CHAPTER VI

4.3 ISOLATION OF HEPARIN LIKE SUBSTANCES FROM DIFFERENT BODY PARTS OF NEMIPTERUS JAPONICUS AND EXOCOETUS VOLITANS

4.3.1 Introduction

Heparin is the only chemically unique polysaccharide found in mammalian tissues with important biological properties like anticoagulant, antilipaemic agent, and antiviral agent [442]. Heparin contains equimolar amounts of D-glucoronic acid and 2-amino-2-D-glucose residues and is distinguished by having the amino sugars N-sulphated rather than N-acetylated and by its O-sulphate content. There are two problems related to extraction of heparin 1) the release of the heparin from the tissues and 2) the freeing of the extracts of the material interfering with the measurement of heparin [443]. Heparin does not exist in the body fluid in the free state and is usually firmly bound to tissue protein and the principle aim of isolation is heparin with high anticoagulant activity with low toxicity. The incidence of death because of thrombosis is believed two times higher than the next disease cancer. Heparin is the most widely used glycosaminoglycans (GAG) used as anticoagulant therapy in thrombolic processes. Heparin and its derivatives are extensively used in the treatment of myocardial infarction, unstable angina, venous thrombosis and pulmonary embolism and has decreased substantially these deadly heart diseases (about 30 %) when compared to malignant cancer [444]. This explains the efforts to discover and develop specific and more potent antithrombotic agents.
Heparin and heparin like substances appears to be ubiquitous natural product found in most of the mammalian tissues like viscera, lung, skin, kidney, liver, mast cells and basophils. It is reported that variety of tissues from marine life contains heparin or heparin like substances with high anticoagulant activity [424]. Moreover, heparin or heparin like substances have been reported from variety of clams, in blood, muscle, viscera and skin of various fishes, in cartilage of skates and sharks and in the tissues of many crustaceans [424].

The present work is focussed on heparin like glycosaminoglycans isolated from muscle, skin, liver and visceral mass of two commercial fishes *Nemipterus japonicus* and *Exocoetus volitans*.

### 4.3.2 Materials and Methods

Standard heparin, chondroitin sulphate, dermatan sulphate, heparan sulphate were purchased from Sigma Chemical Co., (St. Louis, Mo, USA.). Barium chloride, gelatin, toluidine blue and 1, 3-diaminopropane was purchased from Himedia Chemicals. Vero cell lines were obtained from National Center for Cell sciences, Pune, India whereas, HSV-I Viruses were obtained from National Institute of Virology, Pune, India.

#### 4.3.2.1 Extraction of heparin-like compounds

Extraction of heparin like-compound was done by the method of Holick, *et al.*, [424].

**Reagents**

1. Peroleum ether
2. Sodium sulphate
3. Ethanol
4. Acetone
5. Aluminium sulphate
6. Sodium hydroxoide
7. Cetyl pyridinium chloride

Procedure

The fish skin, muscle, liver and visceral mass were blended, separated, defatted with acetone, filtered and further defatted with petroleum ether. The defatted tissues were air dried at room temperature. 100 gm of dried defatted tissues were grounded and incubated with 2 litres of 0.4 M sodium sulphate (Na₂SO₄) at 55 °C for one and half hour. The pH was maintained at 11.5 using sodium hydroxide (NaOH). After incubation aluminium sulphate (Al₂SO₄) was added to bring down the pH to 7.7 and heated to 95 °C for 1 hour. The sample was then filtered through a cheese cloth and treated with cetyl pyridinium chloride (CPC). To the collected filtrate, 135ml 3% CPC in 0.8 M sodium chloride (NaCl) was added. The suspension was incubated at 37 °C for 18 hours and a white precipitate was formed. It was then centrifuged at 3000 g for 30 min at 4 °C in a refrigerated centrifuge to collect the crude sulphated polysaccharide complex. The precipitate was redissolved in 135 ml of 2 M warm NaCl (40° C) to remove pyridinium salts from the compound. The mixture was filtered through a Whatman No. 1 filter paper and 3 volumes of 95% ethanol to precipitate the crude glycosaminoglycans. The heparin complex was collected by centrifugation at 3000 rpm at 4 °C for 30 minutes. The precipitate was then washed twice with 99.9% methanol and diethyl ether and dried by keeping in a vacuum desiccator.

The amount of heparin complex is estimated by mg/g of dry weight.

4.3.2.2 Purification by acetone fractionation

Acetone fraction was used for further purification as outlined by Dietrich et al., [406].
Reagents

1. Acetone
2. Sodium chloride

Procedure

Crude glycosaminoglycan extract (50 mg) was dissolved in 1 ml of 0.15 M sodium chloride and kept for centrifugation at 5000×g for 30 minutes. After centrifugation, acetone (0.4 volumes) was added to the supernatant. The resulting solution was kept at 5 °C for 24 hours. The precipitate that formed was collected and dried. The operation was repeated by successively adding and increasing volumes of acetone (0.5, 0.6, 0.7, 1.0 volumes). The amount of heparin is estimated by mg/g before and after purification.

4.3.3 Results

4.3.3.1 Extraction and purification of heparin-like compounds

Glycosaminoglycans were extracted from skin, muscle, liver and visceral mass of *Nemipterus japonicus* and *Exocoetus volitans* after defatting with acetone and petroleum ether. The crude heparin like substances were found more in the muscles of *N. japonicus* (1.0 %) and *E. volitans* (1.24%) compared to other body parts. The total yield of crude glycosaminoglycans from the tissues is given in Table 13. Purification was done by acetone fractionation and precipitates were dried and analysed. The yield of heparin like glycosaminoglycan varied from one body part to another. After purification heparin like glycosaminoglycan content decreased to 0.78% and 0.74% for the muscle of *N. japonicus* and *E. volitans* respectively. The total heparin like glycosaminoglycan content for both the fishes after purification is given in Table 14.
Table 13 Total yield of crude heparin from different body parts of *N. japonicus* (NJ) and *E. volitans* (EV)

<table>
<thead>
<tr>
<th>Body parts</th>
<th>Weight (g)</th>
<th>Wt. after Defatting (g)</th>
<th>Heparin Extract (g)</th>
<th>% Heparin Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NJ</td>
<td>EV</td>
<td>NJ</td>
<td>EV</td>
</tr>
<tr>
<td>Muscle</td>
<td>100</td>
<td>100</td>
<td>23</td>
<td>26.8</td>
</tr>
<tr>
<td>Skin</td>
<td>100</td>
<td>100</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Visceral mass</td>
<td>50</td>
<td>50</td>
<td>8.1</td>
<td>8.8</td>
</tr>
<tr>
<td>Liver</td>
<td>50</td>
<td>50</td>
<td>2.5</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table 14 Purification of crude heparin of *N. japonicus* (NJ) and *E. volitans* (EV) by acetone fractionation with three fractionations

<table>
<thead>
<tr>
<th>Body parts</th>
<th>Weight (g)</th>
<th>Acetone Fractionation I (mg)</th>
<th>Acetone Fractionation II (mg)</th>
<th>Acetone Fractionation III (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NJ</td>
<td>EV</td>
<td>NJ</td>
<td>EV</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.0</td>
<td>1.24</td>
<td>0.86</td>
<td>0.82</td>
</tr>
<tr>
<td>Skin</td>
<td>0.86</td>
<td>0.92</td>
<td>0.46</td>
<td>0.75</td>
</tr>
<tr>
<td>Visceral mass</td>
<td>0.35</td>
<td>0.40</td>
<td>0.29</td>
<td>0.34</td>
</tr>
<tr>
<td>Liver</td>
<td>0.27</td>
<td>0.31</td>
<td>0.21</td>
<td>0.22</td>
</tr>
</tbody>
</table>

4.3.4 Discussion

4.3.4.1 Extraction and purification of heparin

The yield of extraction of heparin like substances varied in both fishes and between their body parts. The yield of the crude heparin like substances was more from in the muscles (*N. japonicus*, 1.0% and *E. volitans*, 1.24%) compared to other body parts. The yield was comparatively acceptable
when observed with other studies like ascidian (1.5%) [418], ascidian (1%) [418] ray skin *Raja radula* (1%) [426] and dried red algae (2-3%) [444]. Straus *et al.*, [445] recorded the yield of heparin and other sulphated mucopolysaccharides from thymus as 0.274%. The yield of crude heparin isolated from *Tridacna maxima* and *Perna viridis*, was reported as 2.72% and 2.2% respectively [446]. Jeske *et al.*, [410] reported 0.91% yield of heparin by acetone fractionation in tuna skins and found that fractions precipitated with 0.6 and 0.7 volumes of acetone contained more purified heparin than precipitated with 0.5 volumes. Brito *et al.*, [447] isolated heparin-like glycosaminoglycan with reduced anti-coagulant activity from marine shrimp *Litopenaeus vannamei* and reported yield as 0.9% dry tissue. Volpi and Maccari [448] found yield of glycosaminoglycans isolated from the body of marine clam *Scapharca inaequalvis* as 1.5-1.8%. Saravanan and Shanmugam [432] quantified the heparin yield as 1.72% from marine mollusc *Amussium pleuronectus* and characterised as low molecular weight heparin. Hence, based on the above, it could be concluded that the heparin-like substances of *N. japonicus* and *E. volitans* may be used as an alternative to the mammalian heparin and therefore can be a potential source for the heparin-like substances with good yield.