6. ANTICOAGULANT AND ANTICANCER ACTIVITIES OF THE BLOOD CLAM GAG

6.1. INTRODUCTION

Heparins are found to be predominant glycosaminoglycans, which show potent anticoagulant activity. The heparins isolated from various molluscs and structurally different from human heparin (Lindhardt et al., 1992) and pharmaceutical heparins (Lindhardt et al., 1988; Loganathan et al., 1990) mollusc heparins contain antithrombin dependent anticoagulant activity associated with the presence of the unique 3-0-sulfated glucosamine residue found in the antithrombin pentasaccharide binding site common to all anticoagulant heparin (Burson et al., 1956; Dietrich et al., 1985; Loganathan et al., 1990; Frogmnhagon et al., 1953).

Anticoagulants GAGs are used clinically since 1937, because of its anticoagulant proprieties and its useful to treat thromboembolic disorders. Originally, it was introduced to prevent thrombosis in surgical patients, followed by its use for the treatment of deep venous thrombosis, and preventing complications following vascular surgery, myocardial infarction and extracorporeal circulation. It is estimated that 2–4 patients out of 1,000 receive anticoagulant therapy (Hasui et al., 1995; Groth and Wagenknecht, 2001). In the materials field, anticoagulants are used to improve the hemo-compatibility of medical devices and tissue engineering materials (Baumann, 2001). Recently, the concept of "vascular beautifying" has been promoted by the cosmetics industry (Wang et al., 2004). The clinical reagent heparin is found nearly exclusively in mast cells it represent a specialized member of the widely distributed class of compounds known as heparin sulfate (Nugent, 2000).

The blood anticoagulant activity of heparin is due to the presence of specific pentasaccharide sequence (Petitou et al., 2003). Polysaccharide chains possessing this sequence bind with high affinity to antithrombin and thus drastically increase the rate by which this proteinase inhibitor inactivates enzymes involved in the coagulation process (Pejler et al., 1987). Today anticoagulant GAG has two major clinical uses: (a) to prevent clotting and thrombus formation
resulting from interventions in the circulatory system such as cardiovascular diagnostic procedure, introduction of catheters, surgery of heart and vessels including prostheses; extra corporeal circulations, artificial organ and transplants (b.) to limit or prevent thromboembolism post-operatively, postpartum, hyper coagulant status and in patients with an increased tendency to thrombosis from other cases particularly noteworthy is heparins antiviral activities (Bazjar and Albisetti, 2005).

The anticoagulant heparin is used therapeutically in surgical operations and also in the treatment of arterial embolism. Patients with venous thromboembolism should be treated with a 5000 unit intravenous bolus of heparin followed by either 32000 units per 24 hrs by continuous infusion or 17500 units subcutaneously every 12 hrs and dose should be adjusted to maintain the APPT at 6 hrs. The intravenous or subcutaneous administration of heparin in therapeutic use prolongs the clotting time (Gould et al., 1999). Therefore, dosage (concentration) and clotting time (APTT, PT and TT) are the two important aspects, which need to be studied for the therapeutic applications. In addition to its anticoagulant effect, including binding of growth factors, inhibiting proliferation of smooth, muscle cells, fibroblasts, mesangial cells and hepatic satellite cells (HSCs), angiogenesis modulating activity and effects on lipase (Gould et al., 1999). Warda et al. (2003) determined the correlation between activated clotting time (ACT) and (APTT) in patients receiving intravenous unfractionated heparin therapy and the accuracy of ACT in predicting the level of anticoagulant. The anticoagulant activity was detected by prolongation of activated partial thromboplastin time, (APTT), TT and PT (Athukorala et al., 2009).

Anticoagulation occurs predominately by inhibiting the key coagulation serine proteases, thrombin and factor Xa. This is facilitated by accelerating the activity of major physiological serine protease inhibitor – SERPIN-AT-III (Bjorj et al., 1989). There is lesser inhibition in the case of IXa, XIa, XIIa and kallikrein. Another SERPIN heparin co-factor II (HC-II) has been identified that exclusively inhibits thrombin, but has no significant activity against other coagulation or fibrinolytic proteases. The anti-haemostatic properties of heparin and other sulfated
polysaccharides extend beyond anticoagulation and include fibrinolytic potentiation and anti-lipidimic effects. Through heparin is a primary anticoagulant drug, it has some disadvantages like it is extracted from internal organs of higher animals and purified. Hence its production is difficult and it exhibits hemorrhagic-like side effects. These disadvantages associated with heparin have opened up a new area of antithrombotic research for discovering novel anticoagulant agents. Recently few drugs have been introduced and many are in clinical trials (Shanmugam and Mody, 2000).

The unfractionated heparins are a heterogeneous mixture of highly sulfated GAG with a strong negative surface charge. The heparin exerts many functions including the anticoagulant activity which has been a major impetus for exploring the drug for its multifaceted biological properties and functions at the cellular and the molecular level. Some focus was directed at producing fragments of heparin particularly low molecular weight heparins (LMWH) that had reduced the risk of bleeding for equivalent antithrombic effect when compared with the heparin therapy. But for that the GAGs are otherwise comparable with standard heparin in their basic structure and biologic properties. These GAG consist of chains of alternating residues of D-glucosamine and an uronic acid, either glucose acid or iduronic acid (Poonguzhali, 2011).

Holick et al. (1985) developed a method for the recovery of heparin and heparin like substances from various marine organisms like flounder, crab, mussel and clams. Since mol luscuses heparin contains antithrombin—dependant anticoagulant associated with the presence of unique 3-o- sulfated glycosamine residues in all anticoagulant heparin (Kim et al., 1996). Many marine molluscuses lives yielded GAGs compounds with high anticoagulant activity. Thomas (1951; 1954) obtained such a substance chemically similar to that of heparin from the common surf clam, S. Solidissima. Cifonelli (1972) derived anticoagulant sulfated “Mactin A” and “Mactin B” respectively from (Mactra) spisula and (Artica) islandica. These heparin-like substances exhibited high anticoagulant potency and low toxicity.

Among the molluscuses, some have pronounced pharmacological activities or the properties useful in the biomedical area. It is surprising that some of these
pharmaceutical activities are attributed to these presences of polysaccharide, particularly those that are sulfated (Arumugam and Shanmugam, 2004). An exhaustive assessment showed that a large number of marine invertebrate species contain GAGs; especially the marine molluscs GAGs were exert powerful anticoagulant activity and have lower toxicity (Holick et al., 1985; Dietrich et al., 1989; Saravanan, 2010; Periyasamy et al., 2013).

Over the past decade, cancer is the leading cause of death worldwide and it is characterized by uncontrolled growth and spread of abnormal cells. World Health Organization (WHO) reported that there are 7.6 million deaths in 2008 and it is estimated up to 13.1 million deaths in 2030. The most mystifying diseases of all, claims three times more victims in developed countries than in developing countries. It is due to failure of the mechanisms that usually control the growth and proliferation of cells. Treatment of cancer varies according to each type, has been facing large number of problems. Several ways in the treatment of cancer have been developed. Currently cancer is treated using surgery, radiation, and chemotherapy which are associated with severe side effects (Garcia et al., 2001).

Cancer is a complex disease in which a group of cells displays uncontrolled growth, causing a cell invasion that intrudes upon and destroys adjacent tissues, sometimes leading to metastasis and spread to other locations in the body via lymph or blood. Leukemia is a common hematopoietic cancer worldwide characterized by abnormal proliferation, differentiation and overproduction of white blood cells and their precursors (Ampasavate et al., 2010). Leukemia forms one among the many forms of cancer. It is the result of excessive proliferation of cells or neoplasia of blood forming tissues. In this disease there is considerable increase in the number of white blood cells. Sometimes the number of WBC exceeds 450,000 cells/m³ compared with the normal level of less than 10,000 cells/m³. Different forms of leukemia derive their names from the type of cell that has become malignant were i) chronic lymphatic leukemia ii) chronic myeloid leukemia iii) acute lymphatic leukemia iv) acute myeloid leukemia and v) acute monocytic leukemia (Jaya Prakash, 2009).
Cancer is one of the most dangerous diseases in humans and presently there is a considerable scientific discovery of new anticancer agents from natural products. The search for cancer cure from natural product (plant and animal) has been practiced for over a century and the use of purified chemicals to treat cancer still continues. As the infectious diseases are evolving and develops resistance to existing pharmaceuticals, the marine environment provides a novel source for the development of lead compounds against fungal, parasite, bacterial and viral diseases. The earliest efforts in this field derived from the interests of marine biologists and naturalists who found a number of unique bioactive compounds that were present in diverse marine life. The use of marine agents for medicinal benefits has played an important role in nearly every country (Wang et al., 2003) and during the past century more attention has been focused on discovering anti-tumour agents derived from marine organisms (Lee et al., 2005). A number of agents with marine origin have been identified that inhibit the initiation and progression of tumours, for example, bryostatin 1, didemnin B, and aplidine (Erba et al., 2003).

The treatment of leukemia includes several methods such as chemotherapy, radiotherapy and bone marrow or stem cell transplantation, for the purpose of improving the survival rate and preventing remission and relapse of leukemia. However, there are many anticancer agents are side-effects such as headache, nausea, vomiting and weight-loss after treatment. As a result, marine organisms extracts is an alternative method of leukemia treatment which reduced side effects (Kristen Benkendorff et al., 2009). Bromination was found to increase the anticancer activity of these isatins and greater selectivity was identified toward leukemia and lymphoma cells over breast, prostate and colorectal carcinoma cell lines (Vine et al., 2007). Subhasis Roy et al. (2010) showed that the gastropod extract was able to restrict the induced tumor (sarcoma-180) growth in mice concomitantly with an increased life span of the tumor bearing mice, but without any hematopoietic toxicity. The Brazilian tunicate, *Ascidian didemnum granulatum*, is the source of the aromatic alkaloids granulatinimide and isogranulatinimide, which appear to act as G2 checkpoint inhibitors (Berlineck, 1998). These compounds have been synthesised, and several analogues are being
developed for further testing. In addition, new bisindole alkaloids of the topsentin and hamacanthin classes have been isolated from the Mediterranean sponge Rhaphisia lacazei, and these compounds also showed significant antiproliferative activity against a series of human cell lines in vitro (Casapullo et al., 2000).

Indirubin derivatives are shown to inhibit Stat III signaling, inducing apoptosis in human breast and prostate cancer cells (Nam, et al., 2005). They also suppress tumor necrosis factor (TNF)-induced NF-κB activation in human leukemia and lung adenocarcinoma cells and significantly block proliferation in lung carcinoma, stomach carcinoma and fibrosarcoma cell lines (Sethi et al., 2006). L-glutaminase in combination with or on alternative to asparaginase could be of significance in enzyme therapies for cancer especially acute lymphocytic leukemia (Mayer and Gustafson, 2003). It is also potent anti-leukemia agent and it has been tried as therapeutic agent in the treatment of HIV also (Robert et al., 2001). Zandi et al. (2010) study was designed to assay antitumor activity of the extract from brown alga Sargassum oligocystum, gathered from Persian Gulf seashore, against K562 and Daudi human cancer cell lines. The ecteinascidins are derived from the Caribbean tunicate Ecteinascidia turbinata and also show significant antitumour activity in both murine and human tumour cell lines (Garcia et al., 2001).

Molluscs are said to be pharmacological significant outlet. There are more than thousand of bioactive compounds discovered in molluscs. They are peptides, depsipeptide, sterols, sesquiterpene, terpenes, polypropionate, nitrogenous compounds, macrolides, prostaglandins and fatty acids derivatives miscellaneous compounds and alkaloids. Extraction of anticancer compounds from marine organisms has been in vogue since many years, but the marine molluscs are the source of at least four structurally distinct anticancer agents, which are currently in phase II and III clinical trials (Faircloth, et al., 2006; Newman and Cragg, 2004). In recent past, mollusc have been screened for antitumor, antileukemic, anticoagulant, antimicrobial and antiviral properties world over (Kristen Benkendorff et al., 2009; Dietrich et al., 1989; Periyasamy et al., 2013; Rajagnanapathy, 2001). Kahalalide F, another marine-derived agent obtained from
the mollusc *Elysia rubefascens*, which is found in Hawaii, showed substantial antitumour activity in preclinical models and is due to start phase I trials in patients with hormone-refractory prostate cancer (Kan *et al.*, 1999). Tyrian purple is generated from the secretions of the hypobranchial glands of molluscs *Dicathais orbita*, after a series of enzymatic, oxidative and photochemical reactions from a choline ester precursor salt of tyrindoxyl sulphate, in addition to the previously described anticancer activities (Westley *et al.*, 2006). The apparent anti-leukemia activity of organic extracts derived from the mollusks (Kristen Benkendorff *et al.*, 2009; Yamada, *et al.*, 2000) suggests that there may also be some chemical basis to the anti-leukemia application of the blood clam remedy. A number of these anticancer agents reduce pain in animal models and several are now in preclinical and clinical development for the treatment of severe pain often associated with diseases such as cancer. In the present study, the anticoagulant and anticancer activities of crude and purified sample of anticoagulant GAG from blood clam *A. granosa* are evaluated.
6.2. MATERIALS AND METHODS

6.2.1. Anticoagulant assay (The US pharmacopoeia method, 1995)

The anticoagulant activity of GAG from A. granosa was measured by using human blood plasma (collected from healthy donor from laboratory) as a source (USP method, 1995). The activity was expressed as USP units/mg.

The assay was based on the increase in the recalcification clotting time of blood plasma with increasing concentration of heparin. The citrated blood plasma to which heparin has been added was recalcified by the addition of calcium chloride reagent. The assay was performed by comparing grades of clotting in assay samples with grades of clotting in set of standards.

6.2.2. IU method

The activated partial thromboplastin time (APTT) and prothrombin time (PT) of fractionated and purified GAG from A. granosa were assayed by adopting the method of Mauary et al., (1995) using human blood as a source. The activity was expressed as USP units/mg.

APTT and PT measurements were performed using the kit obtained from Instrumentation Laboratory (Lexington, USA) by using HS (bovine- kidney-Sigma) as a standard. The activity was expressed as IU/mg.

6.2.3. Cell line culture

Vero cells (African green monkey kidney cell line), Molt-4 (Human Acute Lymphoblastic Leukemia) cell lines were obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in the Department of Virology, King Institute of Preventive Medicine, Guindy, Chennai. The vero cells were cultured method described by Sreedevi (2013). The human leukemia molt-4 cell line was maintained at 37°C in a 5% CO2 humidified atmosphere. They cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (Biosciences, Mumbai), Penicillin/Streptomycin and 200nM L-glutamine (Sigma) (Acharya et al., 2010).
6.2.4. Cytotoxicity assay

Cell viability was measured by the MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-
diphenyl tetrazolium bromide, Himedia, India.) method (Song and Du, 2012). Confluent cultures (Vero cells) in 96-well plate were exposed to different concentrations of the blood clam crude and purified GAG (10-500μg) with three wells for each concentration. Then 10μl of MM containing MTT (final concentration 0.5 mg/ml) was added to each well. After 2 h of incubation at 37°C, the supernatant was removed and 200μl of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595nm. The inhibition 50% (CC50) was calculated as the compound concentration required for reducing cell viability by 50%.

6.2.5. Anticancer activity in cell line

The anti-leukemic activity of crude and purified GAG from A. granosa was checked in vitro (Molt-4 Human Acute Lymphoblastic Leukemia cell line) studies followed the standard protocol of anti-proliferation technique using Microculture Tetrazolium Assay (MTT) described by Anne Monks et al. (1991).

6.2.6. Statistical analysis

The experimental data were subjected to Analysis of Variance (ANOVA) using SPSS software (Version. 16). Duncan’s multiple range test (DMRT) was used to determine the difference among means at the level of <0.05.
6.3. RESULTS

6.3.1. Anticoagulant activity USP method

The anticoagulant activity was found to be 19.4 USP units/mg and 93.1 USP units/mg in the crude and purified GAG from A. granosa using the Sephadex G-100 column chromatography (Table 6).

6.3.2. IU method

The APTT was reported as 49 IU/mg in crude GAGs A. granosa respectively, where as the Sephadex G-100 column purified GAG of A. granosa showed 107 IU/mg (Table 6).

The PT observed as 32 IU/mg in crude GAGs A. granosa respectively, whereas the Sephadex G-100 column purified GAG of A. granosa showed 67 IU/mg (Table 6).

Table 6. Anticoagulant activity of GAG extracted from A. granosa

<table>
<thead>
<tr>
<th>Source</th>
<th>A. granosa</th>
<th>Anticoagulant activity</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>USP units/mg</td>
<td>APTT activity (IU/mg)</td>
<td>PT activity (IU/mg)</td>
<td></td>
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<tr>
<td>Human blood</td>
<td></td>
<td>19.4</td>
<td>49</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Purified</td>
<td>93.1</td>
<td>107</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>

6.3.4. Cytotoxicity of crude and purified GAG

The crude and purified GAG from A. granosa was initially evaluated for their effects on cell viability through cytotoxic test. Cytotoxic effect of crude and purified GAG was observed for 72 hrs of incubation period and the IC50 value was calculated as 320μg/ml and 250μg/ml respectively (Fig. 20 & 21).
Fig. 20. Cytotoxicity of effect crude and purified GAG against Vero cell line

Control cells  Crude GAG treated cells  Purified GAG treated cells

Fig. 21. Cytotoxicity of effect crude and purified GAG against Vero cell line
6.3.5. Anticancer activity

The present study revealed that the blood clam crude GAG and purified GAG showed anticancer activity of leukemia cell line in a dose dependant manner. The crude GAG showed a maximum activity of 61.68% at 200μg/ml. Whereas, the purified GAG showed 70.42% of inhibition at the same concentration. The lowest inhibition of 13.54% and 17.76% was observed at 25μg/ml for crude and purified GAG respectively (Table. 7 & Fig. 22 & 23).

**Table. 7. Anticancer activity of crude and purified GAG against molt-4 leukemia cells**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Name of the sample</th>
<th>Percentage of inhibition/Concentration μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td><strong>Molt-4 Leukemia cell</strong></td>
<td>Crude GAG</td>
<td>99.86</td>
</tr>
<tr>
<td></td>
<td>Purified GAG</td>
<td>99.86</td>
</tr>
</tbody>
</table>

![Graph showing anticancer activity](image)

**Fig. 22. Anticancer activity of crude GAG and purified GAG against leukemic cell**

125
Fig. 23. Anticancer activity of crude GAG and purified GAG against leukemic cell
6.4. DISCUSSION

Heparin preparation is normally heterogeneous and contains components with significant differences in chain length and degree of sulfation. Such structural variations cause the heparin molecules to interact differently with the coagulation enzymes, resulting in blood coagulation via complex and different pathways (Hrish et al., 1992). Among all the coagulation enzymes, factors Xa, IIa (thrombin) are the key proteases involved in the regulation of the coagulation process. Thus, comparison of inhibitory effects of the fractions of heparin induced anti-Xa and anti-IIa activities seems appropriate in evaluating the specificity and efficacy of such as heparin antagonist (Li-Chein Change et al., 2001). Anderson et al. (1976), who found that their heparin fractions of lowest molecular weight had a relatively higher anti-Factor Xa activity than anti-coagulant activity.

The GAGs particularly heparin is known for its anticoagulant activity. It was used to quantify the ability of heparin in the inhibition of coagulation of whole sheep blood plasma following the United States pharmacopoeia (USP) method. The heparin isolated from marine clams and mussels has identical structural features and anticoagulant activity like the mammalian polysaccharides (Pejler et al., 1987).

In the present study, the anticoagulant activity was studied in the human blood following the United States pharmacopoeia (USP) method. The anticoagulant activity was found to be 19.4 USP units/mg and 93.1 USP units/mg in the crude and purified GAG from A. granosa. The anticoagulant activity of crude GAG of A. granosa was low when compared to that the anticoagulant activity ranging from 130-150 USP units/mg for extracted products of Spisula solidissima and Cyprina islandica showed by Burson et al. (1956), Somasundram et al. (1989) isolated heparin from the bivalve molluscs, K. opima with the anticoagulant activity of about 92 units/mg, Dietrich et al. (1989) investigated the anticoagulant activity of heparin from two species of mollusks, D. striatus and T. mactriodes as 180 units/mg and 220 units/mg respectively, Vijayabaskar (2008) reported the extraction of GAGs from K. opima and D. cuneateus showed anticoagulant activity of 160 USP units/mg and 154 USP units/mg, after the partial
purification through DEAE cellulose column chromatography, the yields of anticoagulant activity were found to be 180 USP units/mg and 175 USP units/mg, the bivalve showed the anticoagulant activity of the crude and purified samples 58 USP units/mg and 114 USP units/mg correspondingly in D. faba reported by Periyasamy et al. (2013) and also the gastropods showed the anticoagulant activity of the crude samples 134 USP units/mg and 78 USP units/mg correspondingly in B. spirata and P. glaucum respectively.

The anticoagulant effect of heparin is closely associated with their molecular weight (Ma et al., 2002). Commercially available low molecular weight heparins are less potent than UF II in term anticoagulation (Faried et al., 2000). On a weight basis, the molluscan heparin exhibited four times more inhibitory activity than unfractionated heparin that has about the same activity as the AT III-high affinity heparin (Paiya et al., 1995). Earlier high anticoagulant activity was also reported for purified GAGs from Anodonta cygnea (Lopes-Lima et al., 2005), Mytilus galloprovincialis (Volpi and maccari, 2003), Aplysia californica and Holpe aspersa (Hoving and Linker, 1998). Moreover benzenamine derivatives and their pharmaceutically acceptable salts, inhibit the enzyme, factor Xa, there by being useful as anticoagulants (Guilford, 2001).

In the present study, the anticoagulant activity was found to be 19.4 USP units/mg and 93.1 USP units/mg in the crude and purified GAG from A. granosa is low when compared to the anticoagulant activity of shrimp heparin measured by the USP assay (95, 100 IU/mg). The biological activity of heparins in invertebrates remains enigmatic. The classes of crustaceans and Mollusca do not possess any blood coagulation system similar to that of mammals and other vertebrates and thus the presence of compound that act specifically upon the protein of blood coagulation system is indeed remarkable (Dietrich et al., 1999). The anticoagulant activity of heparin differs from species to species due to their interaction with enzymes and inhibitors of the coagulation systems (Mulloy et al., 2000). In this respect, polysaccharides with lower anticoagulant activity than heparin could exhibit a potent antithrombotic effect with less hemorrhagic risk (Cassaro and Dietrich, 1997).
Heparin and heparin-like GAGs, which are present in some invertebrates, molluscs showed high anticoagulant activity and share most of the structural properties with mammalian heparins (Pejler et al., 1987). In the present study, the anticoagulant activity was found to be 19.4 USP units/mg and 93.1 USP units/mg in the crude and purified GAG from A. granosa. The anticoagulant activity of crude GAG of A. granosa was high when compared to that Benny (1996) recorded 26 USP units/mg in Hemifusus pugilimus, In vitro anticoagulant activities of heparin from bovine intestine, lung and pancreas were found to be 140, 130 and 140 USP units/mg respectively (Nader et al., 2001), Arumugam and Shanmugam (2004) reported the anticoagulant activity crude and fractionated sample of GAG from T. attenuate - 37 USP units/ mg and 78 USP units, the anticoagulant activity of crude and purified sample of GAG from A. pleuronectus to be 15.38 USP units/mg and 83.99 USP units/mg evaluated by Suganthi (2007), Vidhyanandhini (2010) studied the anticoagulant activity crude and purified was estimated at 22.52, 20.00 and 18.60 USP units/mg; (amberlite) 86.32; 83.06; 92.43 USP units/mg and barium acetate 80.36; 75.92; 89.68 USP units/mg of K. opima. Saravanan (2010) reported that the anticoagulant activity was found to be 52.09 USP units/mg and 61.02 USP units/mg in the fractionated GAG from A. pleuronectus using DEAE-Cellulose column and Amberlite column, the Sephadex G-100 purified GAG sample showed the anticoagulant activity was 70.50 USP units/mg. The present study showed that anticoagulant activity of GAG from A. granosa GAG is better than the GAG from other mollusces. Their difference in activity can be attributed to the higher sulfate content in A. granosa. From the above it could be understood that whole body tissue of A. granosa is also comparatively a good potential source of anticoagulant compounds.

The anticoagulant and antithrombotic are the most widely studied properties of sulfated polysaccharides. The anticoagulant activity of GAGs does not merely depend on sulfate content and charge density rather the structural requirements and their interaction of these polysaccharides with coagulation cofactors are said to be responsible (Melo et al., 2004). In the present study, the anticoagulant activity (APTT) was reported as 49 IU/mg in crude GAGs A. granosa respectively, where as the purified GAG of A. granosa showed 107
IU/mg. The anticoagulant activity (APTT) of sulfated polysaccharide isolated from the green seaweed _Caulerpa cupressoides_ was calculated as APTT at 24.62 IU/mg (Rodrigues et al., 2011). Likewise, Farias et al. (2000) reported the anticoagulant activity of crude polysaccharides and purified sulfated galactans (fractions I, II & III) isolated from _Botryocladia occidentalis_ as 30 IU/mg and 22, 150 & 130 IU/mg respectively, the anticoagulant activity (APTT) of amberlite fractionated and barium acetate purified GAGs was calculated as 64.10 IU/mg and 35.25 IU/mg in _M. casta_ determined by Vidhyarandhini (2010). The variation in the anticoagulant potential may be due to the difference in molecular weight, nature of the sugar residue and the position of sulfate group in the sulfated polysaccharides. Generally, high APTT activity of anticoagulants is due to the inhibition of the intrinsic and/or common pathway, whereas prolongation of TT (Thromboplastin Time) indicates inhibition of thrombin activity or fibrin polymerization (Roberts and Escobar, 2002). The present study reveals the anticoagulant activity potential of the GAGs from _A. granosa_.

The major inhibitors of serine proteases involved in blood coagulation are antithrombin and heparin cofactor II. Their mechanisms of action are profoundly influenced by GAGs, which accelerate the rate of inhibition by binding to the inhibitors and, albeit with some exceptions, to their target enzymes (Bourin and Lindahl, 1993). The anticoagulant activity of GAG was tested _in vitro_ by APTT assay and the values compared with those of standard heparan sulfate (−170 IU/mg). APTT related to the intrinsic coagulation phase in plasma. GAG had 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).

In the present study, the anticoagulant activity (PT) observed as 32 IU/mg in crude GAGs _A. granosa_ respectively, whereas the purified GAG of _A. granosa_ showed 67 IU/mg. The anticoagulant activity of GAGs from _A. granosa_ was investigated by the classical coagulation assays PT, using HS (−170 IU/mg) as a reference. PT is related to the extrinsic coagulation phase in plasma. These tests are
often referred to as functional tests because they monitor clot formation. The prolongation of PT suggested inhibition of the extrinsic coagulation pathway. When compared to HS the weakest effect was observed in the PT assay for the GAGs from *A. granosa*. Since the anticoagulant effect of heparin is not mainly mediated by a modulation of the extrinsic system, it appears that GAGs is a low inhibitor of the extrinsic pathway. But the present anticoagulant activity (PT) of purified GAG of *A. granosa* was high when compared to that Sanavan (2010) reported the anticoagulant activity (PT) observed as 41 IU/mg, 57 IU/mg and 63 IU/mg in fractionated and purified GAGs *A. pleuronectus* respectively. Providentially, in the present study showed that the GAGs showed better anticoagulant activity in lower concentration.

Thus results of the present study clearly brings out the anticoagulant activity of the GAG from *A. granosa* and further research on the influence and role of chemical composition, molecular weight etc. of the GAG from *A. granosa* shall pave the way to develop a drug which can increase the efficacy of conventional chemotherapy drugs used to treat thromboembolic disorders.

Cancer is a major public health burden in both developed and developing countries. An attempt has been made to review some medicinal plants used for the prevention and treatment of cancer. Medicinal molluscs have been on the forefront whenever we talk about anticancer remedies, molluscan medicines have a vital role in the prevention and treatment of leukemia cancer (Rorsener *et al.*, 1986). With advanced knowledge of molecular science and refinement in isolation and structure elucidation techniques, various anticancer compound has been identified, which execute their therapeutic effect by inhibiting cancer-activating enzymes and hormones. Several anticancer agents Dolastatin-A, Ulapualide-A, Chromodorolide-A and Siphonarin-B derived from molluscs are in clinical use all over the world (Petit *et al.*, 1989; Rorsener *et al.*, 1986; Morris *et al.*, 1990). A number of promising anticancer agents are in clinical or preclinical development (Rajeev Kumar Jha and Xu Zì-róng, 2004).

In most cases there is little scientific data available to support the anticancer activity of bioactive compounds and few have been tested for safety and
effectiveness using rigorous methodologies. Previous studies on, crude preparation as well as purified components of venom isolated from American, Asian and European snakes have been shown to inhibit the growth of mouse sarcoma melanoma and leukemia cells as well as human hepatoma and breast cancer cells (Chinang et al., 1992). Flavopirido is currently in phase I and phase II clinical trials against a broad range of tumors, including leukemia, lymphomas and solid tumors (Christian et al., 1997). A racemic mixture of harringtonine and homoharringtonine has been used successfully in China for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia (Cragg and Newman, 2005). According to other recent study fucoid rich sulfated polysaccharides from Ecklonia cava has antiproliferative effects on murine colon carcinoma (CT-26), human leukemia monocyte lymphoma (U-937), human promyelocytic leukemia (HL-60), and mouse melanoma (B-16) cells lines (Athukorala et al., 2009). In addition, fucoidan extracted from C. okamuranus TOKIDA induces apoptosis of human T-cell leukemia virus type 1-infected T-cell lines and primary adult T-cell leukemia cells. Cancer cells in similar pattern to that of commercial fucoidan was reported (Synytsya et al., 2010). Ulapualide-A, a sponge-derived macroline isolated from the nudibranch Hexabranchus sanguineus exhibits cytotoxic activity against L 1210 murine leukemia cells, which exceeds that of clinically useful amphotericin-B (Rosener et al., 1986).

Marine invertebrates are subjected to massive screening for a variety of bioactive substance since utilization of marine biontix substances have been realized to be more powerful than their pharmacologically important terrestrial counterparts. Bioactive substances from marine biota have also been found used as special tools in pharmacological/ biomedical research. In recent years great attention has been paid to the bioactivity of natural products from mollusks, since numerous compounds from them have been identified as potential sources of drugs (Emerson Kagoo and Ayyakkam, 1992). Though about 6,800 compounds have been isolated, majority of them are not tested for their biological activities. Potent drugs play a key role in the world economy, since two third of the population depends on the medicines (Molinski et al., 2008).
In the present study, the cytotoxic effect of crude and purified GAG from A. granosa was observed for 72 h of incubation period and the IC50 value was calculated as 320µg/ml and 250µg/ml respectively. Previously, staurosporine showed potent cytotoxic activity with ED50 value of 0.0024 µg/mL for the KB system and <0.08 µg/mL for the P-388 studied by Morioka et al. (1986). In addition, significant differential in vitro cytotoxicity of apilidine in primary cultured lymphocytes and in transformed cell lines was observed (Bresters, 2003), which may explain why both diemmin B and apilidine show minimal haemotoxicity in vivo (Gomez et al., 2003). The cytotoxicity of (+)-discodermolide is apparent at concentrations too low to cause cell-cycle arrest, in which aberrant mitosis, altered induction of apoptosis and a significant alteration of microtubule dynamics can be observed (Honore, 2003). Mustahil et al. (2013) evaluated the plant (Aegle marmelos) extracts and isolated compounds were subjected to cytotoxic activity screening against CEM-SS (human T-lymphoblastic) cancer cells. Both chloroform and methanol extracts of roots exhibited strong cytotoxicity with IC50 values 8.8±0.30 and 6.8±0.42 µg/mL, respectively while the petroleum ether extract showed moderate cytotoxic activity with IC50 value 22.0±0.90 µg/mL. The MIC50 for L-Glutaminase was accrued within 24 hrs with the concentration of 100 g/ml but only in 48 hrs of incubation only MIC50 was observed (Jaya Prakash, 2009). The present results are proved that the very lower cytotoxicity at higher concentration this compound. Finally the present study can help others to explore molluscan extracts to further extent and its use in various other disease and toxicity studies along with clinical trials.

The ocean remains as untapped source for many drugs and contemporary studies which indicate that, pharmacologically active substances could be isolated from marine organisms (Baslow, 1969). The vast source of nature includes terrestrial and marine plant, microorganism, vertebrates and invertebrates (sponges, ascidians, molluscs and echinoderms) etc. it is important to consider that the major anti-infective, anti-cancer and anti-leukemic compounds are of natural origin. In the present study, revealed that the crude GAG and purified GAG showed anticancer activity against mol-4 leukemia cell line and the crude GAG showed a maximum activity of 61.68% at 200µg/ml, whereas the purified GAG showed
70.42% of inhibition at the same concentration. The lowest inhibition of 13.54% and 17.76% was observed at 25μg/ml for crude and purified GAG respectively. Previously, Staurosporine (extracted from *Streptomyces staurosporeus*) exhibited in vitro activity against several different type of tumors such as human neuroblastoma cell line (NB-1), HeLa S3 cells, B16 melanoma cells and P-388 leukemia cells (Tamaoki et al., 1986). Molinski, et al. (1989) studied the varamine A and B (from ascidian *Lissoclinum vareau*) exhibited cytotoxicity towards L-1210 murine leukemia cells with IC50 values of 0.03 and 0.05 μg/mL. Charyulu et al. (1989) evaluated the diplamine (tunicate *Diplosoma* sp) was found to be cytotoxic towards L-1210 murine leukemia cells with IC50 value of 0.02 μg/mL.

Maedamines A and B (extracted from marine sponge *Suberea* sp) exhibited in vitro cytotoxicity against murine leukemia L-1210 cells with IC50 values of 4.3 and 3.9 μg/mL, respectively and epidermoid carcinoma KB cells with IC50 values of 5.2 and 4.5 μg/mL reported by Hirano et al. (2000). Endo et al. (2007) extracted Hyrtinated A from marine sponge *Hyrtios* sp and it exhibited in vitro cytotoxicity against murine leukemia L-1210 and human epidermoid carcinoma KB cells with IC50 values of 1.0 and 3 μg/mL, respectively. Venom from *Leiurus quinquestriatus* inhibited growth of breast and prostate cancer cells in vitro (Omran, 2003). Scorpion venom inhibited growth of U937 and k562 cells at concentration of a 10-200 μg/ml (Gupta et al., 2007). Scorpion venom has demonstrated to hamper proliferation of prostate cancer cells and human leukemia cells (Dasgupta et al., 2007). The proliferation of either Jurkat or U937 leukemic cell lines inhibitory concentration (IC50) values of *Houttuynia cordata* extracts were between 403.3 to 445.1 mg/ml and 386.2 to 416.4 mg/ml, respectively reported by Pawinwongchai and Chanprasert (2011). In the present investigation anti-leukemic activity was considerably high or low when compared to that of the other sources. These results are again proved that the anti-leukemic activity is concentration dependent manner and the molluscs GAG possessing anti-leukemic activity.

Chemotherapeutic treatment strategies attempted directly to inhibit proliferation of cancer cells or selectively removed the transformed cells by
inducing apoptosis or eliminated the cause of the growth advantage. Unlike cancer cells, normal cells had intact programmed cell death mechanisms. Most treatments designed to kill cancer cells, which also affected the normal cell, resulting unwanted effects affecting organs included gastrointestinal tract, bone marrow etc., (Marks et al., 2000)

Cancer cell cultures had become useful models to evaluate gene expression and to establish in vitro experiments aiming of the control of tumour cell growth (Correa et al., 2002). The compounds may be tested on tumour bearing animals, against microbial systems or cell cultures. The anti-cancer property of molluscs extracts was well established fact and therapeutically used for human leukemia (Kirsten Benkendorff et al., 2011). In the present study, revealed that the crude GAG and purified GAG showed anticancer activity against leukemia cell line and the crude GAG showed a maximum activity of 61.68% at 200μg/ml, whereas the purified GAG showed 70.42% of inhibition at the same concentration. The lowest inhibition of 13.54% and 17.76% was observed at 25μg/ml for crude and purified GAG respectively. Early, Dolastatin 10 is a linear peptide isolated from the sea hare 

Dollabella auricularia and it is a well known antitumour agent with ED50 = 0.046 μg/ml against P 388 cells. Dolastatin 10 is in Phase I clinical trials as anticancer agent for use in the treatment of breast and liver cancers, solid tumours and leukemia (Yamada et al., 2000). Kirsten Benkendorff et al. (2011) determined the DMSO solutions of the chloroform-soluble crude extract from muricid 

Diacathais orbita egg masses were found to reduce the production of formazan relative to solvent controls in all cell lines at a concentration of 1mg/mL, with additional effects at lower concentrations with all solid tumor and U937 lymphoma cells and there was a significant reduction in cell proliferation by almost 80% when treated with the semi purified extracts at 1 mg/mL. Mollusc’s extracts and their bioactive compounds present in them which are responsible for anticancer activity have to be screened for their valuable information. In the present investigation anti-leukemic activity was considerably the purified GAG was high when compared to that of the crude GAG and the purification process helped to enhance the activity of the isolated crude GAGs. Based on the present study, A. granosa GAGs are viewed as promising anti-leukemic activity. These results suggest that purified
molluscan GAGs preparation from estuarine blood clam *A. granosa* might have potential clinical applications an anticancer in near future.

Extracts from marine sources have been shown to provide protection against many diseases including cancers (Apryshko *et al.*, 2005). The present *in vitro* studies demonstrate the anti-leukemic activity of extracts from blood clam *A. granosa*, a bioactive compound GAG. This result indicated that the extract had selective inhibition on leukemia cell lines. Hence, a great deal of interest has been developed to isolate novel bioactive compounds from estuarine blood clam because of their numerous health beneficial effects.