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Studies on molluscan glycosaminoglycans (GAG) from backwater clam *Donax cuneatus* (Linnaeus)

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ABSTRACT

Objective: To investigate the potent and specific anticoagulant activity of molluscan glycosaminoglycans (GAGs) isolated from whole clam tissue *Donax cuneatus (D. cuneatus)*. **Methods:** Purification of few milligram quantities of GAGs from this tissue sample permitted a thorough examination of its anticoagulant activity characterization, which was partially purified by fractionation by anion exchange chromatography using DEAE cellulose column. The isolated crude and partially purified fractionated sample was showing metachromatic shift while using azure-A. The sample also exhibited prominent of biological and anti-fXa anticoagulant activity assays. Mobility was analyzed by two different buffer systems using agarose gel electrophoresis. **Results:** The fractionated molluscan GAG was also found to have similar peaks as that of standard heparin when assessed by the FT-IR spectrum. Finally molecular weight was determined by the gradient PAGE for crude and fractionated-II GAG, which were found to be 65 000 Da and 50 000 Da, respectively. **Conclusions:** The bivalve GAG was subjected to fractionation for further purification and its chemical components were analyzed. The fractionated clam heparin also showed substantial *in vitro* anticoagulant activity than that of commercial heparins.

1. Introduction

Glycosaminoglycans (GAGs) are a family of linear anionic polysaccharides that are typically isolated as proteoglycans linked to a protein core. Heparin and heparan sulfate have been the subject of intensive study because of their well-recognized ability to bind many different proteins that regulated a varity of important biological processes. Heparin and heparan sulfate GAGs are comprised of alternating 1-4 linked glycosamine and uronic acid residuces. Heparin, a sulfated glycosaminoglycan present in several mammalian and other vertebrates' tissues has been widely used in medicine for more than 75 years because of its anticoagulant, antithrombotic and antilipaemic activites. The heparins like compounds are also present in some invertebrates. Still date the heparin is prepared from terrestrial mammalian tissues for commercial use[1-5]. There is an increased demand, but the resource are depleting. So, it is the right time to look for alternative sources for heparin production especially from marine organisms. Marine molluscs are promising source for heparin production[6-9].

Heparin had been commonly used for prevention and treatment of venous thrombosis[10]. Normally the heparin administered intravenously is of high molecular weight ranging between 15 000 to 30 000 Da, but this is not helpful in treating the patients with deep vein thrombosis (DVT), pulmonary embolism (PE) and coronary thrombolysis. Since molecular weight is one of the limitations that would prevent the drug action against specific target sites, presently low molecular weight heparin (LMWH) has been used for its higher potency in treating DVT, PE and coronary thrombolysis[11]. It was also found quite successful in administrating LMWH through subcutaneous mode to treat DVT[12]. Another added advantage is that LMWH can be given orally due to its greater bioavailability[13].

Heparin and heparin like substances appear to be rather ubiquitous natural products that are found in most mammalian tissues including viscera, lung, skin, kidney, liver, muscle, mast cells and basophils[14].

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Their strategic location and highly charged nature make them important biological players in cell-cell and cell-matrix interactions that take place during normal and pathological events, related to the cell recognition, adhesion, migration and growth[15-19]. However heparin and heparin like substances are not only found in higher life forms, but, also in lower invertebrates[20], lobster[21] and tunicates[22,23]. A large number of animal species contain GAGs and the marine mollusks are particularly rich of these polysaccharides[8,21,24,25]. A compound named fucoidan derivatives had been isolated from brown seaweed *Laminaria japonica (L. japonica)*[26]. The reported chemical analyses and anticoagulant activities of this glycosaminoglycan were distinguishable from those of heparin. Among several invertebrates, the presences of heparin-like GAGs compound were most probably observed in certain mollusks[27-29]. Present paper describes the extraction, partial purification and validation of molluscan GAG isolated from *Donax cuneatus* (*D. cuneatus*) and its mobility was analyzed by agarose gel electrophoresis using two different buffer systems.

2. Materials and methods

2.1. Extraction of molluscan glycosaminoglycans (GAGs)

The common wedge clam was collected from the intertidal region of the Pondicherry coast, South east coast of India (11o 52′N; 79o 49′E) by hand picking. The shells were removed, 1 kg of the whole meat was ground with one lit of 0.9 M NaCl in a blender and 0.4 M sodium sulfate (3.5 L/kg of tissue) was added to it. The whole content was incubated in a water bath at 55 ℃ for 1 h 30 min and was maintained at pH 11.5 using 10% NaOH solution. Then the pH of the solution was reduced to 7.7 using aluminium sulfate and was heated to 95 ℃ for one hour.

After the above process the solution was allowed to cool over night, the samples were centrifuged and the supernantant was collected. Cyteyl pyridinium chloride (CPC) (3% of 0.8 M NaCl) was added to the supernatant until a complete white precipitation of the complex appeared at 40 ℃ incubation for a period of 24 h. The sample was subjected to centrifugation at 3 000 rpm for 90 min and thus the crude heparin complex was obtained. The precipitate was redissolved in 2 M NaCl at 40 ℃ to dissociate CPC salt from heparin and 2 volumes of 95% methanol was used to precipitate the crude heparin. Hexoamine and uronic acid was determined by using acid hydrolysis (4 M HCl 100 ℃ for 6 h) by Tsuji[30].

2.2. Partial purification of crude GAG using DEAE cellulose column

Standard heparin sodium salt and isolated molluscan heparin were passed through a DEAE cellulose column (1.5 cm× 50 cm), with a flow rate of 12 mL/h. The three different molar solutions of NaCl (1.0 M, 2.0 M and 3.0 M) were used for fractionating the sample. The samples were collected for every hour from the column. All the fractionated samples were dialyzed exhaustively against distilled water using dialysis membarane-50 and the samples were lyophilized and subjected to the following assays.

2.3. Uronic acid, hexosamine and other elements estimation

Uronic acid was determined colorimetrically by the Bitter and Muir method[31]. 5 mL of sulphuric acid reagent (0.025 M sodium tetraborate in concentrated sulphuric acid) was taken in each tube stored at 4 ℃ and 1 mL of fractionated sample was carefully layered over the acid. The tubes were shaken gently and then vigorously with constant cooling. After cooling, the tubes were heated for 10 min in a boiling water bath and again cooled to room temperature. 0.2 mL of carbozole (0.125% in ethanol) reagent was added and heated in a boiling water bath for 15 min.

The optical density was measured at 530 nm. Glucuronolactone was used as a standard (4-40 μg/mL) and the solution was saturated with benzoic acid. Hexosamine content was determined colorimetrically after hydrolysis with 2 M HCl for 2 h at 100 ℃ using the method of Tsuji[30]. The elements carbon, hydrogen, nitrogen and sulfate in tested samples were analyzed using micro-elemental analyzer Carb EPBA mode 1106. The amount of sodium was measured by the Flamephotometer systronics MK III.

2.4. Optical rotation

Molluscan GAG (0.40 g) was dissolved in double distilled water and the optical rotation was measured with automatic polarimeter (AUTOPOL IV, Rudolph).

2.5. Metachromatic activity assay

Lyophilized heparin samples were dissolved in double distilled water and aliquots (5 μL) were removed over the three different molar eluted salt solutions and were mixed with 10 mL of 0.02 g/L of azure-A dye solution separately and the absorbance was measured at 620 nm within 30 min[32].

2.6. Biological anticoagulant assay

Biological activity of all the three different molluscan crude GAGs and fractionated (FI, FII and FIII) samples were determined by comparing the concentration necessary to prevent the clotting of sheep plasma using USP[33] method. Gradual amount of the standard preparation were added to the cleaned 13 mm×100 mm test tube, selecting the amounts so that the maximum grade did not exceed 0.8 ml and would be made up with saline solution to varying levels of concentration and

that they corresponded roughly to geometric series in which each step was approximately 5% greater than the next lower. Then one mL of the prepared sheep plasma was added, followed by the addition of 0.2 mL of calcium chloride solution and stopped immediately and the time was also noted simultaneously.

The contents of the tubes were mixed by inverting three times in such a way that the entire inner surface of the tube was wet. In the same manner, the assay preparation was set up. The entire process of preparing and mixing the tubes of both the standard preparation and the assay preparation were completed within 20 min after the addition of plasma. Accurately one hour after addition of calcium chloride, the extent of clotting in each tube was determined recognizing three grades (0.25, 0.50 and 0.75) between zero and full clotting (1.0).

The volume of standard preparation used in the successive 5 or 6 tubes that bracket a grade of clotting of 0.5, including atleast two tubes with a larger and tubes with a smaller grade than 0.5 were converted to logarithms. The tubes were numbered and listed serially and each grade of clotting observed was tabulated from the log-volumes (X) and their corresponding grades of clotting (Y), the paired averages 'x' and 'y' of tubes 1, 2 and 3; 2, 3 and 4; 3, 4 and 5 and 4, 5 and 6 were computed. If for one of these paired averages, the average grade 'Y' is exacting 0.50 then the corresponding 'X' is the medium log volume of the standard preparation 'Xs'. The medium log volumes of assay preparation 'Xu' was computed similarly as that of the standard. The log potency of the assay preparation in

$$M = Xs - Xu + \log R$$

Where, R = Vs/Vu is the ration of the USP heparin units (Vs) per ml of the standard preparation to the mg (Vu) of heparin sodium per ml of the assay preparation. The volume of M is average to obtain M. The potency of heparin sodium in USP units per mg is P = anti log of M.

2.7. Anti-fXa activity

The anti-fXa is a chromogenic assay intended for the quantitative determination of crude and three fractionated GAGs samples in human plasma by measurement of factor Xa inhibition. To 200 μL of AT-III in an ELISA plate, 25 μL of plasma sample along with standard heparin or molluscan GAGs were added in different dilution concentration (0.8 unit/mL, 0.4 unit/mL and 0.2 unit/mL) mixed and incubated at 37 ℃ for 2 min. 200 μL of factor Xa was added, mixed and incubated at 37 ℃ for exactly one minute after which the complex was formed.

Then substrate for spectrozyme FXa (200 μL) was added and kept for five minutes at room temperature. The complex was incubated for six minutes at room temperature until, the yellow color complex was obtained. Finally, 200 μL of 90% acetic acid was added to stop the reaction. The absorbance was measured at 405 nm against the blank in an ELISA reader. A linear standard curve was plotted with the doses expressed as anti-Xa Units/ml on 'X' axis and OD at 405 nm on 'Y' axis.

According to the OD of every dilution of the sample, the equivalent units of the standard were calculated. The obtained value was multiplied by the dilution factor of the sample and the mean value of the titers was calculated.

2.8. Agarose gel electrophoresis

The crude isolated GAG samples (10 μL and 30 μL) were loaded on an Agarose gel (0.5% w/v) to perform electrophoresis. This experiment was helped to find out the nature of the isolated product. The presences of GAGs were analyzed by using two different buffer systems such as 1,3-Diaminopropane buffer (pH 9) and Acetate buffer (pH 3.6) for one hour at 120 V.

After the electrophoresis, the gel was fixed in 0.1% N-cetyl-N,N,Ntrimethylammonium bromide for 12 h. The gel was dried and stained with 0.1% toluidine blue solution (acetic acid, ethanol and water in the ratio of 0.1:5:5 v/v). After staining, the gel was washed in destaining solution (acetic acid, ethanol and water in the ratio 0.1:5:5 v/v) and the result was documented.

2.9. Gradient polyacrylamide gel electrophoresis (PAGE)

Gradient polyacrylamide gel electrophoresis (PAGE) was performed on a 16 cm × 14 cm vertical slab gel bangalore genei equipment (Cat No: 05-03). The crude and fractionated II with loading buffer (tris-HCl-pH 6.8, bromophenol blue and glycerol) were loaded in the 15%-22% of gradient polyacrylamide gel and the electrophoresis was carried out at a constant current (30 mA). After electrophoresis, the gel was stained briefly in the staining solution (azure-A 0.08% w/v in PBS) and was destained in the destaining solution (methanol, glacial acetic acid and water in the ratio of 10:10:80).

2.10. FT-IR spectroscopy

IR spectroscopy of solid sample was tested using Perkin-Elmer-FT-IR instrument(USA). Fraction-III sample was mixed with 500 μg of dried potassium bromide and then compressed to prepare a salt-disc (3 mm diameter). The disc was analyzed from 450 to 4 000 cm^{-1}.

3. Results

3.1. Physico-chemical properties of molluscan crude and fractionated GAGs

Elementary microchemical analysis of the crude molluscan GAG and Fraction II GAG showed progressive increased in the all elements namely H, N and Hexosamine by considering from the crude GAG to Fraction II, but sulfate, carbon and hydrogen levels have been increased (Table 1).

Table 1.
Physico-chemical properties of molluscan crude and fractionated GAGs from *D. cuneatus*.

Physico-chemical analysis (%)	Crude molluscan GAG (%)	Fractionated GAG -II (%)
Carbon	19.21	24.32
Hydrogen	3.82	4.26
Nitrogen	1.81	2.60
Sulfate	15.40	19.42
Uronic acid	23.90	27.83
Hexosamine	25.70	28.57

Table 2.
Anticoagulant activity of molluscan GAG from *D. cuneatus*.

Sample	Azure-A Metachromatic activity(unit/mg)	Biological activity assay(USP* units/mg)	Anti-fXa activity (IU*/mg)
Crude GAG	7.02 0.19	154.0 8.0	20.07 2.22
Fraction I (1.0 M NaCl)	8.12 0.25	158.0 7.0	27.50 2.12
Fraction II (2.0 M NaCl)	12.65 0.29	175.0 7.5	58.50 2.62
Fraction III (3.0 M NaCl)	10.44 0.24	166.0 6.5	42.64 1.87

*USP-United State Pharmacopoeia; *IU- International Unit.

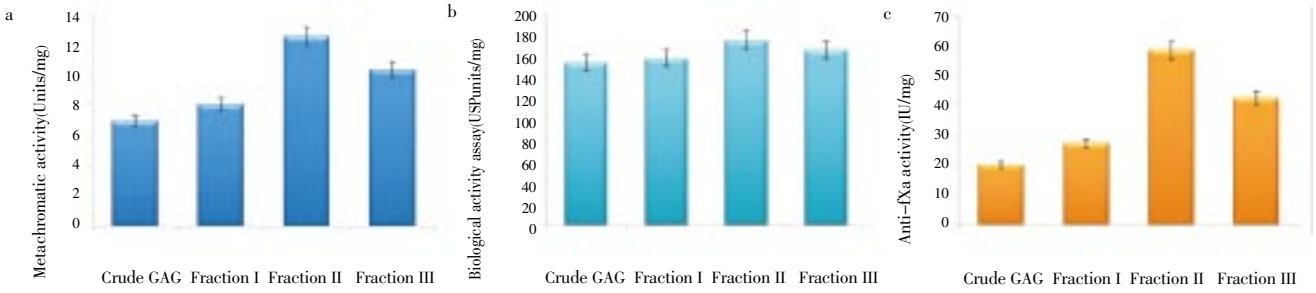

Figure 1. Anticoagulant activity assays (a: Metachromatic activity assay, b: Biological anticoagulant assay and c: Anti-fXa activity assay).

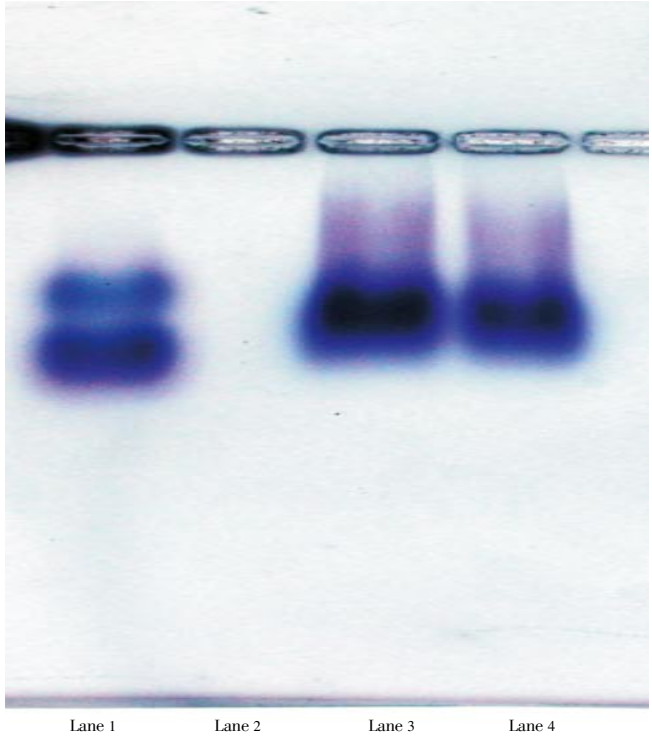

Figure 2. Electrophoretic migration of molluscan GAG from *D. cuneatus*.
Lane 1: Mixture of standard dextran sulphate; Lane 3: 30 μL of isolated molluscan GAG sample; Lane 4: 20 μL of isolated molluscan GAG sample.

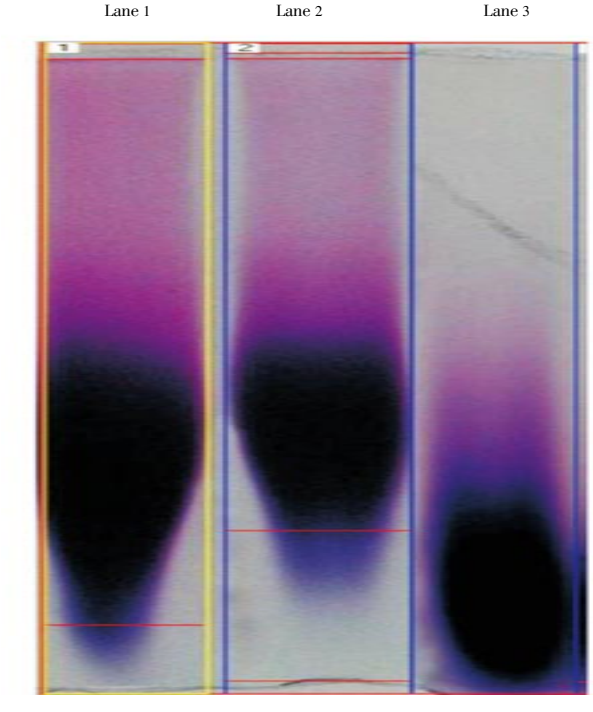

Figure 3. Showing gradient PAGE gel of various heparin bands (Lane (1) standard porcine mucosal heparin; Lane (2) crude GAG from *D. cuneatus*; Lane (3) DEAE fractionated-II).

3.2. Metachromatic, anticoagulant and anti-fXa activity

The fractionated heparin samples were analyzed for metachromatic property against azure-A and were compared with the standard heparin sodium salt. The metachromatic activity of the crude extract was (7.02 ± 0.19) units/mg (Table 2), and the fraction II was showing the proper metachromatic shift (Figure 1a).

The *D. cuneatus* showed anticoagulant activity of (154 ± 8) USP units/mg (Table 2). Figure 1b showed that the fraction II had a maximum activity of (175 ± 7.5) USP units/mg. The anti-fXa assay of *D. cuneatus* showed an activity of (20.07 ± 2.22) IU/mg (Table. 2). DEAE cellulose purified products exhibited by (58.50 ± 2.62) IU/mg for *D. cuneatus* (Figure 1c).

3.3. Agarose gel electrophoresis

The investigation showed that the isolated product has a molecular size of 30 000 (Figure 2 lane 3 and 4). Thus the difference in electrophoretic mobility of the various GAGs is a first indication of distinctive structure of these polysaccharides. The high concentration (30 μL) samples were separated as crispy band (Figure 2, lane 3). These molecules were compared with mixture of standard dextran sulphate (Figure 2, lane 1).

3.4. Gradient Polyacrylamide gel electrophoresis (PAGE)

Molecular weight of molluscan GAG from D. cuneatus was examined by gradient PAGE with azure-A staining. The crude and fractionated-II samples were compared to the known molecular weight standard porcine mucosal heparin. The Figure 3 (lane 2 and 3) showed that the molecular weight of crude and fractionated-II samples were found to be 65 000 and 50 000 Da respectively.

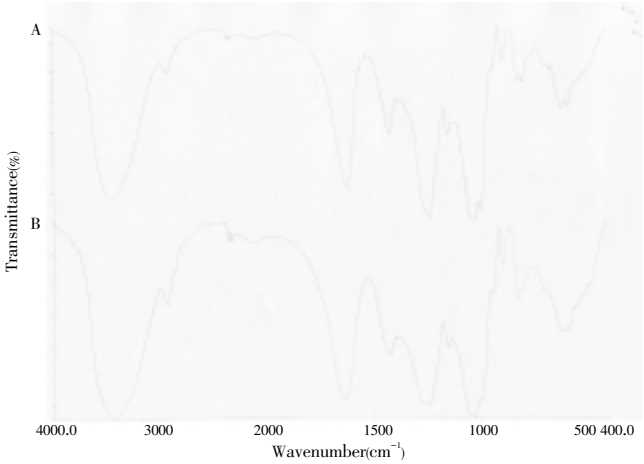

Figure 4. FT-IR analysis of fractionated -III GAG (A) compared to the standard dextran sulphate (B).

3.5. FT-IR spectroscopy

IR spectroscopy of fractionated heparin showed the presence of hydrogen bonds long with acid or amine salt, ionized compound and aliphatic tertiary amine salt. The peaks at 3 448 cm^{-1} and 2 928 cm^{-1} can be attributed to the stretching of N-H bond and C-H stretch in primary and secondary amines and alkane bends. This is similar to the functional groups of standard dextran sulphate. The ranges from 610 cm^{-1} to 680 cm^{-1} can be attributed to the sulfate ion present in dextran sulphate as well as molluscan GAG (Figure 4).

4. 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. Similarly, heparin has been prepared from a number of different species including humans[34], clams[1,6], shrimp[35] and seaweeds[36,37].

The metachromatic activities were found to be similar to that of scup viscera and turkey[4,38]. The process of fractionation helped to enhance the activity of the crude GAG by removal unwanted salt contaminants[6]. The fraction II activity results and such finding was also recorded by Mariana and Barbara[39].

Warda *et al*[40] showed the anticoagulant activity of heparin from porcine intestinal heparan sulfate with a minimal anti-factor Xa activity of <20 unit/mg. Warda *et al*[40] showed that heparin isolated from camel intestine exhibited an anticoagulant anti-factor Xa activity of ~50-60 units/mg, which was similar to that the isolated molluscan GAGs. Fractionated heparin anti-Xa activity of Anomalocardia brasiliana and Tivela mactoides clams showed an increased activity of 90 and 72 IU/mg respectively.

These results may be explained by the protease inhibitor present in plasma that inhibit factor Xa. Factor Xa can be inhibited by both antithrombin III (AT III) and heparin cofactor II (HC II). While AT III is known to bind to a specific pentasaccharide sequence found within molluscan GAGs structure. Based on these studies, molluscan GAGs are viewed as promising anti-fXa specific anticoagulants. This result suggests that purified molluscan GAGs preparation from *D. cuneatus* might have potential clinical applications as anticoagulant in near future. Similar high anticoagulant activity was also reported for purified GAGs from *Anodonta cygnea*[25], *Mytilus galloprovincialis*[41], *Aplysia californica* and *Helix aspersa*[42]. Therefore, molluscans are good alternative source for the production of anticoagulants.

Elementary microchemical levels were within the range reported by Kavanagh and Jaques[43]. The hexosamine and uronic acid values of the crude molluscan GAG and fraction II heparin are with in the range of commercial heparin as reported by earlier workers for heparins isolated from a variety of mammalian tissues[43].

Electrophoresis mobility of the sample on agarose gel was visualized with toluidine blue staining. There was a variation in mobilities among the various isolated products. The toluidine blue was only bound to the sulfated polysaccharide, but was not found to bind with other compounds. The electrophoretic migration of sulfated polysaccharides using two different pH buffer systems in 1, 3-diaminopropane (pH 9) and acetate buffer (pH 3.6) depend on the structure of the polysaccharide, which forms a complex with the diamino buffer[6]. Mariana and Barbara[39] showed that the sulfated polysaccharide had different electrophoretic mobility for different buffer system, depending on the structure of the polysaccharide.

In gradient PAGE analysis the azure-A dye bind to GAGs because of the suitable arrangement of heparin's anionic sites for interaction with the dye dimer and do not bind to proteins or any other compounds[44]. Yeong *et al*[45] obtained an electrophoretically anionic polysaccharide from *Achantina fulica* (African snail) and the purified sample was found to have a molecular weight of 29 000 Da. The fraction II, which showed a high molecular weight than the above.

The IR spectra absorption was read between 450^{-1} and 4 000 cm^{-1} and the absorption ratios were observed at 1 149 cm^{-1} and 897 cm^{-1} which attributed to the stretching of C-H bond and indicating α-glycosidic linkages of polysaccharide fractions[9]. The IR spectral bands at 1 638 cm^{-1} and 1 655 cm^{-1} are indicating the presences of carboxyl with amine and sulphate groups[46]. The standard heparin also showed closer peak intensities of similar configuration. Thus the present investigation showed that *D. cuneatus* could be a possible of anticoagulant in a future.

Conflict of interest statement

We declare that we have no conflict of interest.

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