CHAPTER VIII

4.5 PROPERTIES OF HEPARIN LIKE SUBSTANCES USING ANTICOAGULANT, CYTOTOXIC AND ANTIVIRAL ASSAYS

4.5.1 Introduction

Heparin is heterogeneous with respect to molecular size, anticoagulant activity, and pharmacokinetic properties. Its molecular weight ranges from 3000 to 30 000 Da [466]. The anticoagulant activity of heparin is heterogeneous, because only one third of heparin molecules administered to patients have anticoagulant function, and the anticoagulant profile and clearance of heparin are influenced by the chain length of the molecules, with the higher molecular-weight species cleared from the circulation more rapidly than the lower molecular weight species [467]. This differential clearance results in accumulation of the lower molecular-weight species, which have a lower ratio of antithrombin (AT) to anti-factor Xa activity, in vivo. This effect is responsible for differences in the relationship between plasma heparin concentration (measured in anti-factor Xa units) and the activated partial thromboplastin time (aPTT) [468]. Molecules of heparin with fewer than 18 saccharides do not bind simultaneously to thrombin and AT and therefore are unable to catalyze thrombin inhibition. In contrast, very small heparin fragments containing the high-affinity pentasaccharide sequence catalyze inhibition of factor Xa by AT [469]. Standard practice to adjust the dose of heparin and monitor its effect is usually done by measurement of the aPTT (activated partial thromboplastin time). The partial thromboplastin time (PTT) or activated partial thromboplastin time (aPTT or APTT) is a performance
indicator measuring the efficacy of both the "intrinsic" and the common coagulation pathways. Apart from detecting abnormalities in blood clotting it is also used to monitor the treatment effects with heparin, a major anticoagulant. This test is sensitive to the inhibitory effects of heparin on thrombin, factor Xa, and factor IXa, as there is a relationship between heparin dose and both anticoagulant effect and antithrombotic efficacy, and follows that there is a relationship between anticoagulant effect and antithrombotic efficacy [470]. APTT remains the most convenient and most frequently used method for monitoring the anticoagulant response [469].

Herpes simplex virus (HSV), a DNA enveloped virus, is a common human pathogen with between 60 and up to 95% of certain populations infected with HSV-1. The herpes simplex virus type 1 (HSV-1) is the primary cause of oral-facial and pharyngeal infections and may cause herpetic whitlow, as well as severe and sometimes dangerous infections of the eyes and brain. HSV-1 also accounts for 10 to 15% of all genital herpetic infections. This virus can produce latent infection in the host for life and is reactivated by stimulus to cause recurrent infections and lesions [471]. The number of antivirals approved for clinical use has been increased from 5 to more than 30 drugs [472]. However, as these drugs are not always efficacious or well-tolerated and drug-resistant virus strains are rapidly emerging, there is still a great demand for further drug development including novel modes of action [473, 474]. The inhibitory effects of polyanionic substances on the replication of herpes simplex virus (HSV) and other viruses were reported almost four decades ago. However, these observations did not generate much interest, because the antiviral action of the compounds was considered to be largely nonspecific. Heparin and other sulphated polysaccharides are reported to be potent and selective inhibitors of HIV-1 and HSV replications in cell culture as they are thought to inhibit the very early step of viral replication, ie. virus attachment to the cell surface [475]. A very promising approach is the antiviral screening of products derived from natural sources, such as marine flora and
fauna, bacteria, fungi and higher plants [476-478]. Previous investigations have revealed antiviral activity in significant numbers of algae from various marine environments in the Mediterranean [479-481], Britain [482], India [483], Korea [484, 485], China [486] and Japan [487].

4.5.2 Materials and Methods

4.5.2.1 Anticoagulant Activity

*In vitro* anticoagulation assay was performed with citrated human platelet poor plasma (PPP) as test system as previously described by Jeske et al. [410].

**Regents**

1. Platelin L aPTT kit
2. Standard heparin
3. Chondroitin sulphate
4. Human plasma

**Procedure**

Human blood was pooled from individual healthy donors and mixed immediately with 3.8% trisodium citrate in volume ratio 9:1. Then the mixture was centrifuged at 2500×g for 15 min to obtain purified plasma. Then 500 μl of the citrated plasma was added along with 50 μl of the extracts (0-100 μg/ml) in five different tubes and were incubated at 37 °C for 1 minute. These incubated solutions were analyzed by aPTT assay.

**aPTT assay**

100 μl of the incubated solutions (standard heparin and samples) were added with 100 μl of aPTT reagent and was incubated at 37°C for 2 minutes. After 2 minute of incubation 100 μl of CaCl$_2$ were added to the mixtures and the clotting times were recorded.
4.5.2.2 Cytotoxicity assay

Cell viability assay was performed by the method of Mossmann [338].

Requirements

1. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide)
2. Vero cell lines

Vero cell viability was measured by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) method. Confluent cultures in 96-well plates were exposed to different concentrations of the heparin like substances, with three wells for each concentration. Plates were incubated at 37 °C in a humidified CO₂ atmosphere (5% CO₂) for 72 hr and then 10 μl of MEM (Minimal Essential Medium) containing MTT (final concentration 0.5 mg/ml) was added to each well. After 2 hr of incubation at 37 °C, the supernatant was removed and 200 μl of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm. The cytotoxic concentration 50% (CC₅₀) was calculated as the compound concentration required reducing cell viability by 50%.

4.5.2.3 Antiviral Assay

The ability of Glycosaminoglycans to inhibit the growth of virus was estimated by the method of Hu and Hsiung [488].

Requirements

1. Vero cell lines
2. HSV-1 viruses
3. MEM medium

Procedure

Different non-toxic concentrations of heparin like substances i.e., lower than CC₅₀ were checked for antiviral property by cytopathic effect
(CPE) inhibition assay against different herpes simplex virus (HSV-1) challenge doses. In CPE inhibition assay, Vero cells were seeded in a 24-well microtitre plate with 10,000 cells per well, incubated at 37º C in a humidified incubator with 5% CO₂ for a period of 48 hr. The plates were washed with fresh MEM was infected with HSV-1 suspension (50 µl) and incubated at 37 ºC for 90 min for adsorption of the virus. The cultures were treated with different dilutions of extracts (0-200 µg) in fresh maintenance medium and incubated at 37º C for five days. Every 24 h the observation was made and cytopathic effects were recorded. Anti-HSV-1 activity was determined by the inhibition of cytopathic effect compared with control, i.e., the protection offered by the test samples to the cells was scored.

4.5.2.4 Statistical analysis

All the assays were conducted with three replicates and data were expressed as mean ± standard deviation. The statistical analysis was performed using SPSS 10.0 software (SPSS Inc. Chicago, IL, USA). The significant difference was determined with 95 % confidence interval (p <0.05).

4.5.3 Results
4.5.3.1 Anticoagulant activity

The anticoagulant properties of fish extracts were assessed by aPTT (activated partial thromboplastin time) using human plasma. The normal values of aPTT for healthy human plasma were 32.5 sec. Heparin like substances from *N. japonicus* and *E. volitans* prolonged clotting time compared to normal saline. The anticoagulant activity of the heparin like substances extracted from muscles of *N. japonicus* and *E. volitans* was significantly (p<0.05) found higher compared to skin, visceral mass and liver (Figure 38). Moreover, heparin like substances extracted from different body parts of both fishes tested on human plasma showed concentration dependent anticoagulant activities and higher concentrations were required to achieve the same effect as with heparin in the aPTT assay.
Figure 38  aPTT- the activated partial thromboplastin time values were determined in citrate-anticoagulated human plasma in the presence of increasing concentrations of *N. japonicus* (a) and *E. volitans* (b) heparin extracts. Values are mean ± SD of three triplicates. Different letters within the same concentration indicate significant differences (*p* < 0.05)
4.5.3.2 Cytotoxicity assay

Heparin like substances from different body parts of *N. japonicus* and *E. volitans* were initially evaluated for cytotoxicity by assessing their effects on cell viability on Vero cell lines at concentrations ranging from 10 µg/ml – 250 µg/ml. In case of *N. japonicus* muscle, skin and visceral mass toxicity was observed after concentration of 200 µg/ml while as for liver cell cytotoxicity was found after 100 µg/ml (Figure 39a). Whereas, in case of *E. volitans*, cell cytotoxicity on Vero cell lines was seen after 200 µg/ml for muscle and visceral mass, while as liver and skin extracts were toxic after 100 µg/ml and 150 µg/ml respectively (Figure 39b). Therefore, heparin like compounds extracted from both the fishes showed weak cytotoxic properties at lower concentrations (Table 18).

**Table 18 Cytotoxic effect of heparin like substances on Vero cell lines at different concentrations of *Nemipterus japonicus* (NJ) and *Exocoetus volitans* (EV)**

<table>
<thead>
<tr>
<th>Extracts</th>
<th>10 µg</th>
<th>25 µg</th>
<th>50 µg</th>
<th>100 µg</th>
<th>150 µg</th>
<th>200 µg</th>
<th>250 µg</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.J skin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N.J muscle</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N.J liver</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N.J visceral mass</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E.V skin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>E.V muscle</td>
<td>-</td>
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<td>-</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>E.V liver</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>+</td>
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<td></td>
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<tr>
<td>E.V visceral mass</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

+ Toxic; - non toxic; C control
Figure 39 *In vitro* cytotoxicity assay of heparin extracts from *N. japonicus* (a) and *E. volitans* (b) at different concentrations. All assays were performed in triplicates. Different letters within the same concentration indicate significant differences (*p < 0.05*).
4.5.3.3 Antiviral Assay

Cytopathic effect of HSV-1 infected on Vero cell lines was observed between concentrations of 10 µg/ml to 25 µg/ml for muscle extract and 10 µg/ml to 50 µg/ml for skin, liver and visceral mass extracts of *N. japonicus*. In case of heparin like substances from *E. volitans* cytopathic effect was found at concentrations between 10 µg/ml to 50 µg/ml for muscle and liver extracts and 10 µg/ml to 25 µg/ml concentrations for skin and visceral mass extracts (Figure 40). However inhibition of cytopathic effect by heparin extracts of both fishes was observed at concentrations above 100 µg/ml in a dose related manner, hence, exhibits their antiherpetic activity *in vitro* as shown in Table 19.

Table 19 *In vitro* anti viral activity of heparin extracted from *Nemipterus japonicus* and *Exocoetus volitans* tissues against HSV-1

<table>
<thead>
<tr>
<th>Body parts</th>
<th>10 µg</th>
<th>25 µg</th>
<th>50 µg</th>
<th>100 µg</th>
<th>150 µg</th>
<th>200 µg</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.J skin</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>N.J muscle</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>N.J liver</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>N.J visceral mass</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>E.V skin</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>E.V muscle</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>E.V liver</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>E.V visceral mass</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>

O → Absence of cytopathic effect
O → Presence of cytopathic effect
C → Cell control.
(a) Uninfected Vero cell lines

(b) CPE of *N. japonicus* muscle at 25 µg/ml

(c) CPE of *N. japonicus* skin at 50 µg/ml

(d) CPE of *N. japonicus* liver at 50 µg/ml

(e) CPE of *N. japonicus* visceral mass at 50 µg/ml
Figure 40 (a) uninfected Vero cell lines and (b-i) cytopathic Effect (CPE) of Herpes simplex virus 1 infected Vero cell lines with different concentration of heparin extracts from *N. japonicus* and *E. volitans*
4.5.4 Discussion

4.5.4.1 Anticoagulant activity

The observed concentration dependent anticoagulant activity of two fishes varied from each other and between organs and was less than mammalian heparin. The aPTT assays indicate the heparin like substances from muscles of *N. japonicus* and *E. volitans* has more anticoagulant activity than skin, liver and visceral mass. The difference in the anticoagulant activity of two fishes and their organs may be ascribed to their high sulphate content and discrete variation of their sulfation pattern [489]. Many reports characterizing heparins from various mollusk species has demonstrated species-dependent variability in potency and structure of heparin [459]. Pereira *et al.*, [490] explained that specific sulfation pattern is required for anticoagulant activity of sulphated polysaccharides, and found that anticoagulant activity of *Gelidium crinale* and *Botryocladia occidentalis* varied though with similar saccharide chain but the later being with higher amounts of 2,3-di-sulphated α-units. Differences in the total amount of sulfation or the relative abundance of the minimal antithrombin binding sequence in the heparin chains may account for lower potency of fish heparin compared with mammalian heparin [410]. It has been reported that heparin from marine vertebrate and invertebrates have anticoagulant effects exclusively mediated by heparin cofactor II (HCII) [421]. Brito *et al.*, [491] reported that anticoagulant activity of heparin like glycosaminoglycans from shrimp was concentration dependent and exhibits a reduced anti-coagulant activity when compared to mammalian heparin. Arumugam *et al.*, [446] found variation in anticoagulant activity in clams *Tridacna maxima* and *Perna viridis* that seem to be related to their sulfation pattern. Therefore, heparin like substances extracted from different body parts of *N. japonicus* and *E. volitans* had effect on the aPTT assay, this being expected because of the presence of
sulphate groups necessary to provide anticoagulant effects, but also dependent on the position of the sulphate groups as proved by other investigations.

4.5.4.2 Cytotoxicity assay

Heparin like substances extracted from body parts of *N. japonicus* and *E. volitans* were subjected to cytotoxicity assay with Vero cells. Cell viability was estimated via an MTT (3-[4,5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazoliumbromide) assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells [338]. The results of cytotoxicity studies obtained indicate samples of heparin like substances were tested at different concentrations ranging from 10 µg/ml to 250 µg/ml, it was observed that toxic concentration varied between both fishes and their body parts. It was found that when the cultures (Vero cells) were exposed to very high concentrations of tested samples, cell viability decreased as, high concentrations of any compound, under normal conditions are cytotoxic to cell cultures [492]. Carlucci *et al.*, [433] found that carrageenan isolated from the red seaweed *Gigartina skottsbergii* did not show any cytotoxic effect till 1000 µg/ml. Caaceres *et al.*, [493] also observed no cytotoxicity with carrageenans extracted from cystocarpic and tetrasporic *Stenogramme interrupta* when cell viability was evaluated in Vero cells in the presence of concentrations up to 1000 mg/ml. Furthermore, Matsuiiro *et al.*, [494] reported that no cytotoxic effects were observed when cell viability was evaluated in monolayers of Vero cells at a concentration up to 1000 µg/ml for sulphated galactan from the red seaweed *Schizymenia binderi*. Similarly, Chattopadhyay *et al.*, [495] reported that Galactan sulphate isolated from *Grateloupia indica* was toxic to Vero cell lines after 900 µg/ml with CC$_{50}$ value of >850 µg/ml. Gemin *et al.*, [496] reported that chemically sulphated galactomannan from *Leucaena*
leucocephala seeds showed no significant toxicity to Vero cell line till 125 μg/ml and high concentration till 250 μg/ml showed lowering of cell viability. Therefore, it was found that heparin like substances derived from both fishes were non-cytotoxic at lesser concentration, when high concentration were toxic as proved by other observations studied on sulphated polysaccharides from different marine sources.

4.5.4.3 Antiviral assay

Heparin like substances extracted from the skin, muscle, liver and visceral mass of both fishes showed potent antipherpetic effect against HSV-1 viruses at above the concentrations of 100 μg/ml. It has been reported that an increasing antiviral activity of polysulfates is related to an increasing degree of sulfation [497, 435]. Moreover, Polyanionic compounds might be expected to interact with the positively charged amino acids in the surface proteins of virus envelope [497]. Furthermore, HSV adsorption is the main target for the antiviral effect of these polysaccharides. HSV-I use the heparan sulphate residues of cellular proteoglycans as the primary receptor for virion binding and adsorption [392] and thus, the glycosaminoglycans seem to mainly interfere with the interaction of virion glycoprotein-heparan sulphate. Amornrut et al., [498] has reported that sulphated β-galactan from clam Meretrix petechialis inhibit HIV replication appear to inhibit syncytia formation. The similar studies were carried in red microalga Porphyridium sp. in which the polysaccharide was tested on HSV-1, the polysaccharide significantly inhibited viral infection at 100 μg/ml and the polysaccharide extract was able to inhibit the development of the cytopathic effect in HSV-infected cells [499]. Recently, light has also been shed on the antiviral activity of marine sulphated polysaccharides derived from seaweeds such as Stoechospermum marginatum [500] against HSV types 1 and 2 (HSV-1, HSV-2). Mohsen et al., [501] reported that sulphated polysaccharide fractions isolated from Sargassum latifolium inhibited HSV-1 in the plaque assay with
the most effective fraction having greater sulphate ester content and molecular weight compared to the other fractions studied. It has been generally observed that antiviral activity of sulphated polysaccharides increases with their molecular weight [435]. Harden et al., [502] evaluated the antiviral activity of extract *P. cartilagineum* against HSV-1 and HSV-2 and concluded that this extract has non toxic and effective virucidal agents. Similarly sulphated xylomannans from the red seaweed *Sebdenia polydactyla* inhibited the propagation of HSV-1 in Vero cells [503]. The activity was abolished by desulfation of the xylomannan and, conversely, oversulphated derivatives exhibited enhanced potency. Usually sulphated polysaccharides differing in structure, sulfation level and molecular weight were shown to inhibit HSV-1 and HSV-2 infection [504] reinforcing the view that, as with other activities, antiviral activity of sulphated polysaccharides is due to a complex interplay of structural features including sulfation level, distribution of sulphate groups along the polysaccharide backbone, molecular weight, sugar residue composition, and stereochemistry [505]. The potency of the heparin like substances extracted from different body parts of both the fishes have proved the presence of anticoagulant property, cytotoxic activity and inhibitory effect on the HSV-1 in the required concentration which was also strongly supported by earlier experiments carried out using sulphated polysaccharides of the marine resources. This potential extracts are needed to takeover to further stage to find out in the *in vivo* studies and other clinical applications.
SUMMARY AND CONCLUSION

In the present study, fish protein hydrolysates produced by three different proteases were studied for their functional properties and it was observed that it varied with respect to enzyme used. Thus, it was found that solubility, emulsifying and foaming properties were dictated by two factors i.e. pH and enzyme used in hydrolysis. Further, protein hydrolysates and peptides displayed different antioxidant activity depending on the in vitro chemical assay system used. Pepsin hydrolysates of both fishes were more effective in reducing and metal chelating assays. Trypsin hydrolysates of muscle, backbone of *N. japonicus*, muscle of *E. volitans* and pepsin backbone were more effective in inhibiting the linoleic acid peroxidation and were more effective in scavenging DPPH, hydroxyl and superoxide radicals. Amino acid composition of more potent hydrolysates proved by lipid peroxidation and free radical scavenging assays was assessed by HPLC and showed the presence of both essential and non-essential amino acids. Further, protein hydrolysates were fractionated into different peptides by anion exchange and sephadex gel chromatography and their antioxidant potentials were examined by scavenging DPPH, hydroxy and superoxide radicals measured by ESR spectroscopy. The peptide could scavenge more effectively DPPH, hydroxyl and superoxide radicals exhibiting its potent antioxidative activity towards inhibiting the formation of reactive oxygen species formed by the peroxidation of polyunsaturated fatty acids. Molecular weight and sequence of peptides were confirmed by ESI MS/MS and presence of antioxidant amino acids in peptide sequences was reported. The identified active antioxidant peptides were further investigated for their cytotoxic and antiproliferative effects on Vero
and Hep G₂ cell lines respectively. These peptides were non-toxic up to the concentrations of 90 μg/ml whereas, showed significant inhibitory effect on Hep G₂ cell lines. Moreover, heparin like substances extracted from different body parts of *N. japonicus* and *E. volitans* were reported rich in sulphate content but showed variation between body parts and fish species chosen. Yield of heparin and anticoagulant activity measured by aPTT was found more in muscles of both fishes and showed dependence of sulphate content. Heparin like substances was tested on cell cytotoxicity on Vero cell lines and was non toxic till 100 μg/ml. Further the inhibition of cytopathic effect has proved in different concentrations of the bodyparts on HSV-1 infected Vero cell lines. Therefore, on the basis of present results additional studies like *in vivo* studies are required to investigated antioxidant peptides and heparin like substances as potential drug agents.

In conclusion, protein hydrolysates of *N. japonicus* and *E. volitans* showed good functional properties and can be used as supplements in food ingredients but further research is needed to identify appropriate functional food applications as well as to overcome problems during incorporating the fish protein hydrolysate or semi-purified fractions into food matrices. Peptides extracted from *N. japonicus* and *E. volitans* showed potent inhibitory effect on free radicals hazardous to human health as verified by various *in vitro* assays. These peptides were tested for their toxicity on Vero cells which proved high concentrations were toxic and can be taken at low concentrations. Further, these antioxidant peptides acted antiproliferative agents against HepG₂ cells. Research should be conducted in assessing antioxidative potential of hydrolysates or isolated peptides using animal models or human clinical trials. These studies are necessary to confirm the results obtained through *in vitro* chemical and cell-based assays. Animal and eventually clinical studies should therefore be conducted with the antioxidative fish protein hydrolysate in future.
Heparin like substances extracted from different body parts of *N. japonicus* and *E. volitans* was found in good yield with various pharmacological properties like anticoagulant, cytotoxic and antiviral. Considering these potentials of heparin like substances, further purification and production of low molecular weight heparin is needed. Further, *in vivo* studies are needed for anticoagulant therapy and active agent against HSV infections.