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Isolation and characterization of Molluscan glycosaminoglycans from Pazhayar, South-East coast of India

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Abstract
Glycosaminoglycans (GAG's) are therapeutic potential natural bioactive compounds. Recent knowledge on the biological roles of these molecules in cancer biology, tumor angiogenesis and metasis has promoted the development of drugs targeting them. In the present study the GAG's were extracted from the gastropod *Harpa conoidalis.* The crude GAGs were fractionated by ion-exchange chromatography and were estimated as 18.166 mg/kg. Different methods were compared and the best results were attained when the GAG were measured by densitometry after agarose gel electrophoresis. The gastropod showed the anticoagulant activity of the purified sample 1.4 and 2.3 folds in activated partial thromboplastin time (APTT) and thrombin time (TT) assays correspondingly and its purity was determined metachromatic activity. The structural characterization of GAG was comparability analyzed with standard by Fourier transform infra-red spectrum (FT-IR).

Keywords: Glycosaminoglycans; *Harpa conoidalis*; FT-IR; Anticoagulant, Metachromatic activity.

1. Introduction
The marine species comprising approximately half of the total global biodiversity and the sea offers enormous amount of novel compounds (De vries *et al.* 1995) [1]. This is because most of the marine animal life is fixed to a substratum, that it produces bioactive metabolites in response to ecological pressures such as competition for space, deterrence of predation and the ability to reproduce successfully (Defer *et al.* 2009) [2] and this biodiversity of the marine environment and the associated chemical diversity constitute a practically unlimited resource of new active substances in the field of the development of bioactive products (Aneiros and Garateix 2004) [3]. It also considered as the largest reservoir of natural molecules to be evaluated for drug activities (Gerwick 1987) [4]. Approximately 16,000 marine natural products have been isolated from marine organism and is reported in about 6,800 publications (Bhakuni 1994) [5].

In the past 30-40 years, several clinically useful drug, investigational drug candidates and pharmacological tools have already resulted from these marine product discovery programs (Faulkner 2001) [6]. Of which some of the marine natural products that have been successfully advanced to the last stages of clinical trials are dolastatin-10, ecteinascidin-743, kahalaide F and conopeptides (Ziconotide) and growing number of candidates have also been selected as promising leads for extended preclinical assessments (Donia and Hamann 2003) [7].

Generally, a large proportion of these natural compounds have been extracted from marine invertebrates, especially sponges, ascidians, bryozoans and molluscs, moreover many of them are currently in clinical trials (Proksch *et al.* 2002) [8]. Many studies have been carried on bioactive compounds from mollusk exhibiting antibacterial, antitumour, antileukemic and antiviral activities (Kamiya *et al.* 1984; Anand *et al.* 1997; Rajaganapathy *et al.* 2000) [9, 10, 11]. The study of marine organisms as a source of biologically active compounds is considered as a very lucrative field, having already led to the discovery of various new pharmacological tools and medicines (Bhakuni 1994; Munro *et al.* 1999; Faulkner 2000a) [5, 12, 13]. The work of Bergman and Feeney at the beginning of the 1950's initiated the study of natural products and in the last few decades, an appreciable number of new compounds have been isolated from marine organisms (Bergman and Freeney 1951; Bhakuni 1994; Faulkner 2001) [14, 5, 6].

Moreover, very different kinds of substances have been procured from these marine organisms because they are living in a very exigent, competitive and aggressive surrounding including polyunsaturated fatty acids (PUFA), polysaccharides, minerals and vitamins, antioxidants, enzymes and bioactive peptides (Kim *et al*. 2008; Pomponi 1999) [15, 16].

The extracellular matrix (ECM) is a gel like material filling the extracellular space in animal tissues that holds cells together and provides a porous path way for the diffusion of nutrients and oxygen to interstitial matrix and the basement membrane. The structure is composed by fibrous proteins (collagen elastin, fibronectin and laminin) glycosaminoglycans (GAGs) formerly named as mucopolysaccharides are important compounds of the extra cellular matrix of connective tissue and also on the cell surface of many cell types and in intra cellular granules (Manjusha 2011) [17]. They occur as long chains of linear acidic polysaccharides which are generally associated with matrix proteins (core proteins) via covalent bond form proteoglycans (PGS) and thereby involved in a wide range of biological process. GAGs can be sulfated (chondroitin-sulfate, dermatan-sulfate, heparin/heparan-sulfate, keratin-sulfate) or not (hyaluronic acid). Furthermore, sulfated glycosaminoglycans can be covalently bound to a protein to form proteoglycans. Hyaluronic acid (HA) is very peculiar because it is neither sulfate, nor covalently linked to a protein to form proteoglycan. Moreover this polysaccharide has a very high molecular weight (up to 8 x 10^6 g/mol in tissue) in contrast to sulfated GAGs (from 10 to 100 x 10^3 g/mol) (Jackson *et al*. 1991; Iozzo 1998) [18, 19]. GAGs interact with a wide range of proteins involved in physiological and pathological processes. They display many biological activities which can influence tissue repair as well as inflammatory response. These are negatively charged linear polysaccharides constructed of repeating disaccharide units with the primary configurations containing an amino sugar (either Gly NAC or Gal NAC) and an uronic acid either glucuronic acid or iduronic acid. It was also noted that the chemical diversity of the sulfated polysaccharides was largely species specific (Berteau and Mulloy 2003) [20]. It is highly polarized and attracts water. Therefore, they are useful to the body as a lubricant or as a shock absorber. Generally, GAGs are found in shells of marine animals.

Marine organisms offer a great diversity of polysaccharides showing interesting biological properties mimicking those described for the mammalian GAGs. Among the different sources of polysaccharides, molluscan derivatives could play an important role in Pharmaceutical fields. However, detailed studies on GAGs from tissues of invertebrates, including marine gastropod are rather limited. This neo gastropod has not been screened for marine natural products chemistry yet and therefore an attempt is made to evaluate the biochemical properties and its pharmacological applications. The *H. conoidalis* found abundance along the mudasal odai coast, Tamil Nadu, India was chosen for the present study with an objective to explore its bioactive potential in specific to GAGs.

2. Materials and Methods

The species *H. conoidalis* was collected from Mudasal odai landing centre along the East coast of Tamil Nadu, India (Latitude 11º29'02.44"N, Longitude 79º46'29.41"E) and brought to the laboratory in ice cold condition. All the animals were washed thoroughly twice with distilled water. The shell was removed and the whole body tissue was cut in to the small pieces and then defatted with different organic solvents by following the method of Holick *et al*. (1985) [21].

2.1 Extraction of Glycosaminoglycans

The procedure of Holick *et al*. (1985) [21] was followed with slight modification to extract glycosaminoglycans from *H. conoidalis.* The whole animal *H. conoidalis* was blended and defatted with acetone, filtered and further defatted with petroleum ether. The defatted tissues were air dried at room temperature. The dried defatted tissues were ground and mixed with 250 ml of 0.4 M sodium sulphate (Na_2So_4) and incubated at 55 °C for 1 h for 30 min (pH 11.5). After incubation, aluminium- di sulphate (Al_2 (So_3) crystals were added to bring down the pH to 7.7 and heated to 95 °C for 1 h. The sample was cooled and centrifuged 2000 rpm. After centrifugation, the supernatant was collected and treated with cetyl pyridinium chloride (CPC) 70 ml of 3% CPC in 0.8M sodium chloride (NaCl) was added to the collected supernatant. The mixture was stirred until the complete formation precipitate. This suspension was further incubated at 37 °C for 24 h and centrifuged at 4 °C for one and half hours in a refrigerated centrifuge to collect the crude GAGs. The precipitate was re-dissolved in 2M NaCl to remove pyridinium salts from GAGs and centrifuged for 30 min at 2500 rpm in refrigerated centrifuge. To this collected supernatant, 3 volumes of 99.9% methanol were added that would initiate precipitation of crude GAGs. The precipitated crude GAGs were collected by centrifugation at 3000 rpm at 4 °C for 30 min. Then the precipitate was subsequently washed with 99.9% ethanol and dried in a vacuum desiccator.

2.2 Purification of Glycosaminoglycans

The crude glycosaminoglycans (0.5 gm) was fractionated by anion exchange chromatography on a column (2.5 x 15cm) of Amberlite IRA-900 (cl-) formed in aqueous solution. The crude GAGs samples was applied to the column and washed initially with water. Then the sample was recovered by stepwise elution, by eluting with 100 ml of 1 M NaCl in the same sodium acetate buffer. The flow rate was maintained as 10 ml/h. the 5 ml of factions were collected and assayed through uronic acid estimation. The fractions containing uronic acid were pooled, dialyzed against distilled water and lyophilized.

2.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis using barium acetate 1, 2 - diaminopropane was performed as described by Volpi and Maccari (2003) [22] with suitable modifications. Agarose gel was prepared at the concentration of 0.5% in 0.04 M barium acetate buffer at pH 5.8. The run was made in 0.05 M 1, 2–diaminopropane buffered at pH 9.0 (with acetic acid for 60 minutes at 500 mA. After migration, the plate was soaked in 0.1% CTAB (Cetyl trimethyl ammonium bromide) solution for at least 6 h, dried and stained with toludine blue. Then the gel was de-stained with water to reveal the presence of glycosaminoglycans band.

2.4 Metachromatic Assay

The amount of GAGs present in the sample was estimated following the method of Grant *et al*. (1984) [23]. The addition of GAGs to Azure-A dye change the color of the solution from blue to violet, a phenomenon termed as metachromasia. A stock solution of Azure dye (0.1%) was prepared and stored at 4 °C up to a maximum period of two weeks. The lyophilized samples were serially diluted (100, 200, 300, 400 and 500 µl) and 10 ml of working standard azure-A solution (0.02g) was added. The absorbance was measured at 620 nm within 30 min

of the time duration after the addition of dye. The activity was calculated from the standard curve prepared by plotting OD values on the X-axis against corresponding units on the Y-axis.

2.5 Anticoagulant Activity

The anticoagulant activity of the sample was estimated by using the Activated partial thromboplastin time (APTT) and thrombin time (TT) clotting assay performed according to the manufacture's specifications as described by Mauray *et al.* (1997) [24]. In this assay, normal human plasma (90µl) was mixed with different amounts of standard and samples from 10µl to 50µl in 0.9% NaCl (10µl) and the APTT was recorded as the time for clot formation in acoagulometer. The anticoagulant activity of the GAGs as expressed in concentration dependent manner.

2.6 Fourier Transform Infra-Red spectrum analysis

The functional group present in the isolated GAGs from *H. conoidalis* was determined using FT-IR spectroscopy (Bio – read FTIR – 40 models, USA). 10 mg of sample was mixed with 100 mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc (10 mm diameter) for reading the spectrum. The absorption was read between 500 and 4000 cm^{-1}.

3. Results and Discussion

In general, the occurrence of heparin in invertebrates seems to be more widespread than suspected in the past. Dietrich *et al* (1985) [25] has been studied the complete characterization of this particular compounds and also there are some evidence for the presence of heparin sulphate compounds in molluscs and invertebrates (Cassaro and Dietrich 1997; Nader *et al.* 1999) [26, 27]. In the present investigation, the amount of Glycosaminoglycans (crude) was estimated as 5.737 gm/kg of dry tissue in *H. conoidalis*. Purification was using by anion exchange and the fractions of peak II from 6-11 and Peak III from 13- 16 collected using column chromatography exhibiting maximum absorbance at 550 nm (Fig. 1) were pooled together, the final yield was found as 18.16 mg/gm.

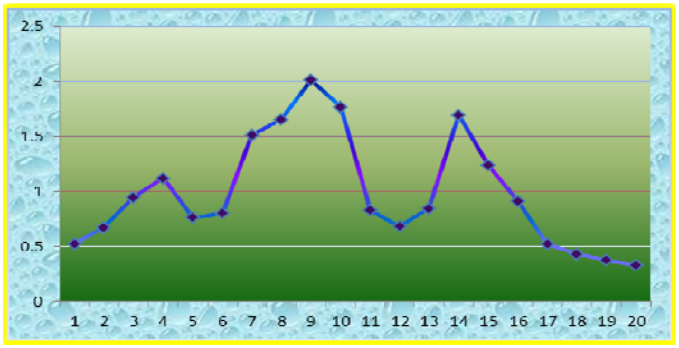

Fig 1: Shows the absorbance spectrum of fractions at 550 nm.

Previously, Dietrich *et al.* (1989) [28] have isolated heparin with a yield of 2.8 and 3.8 g/kg from *Anomalocardia brasiliana* and *Tivela mactroides*, respectively. Somasundaram *et al.* (1989) [29] obtained 7.02 gm/kg of heparin like substances in marine molluscs *K. opima*. 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) [30, 31]. Arumugam *et al.* (2008) [32] had quantified the heparin yield as 2.27 and 2.2 g/kg from *Tridacna maxima* and *Perna viridis*, respectively. Similarly, Vijayabaskar *et al.* (2008) [33] have 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) [34] had 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) [35] have reported that the amount of crude and purified GAG was estimated as 17.2 g/kg and 48 mg/kg of tissue in *A. pleuronectes*. In the present study the net yield of GAGs varies and depends upon the species and extraction method followed. It also showed that mollusks from marine environment might be used as potent source for GAGs extraction.

3.1 Agarose gel electrophoresis

Agarose gel electrophoresis is a useful technique to analysis GAGs in mixture. Separation of polysaccharides using agarose gel electrophoresis was investigated for the first time by Dietrich *et al.* (1976) [36] and Cassaro and Dietrich (1997) [26]. There was a variation in mobilities among the various isolated products in agarose electrophoresis. The toluidine blue was found to bind only with sulfated polysaccharide, but was not found to bind with other compounds (Pavao and Aiello 1998) [37]. Thus the difference in electrophoretic mobility of the various GAGs is a first indication of unique structure of these polysaccharides. Dietrich *et al.* (1989) [28] and Mariana and Barbara (1999) [38] showed that the sulfated polysaccharide had different electrophoretic mobility for different buffer system, depending on the structure of the polysaccharide. In the present study, the result obtained from the agarose gel electrophoresis using a buffer system of 1, 3-diaminopropane revealed that the band mobility of the sample was similar to the standard visualized after staining with toluidine blue peak II and III (Fig. 2). The previous study in the invertebrates made by Arumugam *et al.* (2008) [32] also reported the same migratory pattern of bands for the glycosaminoglycans.

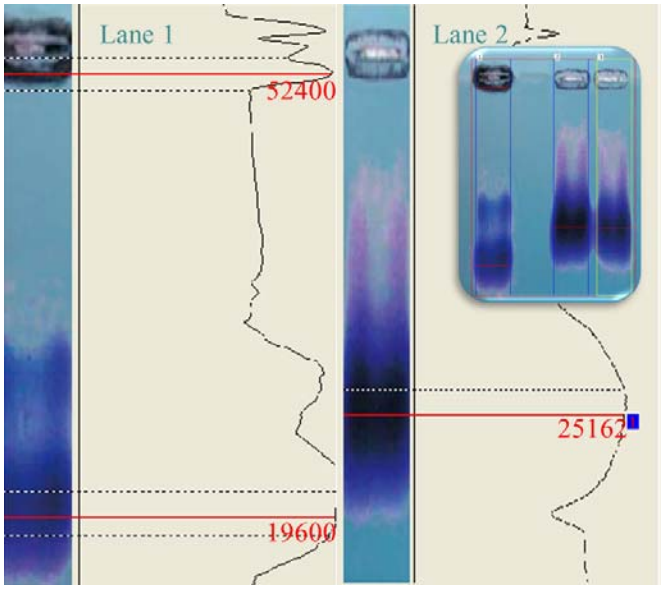

Fig 2: Shows the agarose gel electrophoresis of GAGs. Lane 1 – Standard heparin sulphate; Lane 2 – Purified GAGs sample (Peak II); lane 3 – Purified GAGs sample (Peak III)

3.2 Metachromatic activity

Consequently, the purified GAGs was analysed for metachromatic activity using azure-A and compared with the standard heparin sodium salt. The azure-A assay is a

colorimetric determination based upon dye metachromatic absorbance shift induced by heparin-like substances. Therefore, the metachromatic activity for the purified GAGs from the *H. conoidalis* was calculated as 48.45 IU/mg. Likewise, The total yield of heparin and heparin-like compounds and its activity calculated by metachromatic method were found to be as 73.6 IU/mg (285µg/mg) of wet tissue and 16 IU/mg and 12.65 IU/mg of the sample *Turritella attenuate* and *Donax cuneatus* respectively (Arumugam and Shanmugam 2004 ; Vijayabaskar and Somasundaram 2012) [39, 40] whereas the yield and activity of *H. conoidalis* was found to be higher than the above reported result. Whereas, the metachromatic activity of purified GAGs from the marine bivalves *A. pleuronectes* possessed 172 IU/mg and 12.65 IU/mg (Saravana and Shanmugam 2010) [35]. The yield from the purified sample of *H. conoidalis* was found lower than that of tauttog viscera (13,570 IU/kg), scup viscera (15,461 IU/kg), flounder viscera (10,789 IU/kg), and scallop viscera (9,254 IU/kg).

3.3 Anticoagulant activity

Activated partial thromboplastin time (APTT) related to the intrinsic coagulation phase in plasma. GAGs had considerable effect on the APTT assay, this being expected because sulfate groups are necessary to provide anticoagulant effects and anticoagulant activities of polysaccharides; these are not only dependent on the sulfate content but also on the position of the sulfate groups (Linhardt *et al*. 1992) [41]. In this respect, polysaccharides with lower anticoagulant activity than heparin could exhibit a potent antithrombotic effect with less hemorrhagic risk (Cassaro and Dietrich 1997) [26]. In the case of anticoagulant activity the isolated HS showed significant anticoagulant activity compared with the standard Heparin sulfate. The results showed 1.4 and 2.3 fold higher concentration of sample HS is needed to obtain the same effect of standard heprin sulfate (Fig. 3 and 4). These results are comparatively better with the data reported by (Monsour *et al*. 2009) [42]. Consequently, higher concentration of sulfated polysaccharide is also isolated from the green algae *Arthrospira platensis*, with 5 and 7 folds for APTT and TT assay (Majdoub *et al*. 2009) [43]. Hence the present investigation revealed that the extracted GAGs have prominent anticoagulant activity on both APTT and TT assay.

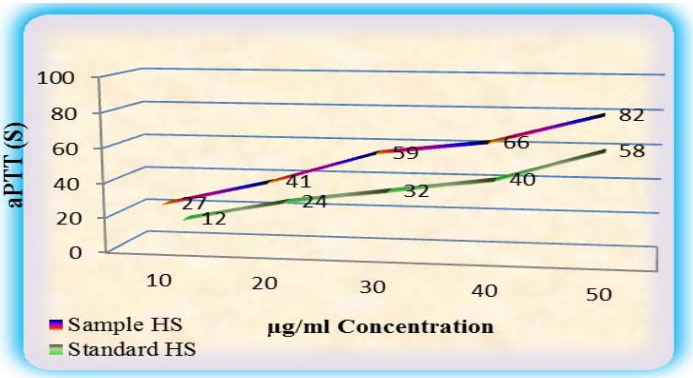

Fig 3: Shows the effect of APTT assay (*H. conoidalis*)

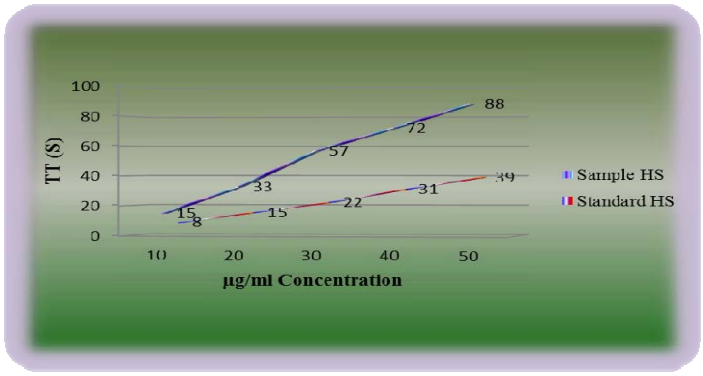

Fig 4: Shows the effect of TT assay (*H. conoidalis*)

3.4 FT-IR

IR spectroscopy is one of the most powerful analytical techniques used for structural elucidation and identification compound. It has been used to examine and provide important data on a wide variety of biological molecules. Thus, the isolated GAGs have been characterized by the FTIR. The assignment of different band patterns of standard, confirmed the presence of important functional groups of Hyaluronic Acid (HA). The revealed functional group of extracted GAGs was compared with heparin standard. Therefore, the similar peak ranges were obtained for both the purified GAGs from the *H. conoidalis* and the standard (Fig. 5 and Table 1).

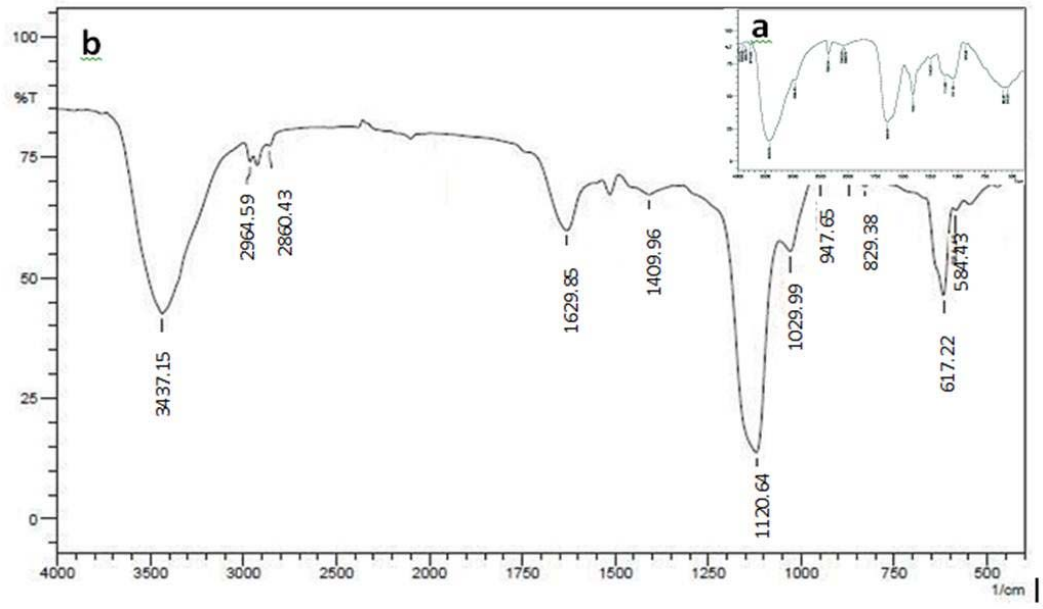

Fig 5: FTIR spectrum of (a) standard Heparin sulphate and (b) isolated GAGs (*H. conoidalis*).

Table 1: Peak assignment of FTIR Spectrum for isolated GAGs and Standard HS.

S.No.	Group frequency (cm^{-1})	Functional group	Group frequency (Cm^{-1})	
			Standard Heparin sulphate	*H. conoidalis*
1.	705-570/ 620-600	Di Sulfides (C-S / S-S Stretch)	580.57	584.43 / 617.22
2.	1090-1020	C-N- Primary amine CN Stretch	1041.56	1029.99
3.	1140-1070	C-O-C Cyclic ethers, large rings, C-O Stretch	1114.86	1120.64
4.	1420-1300	Carboxylate (Carboxylic acid salt)	1406.11	1409.96
5.	1640-1620	Organic nitrates	1639.49	1629.85
6.	2970-2950/ 2865-2845	Methylene C-H asym. / sym. Stretch	2964.59	2964.59 / 2860.43
7.	3570-3200/ 3490-3430	Hydroxyl group H- bonded OH stretch / Heterocyclic amino NH Stretch	3433.29	3437.15

4. Conclusion

Heparin sulphate is obtained from animal tissues and extraction procedures have been developed for the preparation of highly active nontoxic fractions for several clinical uses. This material however, is not homogenous according to chemical and physical chemical criteria, and several of the components exhibit significant anticoagulant activity. This lack of homogeneity causes difficulties in attempts at establishing a definition of the term "Heparin" $(C_{12}\ H_{16}\ NS_2\ Na_3)_{\ 20}$ and molecular weight are varied from 3000 to 30,000da. Heparin and heparin sulphate are the components of connective tissues or cell surface carbohydrates, involved in the cell's interaction with and response to its surroundings. The anticoagulant heparin sulphate glycosaminoglycans are important therapeutic agent used in the prophylaxis treatment of thrombosis. In conclusion, the study shows the prominent anticoagulant activity that could be pave a clear vision in biomedical arena and clearly depicts the advantage of isolated heparin sulphate – like GAGs of *H. conoidalis* that could serve as an alternative source to meet the demand for commercial value.

5. Acknowledgement

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