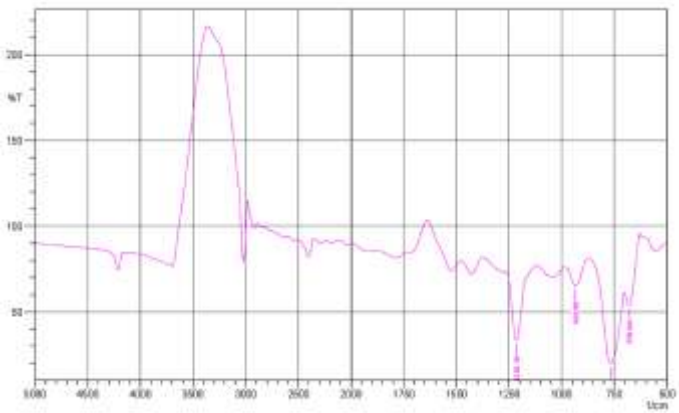


Fig. 3: FTIR spectrum of crude GAGs of *B. spirata*

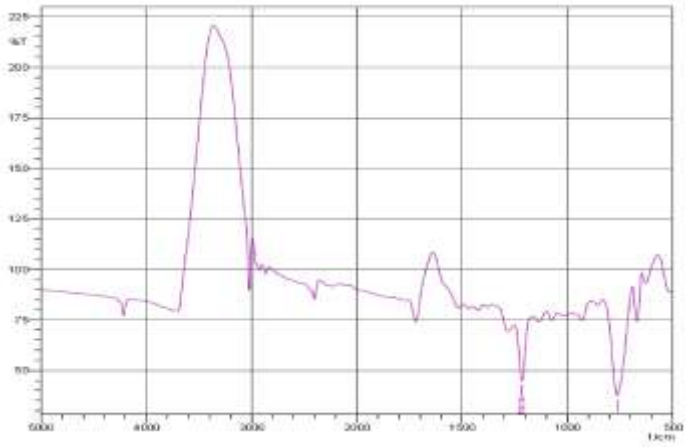


Fig. 4: FTIR spectrum of crude GAGs of *P. glaucum*

DISCUSSION

Heparin and heparin like compounds, which are present in some invertebrate molluscs, showed high anticoagulant activity and share most of the structural properties with mammalian heparins. In the present study, homogenization of body tissue *B. spirata* and *P. glaucum* in acetone and subsequent extraction in acetone/petroleum ether resulted in 50gm of defatted tissue. The heparin obtained from defatted tissue described in the present study has all the features as that of heparin. Similarly, heparin has been prepared from a number of different sources; human [28], clams [21], giant African snail [12]and seaweeds [29]. The use of CPC for quantitative separation of sulphated polysaccharides in tissue extracts in preferred. In the present investigation, the yield of crude heparin-like glycosaminoglycans was found as 8.7 & 5.3 gm/kg in *B. spirata* and *P. glaucum*. Previously [30]have recorded the yield of heparin and other sulfated mucopolysaccharides from thymus as 274 µg/kg. Similarly, [31]isolated the sulfated mucopolysaccharides by using quaternary ammonium salts and reported the yield as 170, 174.0, 843.0, 307.0 and 1090.0 µg/kg dry tissues in different molluscan species such as *Alulocombia ater*, *Perna perna, Mesodesma donacium, Loligo brasiliense* and *Octopus* sp. respectively. [21] isolated heparin with a yield of 2.8g/kg and 3.8g/kg from *Anomalocardia brasiliana* and *Tivela mactroides* respectively. [32]obtained that the 7.02gm/kg of heparin like substances in marine molluscs *Katelysia opima*. [33]Reported that the yield of crude heparin from *Conus amadis* and *Babylonia zeylonica* as 2.6 and 8.16 gm/kg respectively. However, the cephalopods such as *Sepia aculeate* and *S. brevimana* and *Loligo duvauceli* and *Doryteuthis sibogae* showed higher net yield of the heparin like sulfated polysaccharides 21.7 gm/kg, 24.0 gm/kg and 16.5 gm/kg, 8.4 gm/kg respectively [34] [35]. [22]had quantified the heparin yield as 2.27g/kg and 2.2g/kg from *Tridacna maxima* and *Perna viridis* respectively. [36]reported the total yield of the crude heparin was estimated as 5.4g/kg in *Katelysia opima* and 4.1g/kg in *Donax cuneatus* from the defatted tissue. In *B. spirata* and *P. glaucum,* generally the glycosaminoglycans content was found to be low as it is high when compared to other molluscs. Among the two gastropods *B. spirata* showed more anticoagulant compounds than that of *P. glaucum*.

The heparin isolated from marine clams and mussels has identical structural features and anticoagulant activity of mammalian polysaccharide [37]. Heparin with high anticoagulant activity was isolated from the marine molluscs, *Anomalocardia brasiliana Donax striatus* and *Tivela mactroides* [21]. Which showed similar activity like mammalian heparin but differs in molecular weight, the molluscan heparin have a higher molecular weight and high anticoagulant activity [21]. In the present study, the anticoagulant activity of the crude sample of GAG from the whole body tissue of *B. spirata* and *P. glaucum* was reported 134 USP units/mg and 78 USP units/mg. [38] showed the activity ranging from 130-150 USP units/mg for extracted products of *Spisula solidissima* and *Cyprina islandica*. [21]showed that the anticoagulant activity of heparin from two species of molluscs, *D. striatus* and *T. mactriodes* as 180 units/mg and 220 units/mg respectively. [16]recorded 26 USP units/mg in *Hemifusus pugilinus*. In *Turritella attenuata* [39] reported the anticoagulant activity of 37 USP units/mg in crude sample. [34]reported the anticoagulant activity of the crude sample of *S. aculeata* 376.98 USP units / mg and *S. brevimana* 421.72 USP units / mg. [35]reported the activity of the crude sample of *L. duvauceli* was 376.98 USP units / mg and *D. sibogae* was 376.98 USP units / mg. [36]Vijayabaskar (2008) reported the extraction of GAGs from *K. opima* and *D. cuneateus* showed anticoagulant activity of 160USP units/mg and 154USP units/mg respectively. [40] reported the anticoagulant activity of the crude sample of *Meretrix casta* (sheep blood 22.52 USP units / mg) (chicken blood 20.00 USP units / mg) and (human blood 18.60 USP units / mg). In the present investigated two gastropods *B. spirata* and *P. glaucum* tissue is value medicinal due to high quality of anticoagulant compounds. From the above it could be understood that body tissue of *B. spirata* is a very good potential source of anticoagulant compounds than that of *P. glaucum*.

Electrophoresis is a selective tool for the identification of different sulfated mucopolysaccharides from different sources. Agarose gel electrophoresis is the only technique to conform the presence of GAGs. The staining solution (toluidine blue) was found to bind only with the sulfated polysaccharide and not bind with other compounds [41]. The electrophoratic migration of the sulfated polysaccharide using acetate buffer (pH 3.6) system depends on the structure of the polysaccharide. In this study, the molecular weight of crude sample of GAGs from gastropods, *B. spirata* (lane. 2) and *P. glaucum* (lane. 3) showed one band were compared with commercially available standard markers (Lane 1. Heparin sulfate). The previous study made by [31]in the invertebrates also reported the same migratory pattern of bands for the sulphated polysaccharides particularly in *Octopus* sp. which showed polydisperse sulphated mucopolysaccharides migrating in the region corresponding to all the sulphated mucopolysaccharide standards. In these research determined that the crude GAGs sample contains complex mixture of anticoagulant compounds. In the agarose gel electrophoresis using acetate buffer, the anticoagulant GAG is isolated from *B. spirata* and *P. glaucum* showed same level of migration as that of heparin sulfate which was used as standard in the same direction confirming the presence of heparin and heparin-like anticoagulant GAGs in the extracted samples.

In the present study, the anticoagulant GAGs from body tissue of *B. spirata* and *P. glaucum* crude sample showed a major peaks at 1215.15 and 1215.15 cm^{-1} which is said to be for the GAGs groups. The peak at 1113 cm^{-1}indicates the presence of sulphate as also reported by [42]. The sample showed the absorption band for the carboxyl group at 1654 $cm^{-1}$and acetyl amino group at 1400 cm^{-1} which were also reported by [43]at 1615 cm^{-1}(carboxyl group) and 1375 cm^{-1} (acetyl amino groups) in the sulfated mucopolysaccharides isolated

from the skin of *Chimaera* sp. Assignment of IR absorption bands and 1240 cm^{-1}[14][4]and 1430 cm^{-1}in the spectrum of fully o-sulphonated HA were based on the reports by [45]. The peak pattern between the standard heparin and the sample was at 1213.23cm^{-1}in crude sample and 1215.15 cm^{-1}as standard heparin indicating the presence of GAGs group in the samples analyzed. The FT-IR spectral analysis of the anticoagulant GAGs from marine molluscs *B. spirata* and *P. glaucum* showed more or less same number of peaks lying within the same range of values of the standard heparin. This proves that the extracted anticoagulant GAGs in the body tissue from *B. spirata* and *P. glaucum* is also an anticoagulant resembling the heparin and heparin-like substances. The results in this research showed that gastropod, *B. spirata* tissue having GAGs with high quantity of anticoagulant compounds.

The anticoagulant effect of heparin is closely associated with their molecular weight [46]. Commercially available low molecular weight heparins are less potent than UF II in term of anticoagulation [47]. Crude products isolated from marine organisms have served as a source of drugs and starting materials for synthesis of useful drugs. In addition, because of the differences in the environmental conditions, new biochemical entity having biological activity can be evolved by marine organisms. So, it is believed that the studies of new and unique compounds derived from marine organisms will continue to increase our basic knowledge with respect to pharmacology and medicine.

Thus the result of the present investigation provides information about the isolation, anticoagulant activity and characterization of the crude GAGs from a non-conventional source, the gastropods, *B. spirata* and *P. glaucum*. Further the good anticoagulant activity by the anticoagulant compound from this species proves the possibility of its utilization as an additional potent source for the extraction of such anticoagulant compound. Besides the above, the result of the present study is providing baseline data for the future researchers in this line of work and is also throwing more light on the use of the marine gastropods, body tissue by the pharmaceutical technologists for the extraction of useful drugs or the active principles or functional units for the synthesis of drugs in future.

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