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Isolation and characterization of anticoagulant compound from marine mollusc *Donax faba* (Gmelin, 1791) from Thazhanguda, Southeast Coast of India

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Glycosaminoglycans (GAGs) are linear polysaccharides found in the extracellular matrix and biological fluids of animals where they interact with hundreds of proteins and perform a variety of critical roles. There are five classes of animal GAGs: heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronan (HA). Many biological functions can be monitored directly by their impact on GAG quantity. In the present study, glycosaminoglycans were isolated from marine bivalve *Donax faba*. The amount of crude GAG was estimated as 12 gm/kg and of tissue in *D. faba*. After purification using gel chromatography, the yield was found to be 0.83 mg/kg. The bivalve showed the anticoagulant activity of the crude and purified samples 58 and 114 USP units/mg correspondingly in *D. faba*. The structural characterization of anticoagulant GAG was analyzed by Fourier transform infrared spectroscopy. Among the marine bivalve, *D. faba* purified showed more anticoagulant activity than that of crude sample. The results of this study suggest that the GAG from *D. faba* could be an alternative source of heparin.

Key words: *Donax faba*, GAGs crude and purified, anticoagulant activity, Fourier transform- infra red (FTIR).

INTRODUCTION

Glycosaminoglycans (GAGs) are long, linear, disaccharide repeats of hexosamine and highly sulphated galactose or hexuronic acids, and are usually found covalently attached to a protein 'core' to form proteoglycans (Kjellen and Lindahl, 1991; Silbert et al., 1997). GAGs are an extremely heterogeneous group of molecules that can be divided into several different general classes, such as haparan sulphate (HS) (and its model analogue, heparin), dermatan sulphate (DS) and chondroitin sulphate (CS), depending on the composition of the sugar backbone and the degree of sugar modification. Proteoglycans/GAGs are expressed by all nucleated cells and several bacterial pathogens such as *Bordetella pertussis* (Menozzi et al., 1994), *Mycobacterium* sp (Menozzi et al., 1996), and *Listeria monocytogenes* (Alvarez-Domingvez et al., 1997), encode surface proteins that recognize GAG, that is, GAG- binding adhesions (Rostand, 1997). The GAGs, HS and heparin have been implicated as participants in a variety of physiological processes including cell-cell recognition, blood coagulation, infection, and cell growth and differentiation. Both are polysaccharides that consist of repeating disaccharide units composed of uronic acid and an amino sugar. HS, which is sulfated lesser than heparin, is nearly ubiquities in the animal kingdom and is often a cell surface marker. On the other hand, heparin is found only in Chordata, Mollusca and Arthropoda (Nader et al., 1999) and over 80% of its glucosamine residues are N-sulfated (Gallagher and Walker, 1985). Heparin

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has been used for anticoagulant therapy for many years. Its anticoagulant effect appears to be mediated mainly through antithrombin III (AT III), which is a plasma protein and the main coagulation inhibitor in the blood.

Antithrobin III or heparin cofactor, as it is also called, inhibits thrombin and the activated forms of the coagulation factors IX, X, XI and XII. In the absence of heparin, the inhibition reactions are slow, but the addition of heparin strongly accelerates them. Several models have been proposed to explain the effect of heparin on the inhibition of thrombin by ATIII. Heparin is known to bind the ATIII; one widely accepted model assumes that heaprins forms a complex with AT III and transforms it into a more rapidly acting inhibitor (Holmer et al., 1979). Currently, commercial heparin preparations are obtained from mammalian sources, either from porcine, bovine intestine or bovine lung. Non-animal sources of heparin for pharmaceutical use are currently not available. However, the occurrence of the heparin is not restricted to mammals. Several heparin and heparin like polymers have been described in invertebrate animals such as crustaceans (Hoving and Linker, 1982), mollusks (Cavalcante et al., 1985) and ascidians (Cavalcante et al., 2000) also. It is real fact that the importance of marine organisms as a source of new substance is growing with marine species comprising approximately a half of the total global biodiversity, the sea offers an enormous resources for novel compounds, and it has been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity. A very different kind of substances have been obtained from marine organisms among other reasons because they are living in a very exigent, competitive and aggressive surrounding very different in many aspects from the terrestrial environment, a situation that demands the production of quite specific and potent active molecules (Aneiros and Garateix, 2004).

Molluscs contribute significantly to the total marine fish catch of the world. Marine bivalves are abundant in coastal and estuarine waters of India. The bivalve's fishery is constituted mainly by clams, mussels and oysters; molluscan fishery is not well-organized along the Indian coast. Molluscs are exploited in large quantities by traditional methods and sold live in the market for human consumption. The economically important species of marine bivalves are green mussel (*Perna viridis*), estuarine oyster (*Crssostera madarasensis*) and clam (*Meretrix casta*, *M*. *meretrix*, *Donex* sp. *Phapia malabarica*, *Villorito cyprinoids*) (Chattergi et al., 2002). Among the mollusks, some have pronounced pharmaceutical activities or other properties useful in the biomedical area. It is surprising that some of these pharmacological activities are attributed to the presence of polysaccharides, particularly those that are sulfated (Arumugam and Shanmugam, 2004). Hence, the attempt has been made to isolate, characterize and anticoagulant activity of the GAG from the marine bivalve *Donax faba* using chromatography. The GAGs were purified by gel chromatography using sephadex G-100 column. The structure analysis of crude and purified GAGs was determined through Fourier Transform- Infra Red spectrum.

MATERIALS AND METHODS

Isolation of glycosaminoglycans

The molluscs *D*. *faba* (Class-bivalvia; Family- Donacidae) was collected from the Thazhanguda coastal waters (Latitude, 11° 45' 0N; Longitude, 79° 45' 0E) Cuddalore, Southeast coast of India. Shells were opened and whole tissues were taken. They were blended in 0.4 M sodium sulfate solution (Na_2SO_4; 3.5 1/kg of the tissue) and kept at 55°C 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°C 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 30°C for 30 min. The precipitate was washed with ethanol and methanol through centrifugation and vaccum dried.

Purification of GAGs - Gel chromatography

GAGs were purified on a 5 × 90-cm column of sephadex G-100 (Sigma). The elution rate was approximately 60 ml/h and 15-ml fractions were collected. The active fractions were pooled and extensively dialyzed against distilled water and freeze dried (Laurent, 1978).

Anticoagulant activity

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

Fourier transform- infra red spectrum analysis (FTIR)

FT-IR spectroscopy of crude and purified GAG sample of *D. faba* relied on a Bio-Rad FT-IR- 40 model, USA. The sample (10 mg) was mixed with 100 mg of dried KBr and compressed to prepare a salt disc (10 mm diameter) for reading the spectrum further.

RESULTS

Estimation of GAGs

The amount of crude GAGs was estimated as 12 g/kg of tissue in *D. faba*. After purification using gel chromatography, the GAGs yield was found to be 0.83 mg/kg (Table 1).

Anticoagulant activity

By United States pharmacopoeia method, the anticoagulant activity of the *D. faba* crude and purified sample was reported to be 58 and 114 USP units/mg (Table 1 and

Table 1. The yield of glycosaminoglycans and their anticoagulant activity from *D. faba*.

Source	Net yield [GAGs (gm/kg)]		Anticoagulant activity [USP (units/mg)]	
	Crude	Purified	Crude	Purified
D. faba	12	0.83	58	114

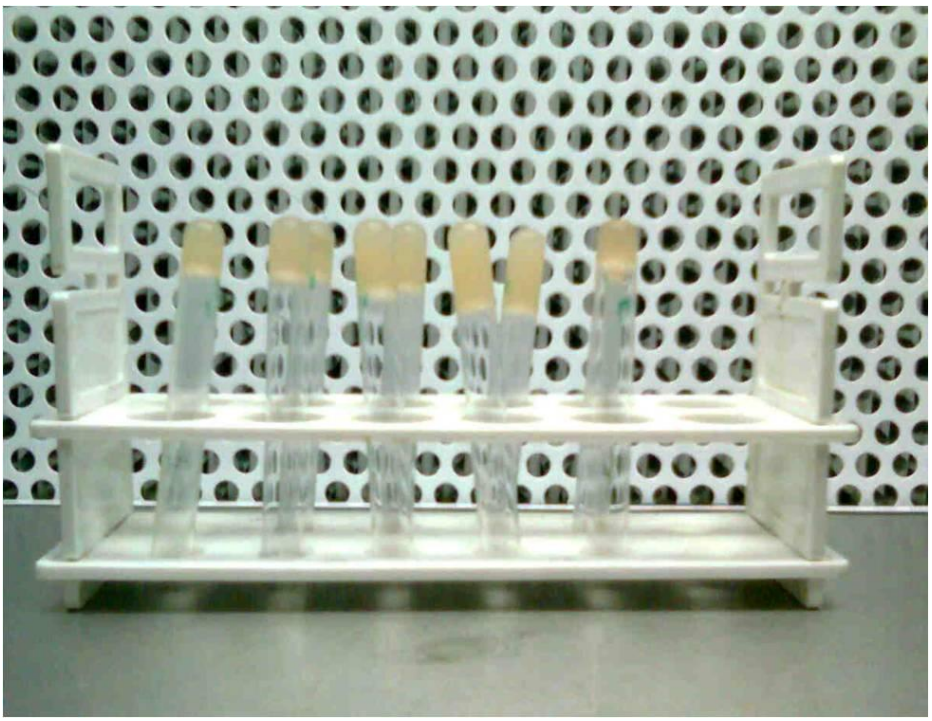

Figure 1. Anticoagulant photo.

Figure 1).

Fourier transform – infra red (FTIR) spectral analysis

FT-IR spectrum of GAGs of *D. faba* was compared with standard heparin sulphate (Figures 2, 3 and 4). The IR spectrum of standard heparin sulphate contained 17 peaks at 3973.36 to 545.85 cm^{-1}; among them 4 major peaks were 3433.29, 1639.49, 1406.11 and 1041.56 cm^{-1} (Figure 2); whereas, the IR spectrum of crude GAG from *D. faba* presents 23 peaks, among them six are major peaks 3437.15, 1639.49, 1251.8, 1197.79, 1128.36 and 1078.21 cm^{-1} (Figure 3). The IR spectrum of purified GAG from *D. faba* presents 17 peaks, and the four major peaks were 3423.65, 1635.64, 1604.77 and 1404.18 cm^{-1} (Figure 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 (Linhardt et al., 1992), clams (Shuhei et al., 2011), shrimp (Muzaffer Demir et al., 2001) and seaweeds (Mahanama De Zoysa et al., 2008). 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. Homogenization of a 1 kg portion of whole *D. faba* in acetone and subsequent extraction in acetone /petroleum ether resulted in 250 g of defatted tissue. The heparin obtained from defatted tissue described in the present study has all of the features as that of heparin. Similarly, heparin has been prepared from a number of different species including (Linhardt et al., 1992), clams (Pejler et al., 1987; Dietrich et al., 1989) and seaweeds (Wladimir et al., 2000). Extraction of the defatted soft body tissue of the giant African snail and subsequent purification of its GAGs showed that this tissue contained a large amount of GAG and free from impurities (Kim et al., 1996).

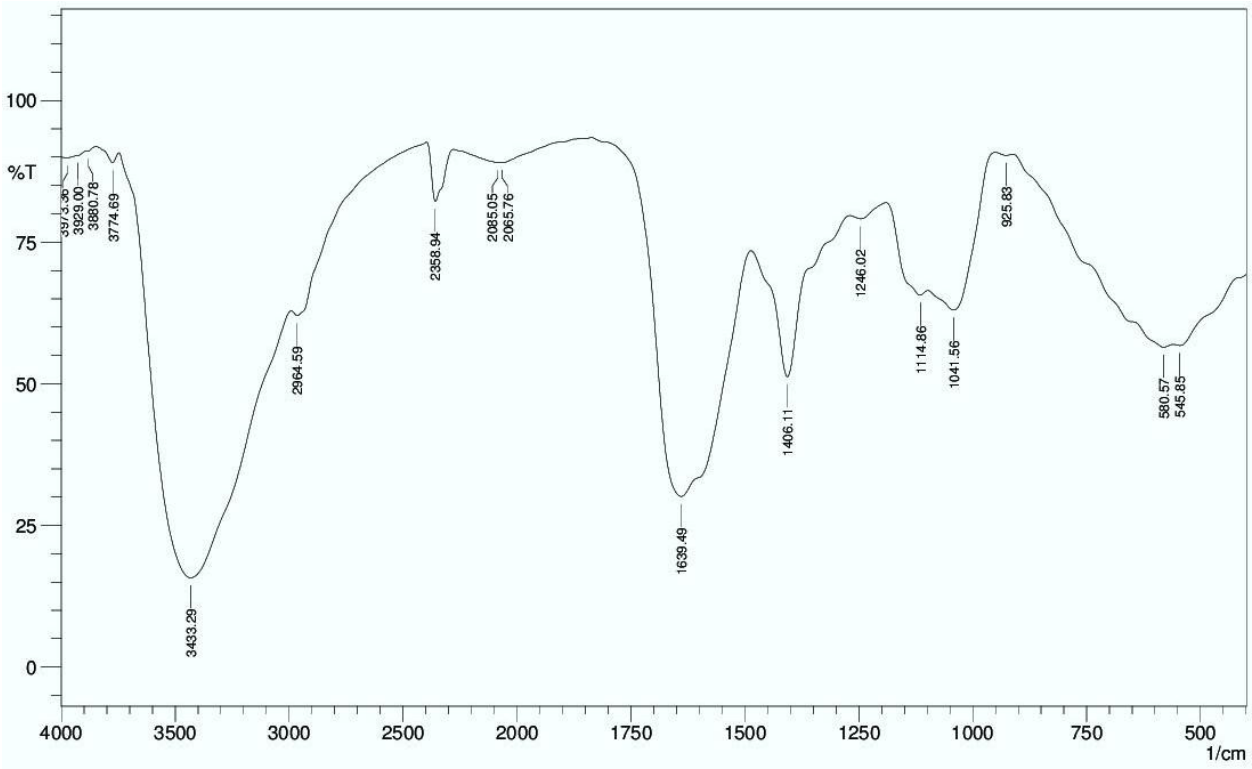

Figure 2. FTIR spectrum of standard.

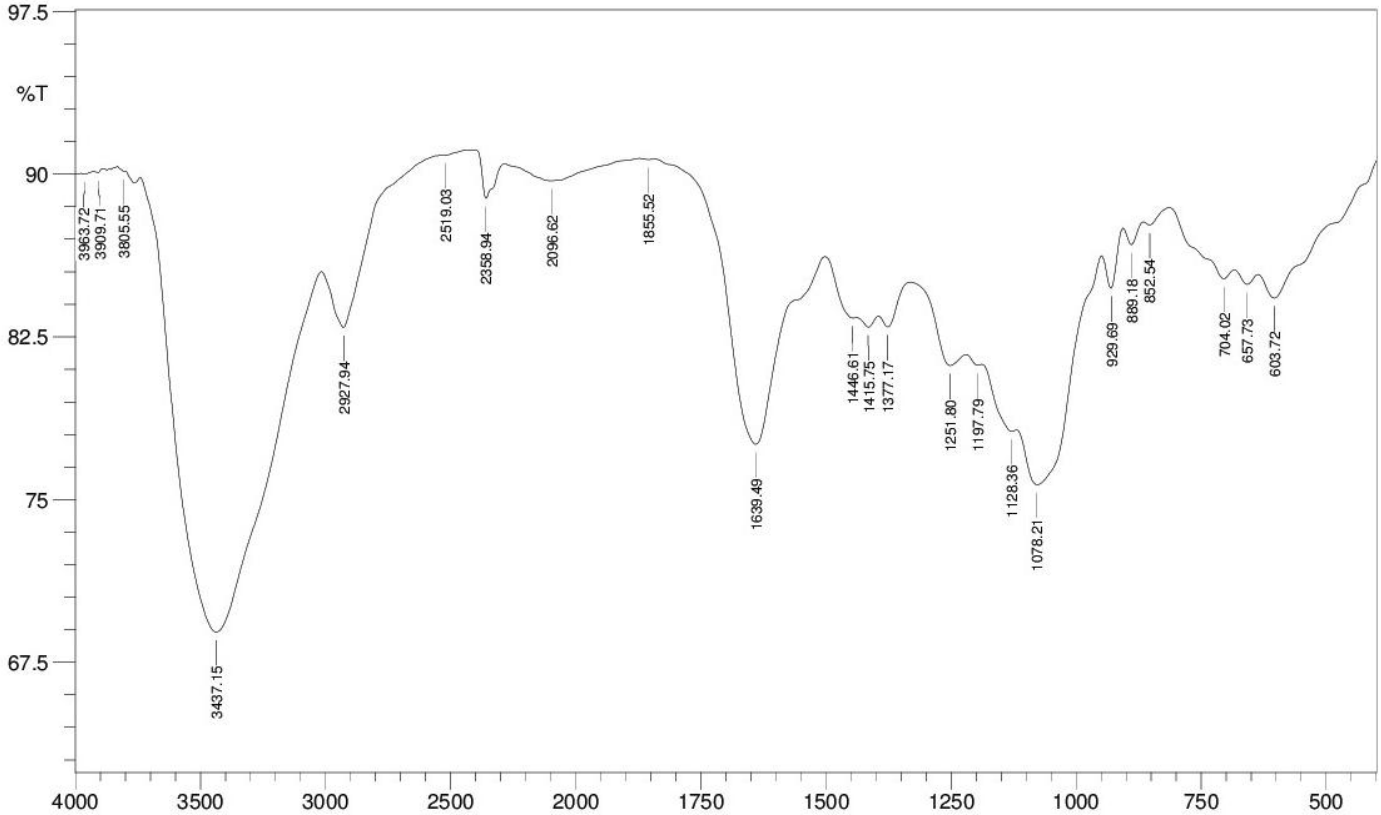

Figure 3. FTIR spectrum of crude GAGs.

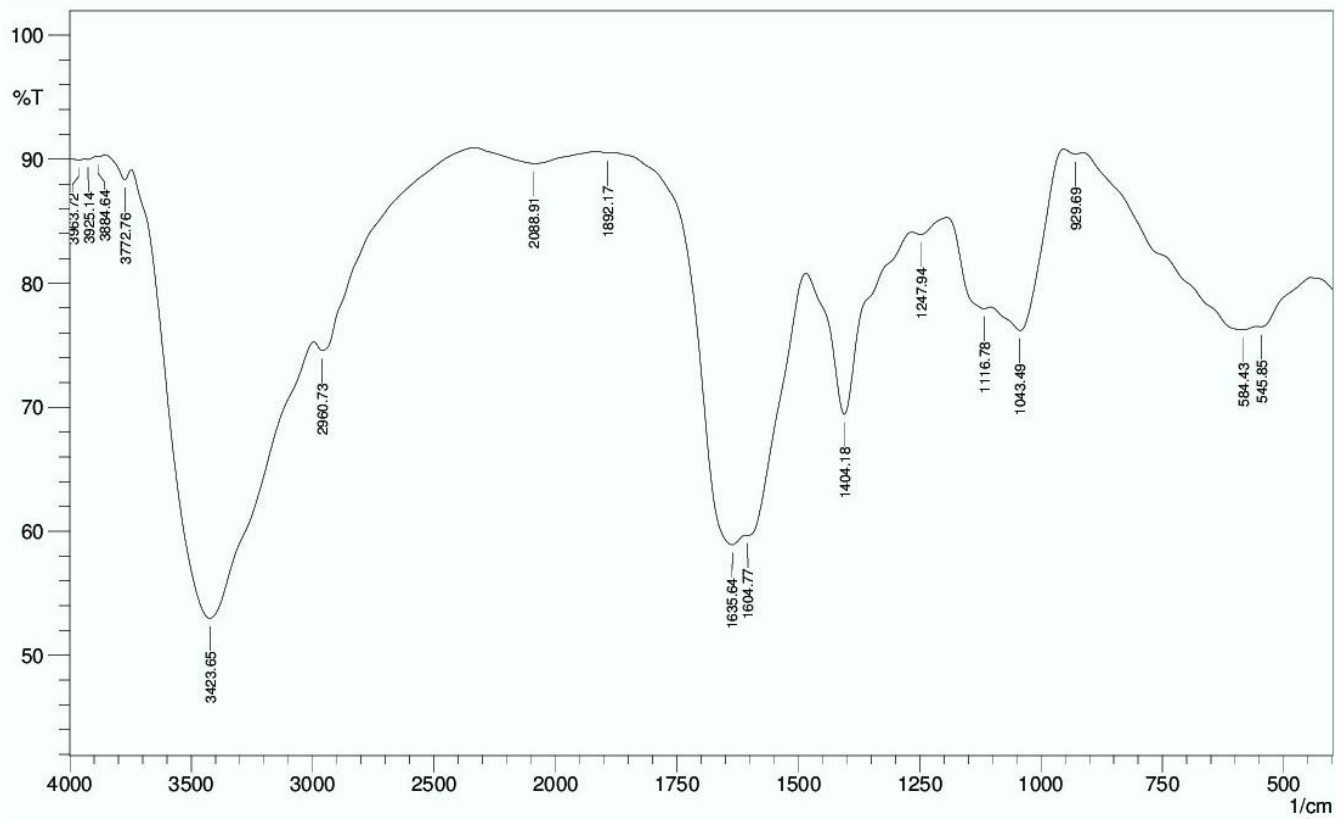

Figure 4. FTIR spectrum of purified GAGs.

The use of cetylpyridinium chloride (CPC) for quantitative separation of sulphated polysaccharides in tissue extracts in preferred. In the present investigation, the yield of crude and purified GAGs was found as 12 and 0.83 gm/kg in *D. faba*. Previously, Dietrich et al. (1989) isolated heparin with a yield of 2.8 and 3.8 g/kg from *Anomalocardia brasiliana* and *Tivela mactroides*, respectively. Somasundaram et al. (1989) obtained 7.02 gm/kg of heparin like substances in marine molluscs *Katelysia opima*. The purification of heparin-dermaton sulfate and chondrotin sulphate from mixtures by sequential precipitation with various organic solvents was reported by Volpi (1996). 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, 24.0, 16.5 and 8.4 gm/kg, respectively (Mahalakshmi, 2003; Barwin vino, 2003). Arumugam et al. (2008) had quantified the heparin yield as 2.27 and 2.2 g/kg from *Tridacna maxima* and *Perna viridis*, respectively. Vijayabaskar et al. (2008) reported that the isolated glycosamnioglycans (GAG) and purified from both bivalves were estimated as 5.4, 4.1 and 1.4, 1.1 gm/kg wet tissue in *K*. *opima* and *D. cuneatus*. Vidhyanandhini (2010) reported that the amount of crude GAGs was estimated as 9.85 gm/kg and after purification by using amberlite and barium acetate, the yield was found to be 33 and 148 mg/kg of *K. opima*. Saravanan and Shanmugam (2010) reported the amount of crude and purified GAG was estimated as 17.2 g/kg and 48 mg/kg of tissue in *A. pleuronectus*. The result of the present study clearly shows that the yield of heparin and heparin- like glycoasaminoglycans is higher and lower than those of previously reports. Hence, it could be concluded that the molluscs might be used as potent sources for the extraction of heparin and heparin -like glycosaminoglycans.

The heparin isolated from marine clams and mussels has identical structural features and anticoagulant activity of mammalian polysaccharide (Pejler et al., 1987). Heparin with high anticoagulant activity was isolated from the marine molluscs, *Anomalocardia brasiliana*, *Donax striatus* and *Tivela mactroides* (Dietrich et al., 1985), which showed similar activity like mammalian heparin but differ in molecular weight; the molluscan heparin have a higher molecular weight and high anticoagulant activity (Dietrich et al., 1989). The biological activity of heparins in invertebrates remains enigmatic. The classes of Crustacea and Mollusca do not posses any blood coagulation system similar to that of mammals and other vertebrates and thus the presence of compounds that all specifically upon the proteins of the blood coagulation system is indeed remarkable (Dietrich et al., 1999). The anticoagulant activity of heparin differs from species to species due to their iteration with enzymes and inhibition of the coagulation

system (Mulloy et al., 2000). The anticoagulant activity of the crude and purified sample of GAG from the whole body tissue of *D. faba* was reported as 58 and 114 USP units/mg. This variation might be due to the presence of non-anticoagulant substance in the samples since the activity of heparin depends upon the amount of impurity carried over in the isolated products. Burson et al. (1956) showed the activity ranging from 130 to 150 USP units/mg for extracted products of *Spisula solidissima* and *Cyprina islandica*. Arumugam and Shanmugam (2004) reported the anticoagulant activity crude and fractionated sample of GAG from *T. attenuate* - 37 and 78 USP units/mg in *T. attenuate*. The anticoagulant activity of crude and purified sample of GAG from *A. pleuronectus* were 15.38 and 83.99 USP units/mg (Suganthi, 2007).

The crude and purified sample of *E. berryi* were 415 and 483.1 USP units/mg (Shanmugam et al., 2008). Vijayabaskar et al. (2008) reported that the extraction of GAGs from *K. opima* and *D. cuneateus* showed anticoagulant activity of 160 and 154 USP units/mg, and after the partial purification through DEAE cellulose column chromatography, the yields of anticoagulant activity were found to be 180 and 175 USP units/mg (Vidhyanandhini, 2010). The anticoagulant activity of crude and purified samples were estimated at 22.52, 20.00 and 18.60 USP units/mg for amberlite; 86.32, 83.06, and 92.43 USP units/mg for barium acetate and 80.36, 75.92, 89.68 USP units/mg for *K. opima*. From the aforementioned, it could be understood that whole body tissue of *D. faba* is also comparatively a good potential source of anticoagulant compounds. The molluscan GAGs were found to be structurally similar to the standard heparin as assessed by the FT-IR spectra. In the present study, the anticoagulant GAGs from whole body tissue of *D. faba* crude and purified sample showed a major peaks at 3437.15, 1639.49, 1251.8, 1197.79, 1128.36 and 1078.21 cm^{-1} which is said to be for the GAGs groups (Saravanan and Shanmugam, 2010).

For FT-IR spectrum of purified sample, the sulfate band stated from 1139.92 cm^{-1} and extended down to 995.12 cm^{-1}. The acetyl amino group was represented by a band at 1474.78 cm^{-1} and the carboxylic group at 1552.66 cm^{-1}. This was also well supported by the study of Rivera et al. (2002) who also claimed that the characterization of traces of contaminants in crude heparin by conventional physico-chemical techniques such as size-exclusion or ion-exchange chromatography is relatively difficult. The peak pattern between the standard heparin and the purified sample were at 3433.29, 1639.49 cm^{-1} and 3437.15, 1639.49 cm^{-1} as indicating the presence of GAGs group in the samples analyzed. The FT-IR spectral analysis of the anticoagulant GAGs from *D. faba* showed more or less same number of peaks, lying within the same range of values of the commercial heparin used as a standard. The results in this finding show that bivalve, *D. faba* tissue had GAGs with high quantity of anticoagulant compounds. Thus, the result of the present investigation provides information about the isolation, purification and characterization of the heparin and heparin like glycosaminoglycans (heparin and heparin sulfate) compound from their chemical characteristic features. Further, it will pave the way for future researchers to take up research in this line to characterize fully the heparin and heparin like GAGs of molluscan source using chromatography different fraction, NMR, MS and other advanced techniques.

Besides the aforementioned, it could also have a very good anticoagulant activity as the anticoagulant compound from this species proves the possibility of its utilization as an additional potent source for the extraction of such anticoagulant compound since the anticoagulant activity of the presents isolated heparin and heparin like GAGs from *D. faba* was even more than that of many heparins obtained from commercial sources.

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