2. REVIEW OF LITERATURE

2.1. Biochemical composition

Knowledge on biochemical composition of any edible organisms is extremely important since the nutritive value is reflected in its biochemical contents (Nagabhushnam and Mane, 1978). A new species should be recommended for human consumption only after assessing the nutritive value of the species with regards to its nutritional qualities (Ajaybhaskar, 2002). The demand for protein rich food is increasing, especially in developing countries, stimulating the exploration of unexploited or non-traditional resources. The marine molluscs are delicious and rich in protein and are next in importance to fishes and prawns. Their nutritive value can be readily assessed by estimating the levels of protein, carbohydrate and fat, in their body tissues. As the bivalves mostly constitute the shell fishery, several studies were dealt with bivalves’ rater, then gastropods (Ansari et al., 1981; Deshbande and Nagabhsanam, 1983). In which, the marine bivalves are commercially valuable species and easy to cultivate in coastal areas. Consumption of marine bivalves (Meretrix casta, Perna viridis, Crassostrea gigas, Ostrea edulis and Donax cuneatus) provides an inexpensive source of protein with a high biological value, essential minerals and vitamins (Shaffe, 1978; Mann, 1979; Shanmugam et al., 2007). Lakshmanan and Nambisan (1980) reported the biochemical composition in Villorita cyprinoides and M. casta. Rajan et al. (1984) observed the biochemical composition of the sexually mature Katelysia opima from the Porto Novo waters. Srikar and Mishra (1989) studied the biochemical content in the departed and shucked clam meat of M. meretrix, M. casta and K. opima.

Molluscs also provide high quality protein with all the dietary essential amino acids for maintenance and growth of the human body. Shafee (1978) determined the variations in the total protein, carbohydrate, lipid, ash and caloric content of the mature and immature green mussel, Perna viridis from the Ennore estuary of Madras coast. Adachi (1979) studied the seasonal changes of the protein level in the adductor muscle of the clam Tapes philippinarum. A female specific
protein from the haemolymph and a related vitaline like protein from the ovary of *C. gigas* were identified by Suzuki *et al.* (1992). Pevicic *et al.* (1993) studied the quantitative determination of metallothionein-like proteins in mussels. The effect of dietary protein content on the growth of juvenile mussels *Mytilus trossulus* were investigated by Kreeger and Langdon (1993). Observation on metallothionein like proteins *M. balthica* and their effects during metal exposure are carried out by Mouneyrac *et al.* (2000). Stress proteins in *M. galloprovincialis* to assess pollution along the Galician coast were investigated by Porte *et al.* (2001).

Carbohydrates are defined as aldehyde on ketone derivatives of higher polyhydric alcohols. They are either simple or complex, and are major sources of energy in all human diets (Ajiyabaker, 2002). Gabbott (1976) also reported wide seasonal variation in carbohydrate level (glycogen content) of marine mussels. Nagabhushanam and Talikhdar (1977) made an observation on the seasonal variation in glycogen of the wedge clam *D. cuneatus*. The biochemical composition and its seasonal changes of various clams were studied by Ansell *et al.* (1980). The influence of seasonal and environmental variations in carbohydrate level in the haemolymph of *C. viriginca* was monitored by Fisher and Newell (1986). The ratio of carbohydrate was less when compare to the other nutrients such as proteins and lipids in animal tissues, especially in aquatic animals (Babu *et al.*, 2010).

Lipids are the major sources of metabolic energy and essential materials for the formation of cell and tissue membranes (Sargent, 1995). Seasonal observation on diet and stored lipids in the horse clam *Tresus capax* were studied by Reid (1969). Lipid content of bivalve molluscs was strictly dependent on the diet; eventhough there is some evidence that several molluscs have lipid levels typical for each species (Watanabe and Ackman, 1974). De Moreno *et al.* (1977) studied the lipid metabolism of the yellow clam *Mesodesma mactroides* where composition of the lipids was discussed. The effect of dietary lipids on the growth tissues composition of metabolism of *C. virginica* was monitored by Triden and Castell (1980). Nair and Gopakumar (1984) were estimated the lipid and fatty acid composition of *P. viridis*. Deslous Paoli *et al.* (1988) estimated the total lipid
content and their energetic values of *M. edulis*. Lipids of marine origin are rich sources of Omega-3(n-3) polyunsaturated fatty acids and they have pronounced hypocholesteolemic effect when supplemented in human diet (Ajiyabaker, 2002). Various tissue lipids from the marine bivalves, *Megangula venulosa* and *M. zymnoensis*, were analyzed for comparison (Kawashima and Ohnishi, 2003).

The nutritional quality of protein is connected to the quantity of the essential amino acids in food (Acton and Rudd 1986). For nutritional purpose, amino acids may be divided into groups, the essential amino acids (EAA) and the non-essential amino acids (NEAA) (Tacon, 1990). Protein as a source of amino acid nitrogen during hyperosmotic volume regulation in *M. edulis* was explained by Deaton et al. (1984). The amino acid composition of periostral proteins from the *Calyptogena magnitum* and *Bathymodiolus thermophilus* were studied by Hunt (1987). Matasushima et al. (1988) observed the effect of metabolic inhibitors on free amino acid accumulation in *Corbicula japonica*. The amino acids in the protein factions of the periostracum of the *P. viridis* and *M. casta* were studied by Yamuna and Balakrishnan (1993). The amino acid metal compositions of the normal and infested oyster *C. gigas* were described by Almeida et al. (1996). Sengor et al. (2008) determined the amino acid and chemical composition of canned smoked mussels (*M. galloprovincialis*).

Fatty acids are aliphatic compounds of lipids, particularly triacylglycerols and phospholipids (Sargent, 1976), have a characteristic pattern in marine invertebrates reflecting ecological conditions and the source of tropic material (Sargent and white, 1981). Watanabe and Ackman (1972 and 1974) studied the effect of unicellular lipids on oyster’s lipids their fatty acid composition of *C. virginica* and *O. edulis*. Paradis and Ackman (1975) observed the occurrence and chemical structure of non-methylene interrupted diatomic fatty acids in *C. virginica*. The fatty acid and tricylglycerol composition of *C. gigas* fed with different algal diets were assessed by Langdon and Waldock (1981). The lipid and fatty acid composition of brown mussel *Glaucoma spicere* were described by Kim (1998). Caers et al. (1999) studied the distribution of lipids and fatty acids in different organs of *Argopecton purpuratus*. Murphy et al. (2003) studied the fatty
acid and sterol composition of frozen and freeze – dried new Zealand green Lipped mussel (*Perna canaliculus*) from three sites in New Zealand. Shamugam *et al.* (2007) studied the valuable fatty acids from *Donax cuneatus* by using gas chromatography.

Molluses are highly delicious seafood for their nutritive value next to finfishes and crustaceans. B-complex vitamins of various Thai fish products including mussel. Increase in food supply could account for the greater that content in cultured clams (Krzynowet *et al.*, 1983). Van-housan (1986) reported the vitamin content in oysters from north carlina waters. Nettleton and Exler (1992) estimated the vitamin content in *C. virginica*. Vitamins content in mussels were studied by Laihao *et al.* (1999). Dietary supplementation of riboflavin through lipids spray beads to *C. gigas* and their uptake were observed by Langdon *et al.* (2000). Changes in tissue concentrations of the vitamins B1 and B2 during reproductive cycle of *C. gigas* were assessed by Seguineau *et al.* (2001). Seguineau *et al.* (2001b) investigated the vitamins B1 and B2 in eggs of the scallop *P. maximus* which are highly utilized during larval development.

Marine organisms form a good source of minerals. The trace elemental concentrations in relation to salinity in *C. virginica* were described by Rucker and Valentine (1961). The uptake and distribution of Zn in oysters were described by (Romeril (1971). The relationship between Zn, Cu and the basophils of *C. gigas* and *C. virginica* were studied by Ruddell and Rains (1975). Lowe and Moore (1979) studied the cytochemical distributions of Zn and Fe in *M. edulis* and their relationship with itsosomes. The uptake and subcellular distribution of Cd, and Zn in the kidney of *M. mercenaria* were described Carmichael *et al.* (1980). Labal and Wright (1982) assessed the Zn concentrations in gonadal and somatic tissues of *M. edulis*. The significance of environment and chronology on the distribution of elements in the oysters were studied by Swann *et al.* (1984). Fractionation of Cu, Ag and Zn among cytosolic proteins and their variability in *M. balthica* were studied by Johansson *et al.* (1986). The distribution of copper in *M. balthica* was observed by Duffrancaasis *et al.* (2001).
2.2. Isolation, purification and characterization of GAG

The glycosaminoglycan family of compounds includes linear sulfated polysaccharides such as heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate and keratan sulfate. Heparin and heparan sulfate are comprised of alternating 1→4 linked glucosamine and uronic acid residues. Heparan sulfate is composed of primary monosulfated disaccharides of N-acetyl-D-glucosamine and D-glucuronic acid, while heparin is composed of mainly trisulfated disaccharides of N-sulfoty-D-glucosamine and L-iduronic acid (Linhardt and Toida, 1996). Tomohiro Maruyama et al. (1998) described the chondroitin sulfate from bovine tracheal cartilage, with the basic structure (4-O-sulfo-D-GalpNACβ1→4-D-GlcA)n, was chemically modified by O-sulfonation. Dermatan sulfate (DS) is a macromolecule member of a class of natural, structurally complex, sulfated, linear polymers named glycosaminoglycans Volpi and Maccari (2009).

Glycosaminoglycans (GAGs), produced by extraction and purification from different animal tissues, have several fundamental biological activities, as well as pharmacological properties, making them important drugs for use in clinical and pharmaceutical fields (Lane and Lindahl, 1989; Mammen et al., 1991; Volpi, 2006). GAGs have been isolated from the tissues of a large number of vertebrate and invertebrate organisms. Invertebrates were first shown to contain a heparin or heparan sulfate (Burson et al., 1956). The presence of sulfated GAGs in some taxa of invertebrates is now well documented (Dietrich et al., 1985; Arumugam and Shanmugam 2004; Saravanan, 2010), crustaceans (Chavante et al., 2000; Demir et al., 2001), and ascidians (Cavalcante et al., 2000; Pavao, 2002), particular in chondroitin sulfate, dermatan sulfate, heparan sulfate, and heparin. An exhaustive assessment showed that molluscs are particularly rich source of the sulfated polysaccharides (Nader and Dietrich, 1989) and it amounts to 90% of the total GAGs content of the molluscs. But the heparins isolated from molluscs are structurally different from human heparin and pharmaceutical heparins (Loganathan et al., 1990).
Heparin is present in several species of molluscs a compound from the clam *M. mercenaria* (Jordon et al., 1986) exhibits several structural similarities to heparin. Somasundaram *et al.* (1989) extracted the crude heparin-like substance from three species of estuarine bivalve molluscs, such as *Katalysia opima*, *A. rhombea* and *Crassostrea madrasensis*. Zierer *et al.* (2000) extracted sulfated polysaccharides from *Aplysina fulva*, *Dysidea fragilis*, *Chondrilla mucedula* and *Hymeniacidon heliophila* by enzymatic digestion, where the tissues were defatted using acetone. Hernaiz *et al.* (2001) performed extraction of heparan sulfate from liver tissues of bovine after defatting the tissues with acetone and chloroform/methanol. Arumugam (2004) extracted the heparin-like substances from the body tissues of the bivalve *T. maxima* and *P. viridiss* respectively. Barwin Vino (2003) extracted the same from the body tissues of *L. divaricata* and *D. sibogae* and also studied the anticoagulant activity of the polysaccharides extracted from intestinal shell (Gladius); whereas Mahalakshmi (2003) also investigated the same from studying the anticoagulant activity of the polysaccharides extracted from the cuttlebone of these two species of the cuttlefishes. Vijayabaskar (2008) studied the GAGs from the body tissues of the marine bivalves *K. opima* and *D. cuneatus* respectively. Vidhyanandhini (2010) investigated the crude extract and characterized glycosaminoglycans from estuarine bivalve *M. casta*. Periyasamy *et al.* (2013a) studied the isolation and characterization of anticoagulant GAGs from marine mollusc *Donax fava*.

The structural studies of heparin sulfates were made by Linker and Hovingh (1977). Hoglund (1976) studied the comparative biochemistry of invertebrate mucopolysaccharides *V. Insecta* (*Calliphora erythrocephala*). Linker and Hovingh (1977) investigated the uses of degradative enzymes as tools for identification and structural analysis of glycosaminoglycans (GAGs). The distribution of sulphated mucopolysaccharides in invertebrates was described by Cassaro and Dietrick (1977). The structure of heparin sulphate oligosaccharides and their degradation by exo-enzymes was studied by Linker (1979). Dietrich *et al.* (1985) isolated and characterized the heparin with high anticoagulant activity from *Anomalocardia brasiliana*. The anticoagulant activity heparin from clam
(Mercenaria mercenaria) was isolated by Jordan and Mareum (1986). The domain structure of heparin sulfates from bovine organs was described by Maccarana et al. (1990). Jackson et al. (1991) studied the molecular properties, protein interactions and role of GAGs in physiological processes. The overview of low molecular weight heparins and basic clinical aspects of heparinoids were reported by Hirsh et al. (1992). The identification of variable and constant oligosaccharide regions in eight heparan sulfate proteoglycans from different origins were reported by Tersariol et al. (1994) through the sequencing of heparan sulfate proteoglycans. Pavia et al. (1995) made a comparative study on the mechanism of the anticoagulant action of mollusc and mammalian heparins. Cosmi et al. (1997) found the effect of non-specific binding to plasma proteins on the AT activities of unfractionated heparin, low-molecular-weight heparin and dermatan sulfate. The regulated diversity of heparan sulfate was studied by Lindal et al. (1998).

Dietrich and Nader (1974) fractionated and studied the properties of four heparin sulfates from beef lung tissues. Reinhold et al. (1989) studied the structural characterization of sulfated GAGs by fast atom bombardment mass spectroscopy and application to heparin fragments prepared by chemical synthesis. The purification of heparin, dermatan sulfate and chondroitin from mixtures by sequential precipitation with various organic solvents was reported by Volpi (1996). Maximilliano et al. (2000) purified sulphated polysaccharides from marine sponges by using Mono-Q-FPLC column which was equilibrated with Tris-HCl buffer. The fractionation of low-molecular-mass heparin by centrifugal partition chromatography in the ion-exchange displacement mode was studied by Intes et al. (2001). Won-Kyo Jung et al. (2002) studied the anticoagulant protein was purified from the edible portion of a blood ark shell, Scapharca broughtonii, by ammonium sulfate precipitation and column chromatography on DEAE-Sephadex A-50, Sephadex G- 75, DEAE-Sephacel, and Bio gel P-100. Ghosh et al. (2004) purified sulphated polysaccharide from Caulerpa racemosa by size exclusion chromatography with Sephacryl S-1000 column. Marina Cesaretti et al. (2004) extracted heparin from dry tissues of Tapes philippinarum and purified by using QAE Sephadex anion exchange resin. Volpi (2005) purified heparin from mollusc.
Anion exchange resin was used for purification and sodium chloride was used at increasing molarity.

Heparin from the body of *Styela plicata* (Santos et al., 2007) was purified by applying to Qsepharose- FPLC column which was equilibrated with Tris-Hcl buffer. Won-Kyo Jung et al. (2007) studied the novel inhibitory protein against blood coagulation factor Va (FVa) was purified from muscle protein of granulated ark *Tegillarca granosa*, marine bivalve by consecutive FPLC method using anion exchange and gel permeation chromatography. Balamurugan et al. (2009) purified the heparin by using strong anion exchange (SAX) chromatography on an HPLC system. Saravanan (2010) studied the crude *Amussium pluronectus* GAGs were fractionated by ion-exchange (DEAE-cellulose and Amberlite IRA-900 & 120) chromatography; the active fractions were purified in Sephadex G-100 column chromatography. Poonguzali, 2011 to investigate the anticoagulant property of low molecular weight heparin from *Ficus gracilis*, and it was purified by anion exchange chromatography (DEAE- cellulose) and size exclusion chromatography (Sephadex G-25 and Sephadex G-10). Periyasamy et al. (2013a) studied the crude *Donax faba* GAGs were purified in Sephadex G-100 column chromatography.

Holick et al. (1985) determined the amount of heparin present in mussel, by metachromatic dye method. In this method tolidine blue was used to determine, calorimetrically the amount of heparin present in the tissues. Somasundaram et al. (1989) used metachromatic dye method for estimating the heparin concentration present in *Katelysia opima, Anadara rhombea* and *Crassostrea madrasensis*. Saravanan et al. (2009) performed Azure-A assay to estimate the level of sulf group substitution of the fractionated and purified glycosaminoglycans from mollusc *Amussium pluronectus*. Metachromatic activity was expressed as the negative slope of standard curve of absorbance at 620 nm vs heparin concentration in a dye solution.

Benny (1996) estimated the sulphate content in glycosaminoglycans by barium chloride method where potassium sulphate solution was used as standard. Ronghua et al. (2003) studied the anticoagulant compound of alginate sulfate and
its quaterized derivates. Ghosh et al. (2004) estimated sulphate composition present in *Caulerpa racemosa*, by hydrolysing the sample initially and determined using a modified turbidimetric barium chloride method. Heparin with different disaccharide composition was isolated from body of *Styela plicata* by Santos et al. (2007). Volpi and Maccari (2009) observed the glycosaminoglycans from the body of marine clam *Scapharca inaequivalvis* were extracted at dry tissue, composed of dematan sulfate and heparan sulfate, further estimated the iduronic acid and glucuronic acid. Saravanam et al. (2009) estimated sulphate content present in glycosaminoglycans extracted from marine molluse *Amussium pluronectus*. Subhapardha et al. (2013) studied the sulfated chitosan was prepared from the shell of donacid clam *Donax scortum* by followed demineralization, deproteinionization, deacetylation & sulfating with chlorosulfonic acid and estimated sulfate content.

Electrophoretic separation of specific protein and carbohydrate has been one of the most useful techniques used to delineate specific biochemical characters. Starch gel as the supporting matrix, but more recently finer separations of proteins and carbohydrate have been achieved using polyacrylamide disc electrophoresis (Volpi and Maccari, 2002). Volpi (1993) studied the “Fast moving” and “slow moving” heparins dematan sulfate and chondroitin sulfate through qualitative and quantitative analysis by agarose-gel electrophoresis. Maxiliano et al. (2000) characterized sulphated polysaccharides from different species of marine sponges, by agarose gel electrophoresis, polyacrylamide gel electrophoresis. Through binding e detection of sub microgram quantities of GAGs on agarose-gels by sequential staining with toludine blue and stains - AlI (Volpi and Maccari, 2002). Hemaiz et al. (2002) characterized heparan sulphate peptidoglycan from bovine liver by polyacrylamide gel electrophoresis (PAGE). Warda et al. (2003) studied the unsaturated disaccharide compositional analysis was performed on the enzymatically depolymerized GAG and the molecular weight of the isolated GAG was determined by gradient polyacrylamide gel electrophoresis (PAGE). Melo et al. (2004) investigated the mechanisms of anticoagulant activity mediated and determining the molecular masses of sulfated polysaccharide by PAGE.
Marina Cesaretti et al. (2004) studied the isolation and characterized by HPLC, AGE & PAGE analysis of a heparin from the clam *Tapes philippinarum*. Volpi (2005) characterized heparin by subjecting on polyacrylamide gel electrophoresis (PAGE) in the presence of markers. The molecular weight of native heparin is 3 to 30 kDa and the molecular weight of commercial preparation of heparin is 12 to 15 kDa (Francis et al., 2006). Walter et al. (2007) extracted heparin from tuna skin and characterized by gel permeation chromatography using high performance liquid chromatography. Santos et al. (2007) characterized heparin from body of *Stylea pilicata*, by agarose gel electrophoresis. Saravanan (2010) characterized heparin by agarose gel electrophoresis, where the molecular mass of isolated heparin was compared with banding ladder of the standard heparin bands. Isolation and identification (AGE & HPLC) of heparin like substances from the gastropod tissue of *Comus musicus* observed by Balamurugan et al. (2009). Poonguzali, 2011 to investigated the anticoagulant property of low molecular weight heparin from *Ficus gracilis*, the purified heparin was characterized by agarose gel electrophoresis (AGE).

Dietrich et al. (1989) described the characterization of heparin from *A. brasiliiana*, *Donax striatus* and *Tivela mactroides* by NMR spectroscopy chemical analysis. Naumann et al. (1991) suggested the possibility of using infrared spectroscopy for biological sample. Cyril et al. (1999) performed the determination of glucose by Fourier-Transform Infrared spectroscopy. Fourier Transform Infrared spectroscopy (FTIR) has been recognized Lefier et al. (2000), Naumann et al. (1991) as a method of fulfilling these requirements. Maxiliano et al. (2000) characterized sulphated polysaccharides from different species of marine sponges, by NMR spectroscopy analysis. Mulloy et al. (2000) studied the solution-state NMR parameters, fiber diffraction data, crystallographic data and molecular modeling methods employed in the conformation and dynamics of heparin and heparan sulfate. Medeiros et al. (2000) employed NMR spectroscopy for detailed structural elucidation of polysaccharides. The NMR determined solution conformation of heparin has been used to predict the binding sites on the surface of heparin-binding proteins. Hernaiz et al. (2002) characterized heparan sulphate
peptidoglycan from bovine liver by $^1$H-NMR spectroscopy. Ronghua et al. (2003) studied preparation and in vitro anticoagulant activities of alginate sulfate and its quaterized derivate. Their structure was characterized by elemental analysis FT-IR, C-NMR and gel permeation chromatography. Marina Cesaretti et al. (2004) studied the isolation and characterized by $^1$H-NMR spectrum analysis of a heparin from the clam *Tapes philippinarum*. Characterization of sulphated polysaccharide from *Caulerpa racemosa* was done by Ghosh et al. (2004).

Walter et al. (2007) extracted heparin from tuna skin and characterized by using 13C-nuclear magnetic resonance (NMR). He et al. (2007) studied the analysis of *Arca subcrenata* water-soluble polysaccharide was carried out by UV, FT-IR and NMR spectroscopy. Xu et al. (2008) analyzed a water-soluble polysaccharide MP-I isolated from *Mytilus coruscus*. MP-I was characterized by Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) spectroscopy were conducted to elucidate its structure. Balamurugan et al. (2009) determined the presence of heparin from two-dimensional 1H-NMR spectroscopy methods, providing new data on hexasaccharides. Volpi and Maccari (2009) observed the glycosaminoglycans from the body of marine clam *Scapharca inaequivalvis* were extracted and structural characterized by Agarose-gel electrophoresis & $^1$H-NMR and $^{13}$C-NMR analysis. Poonguzali, 2011 investigated the anticoagulant property of low molecular weight heparin from *Ficus graciosis*, the purified heparin was characterized by FTIR, Mass spectroscopy and NMR. Periyasamy et al. (2013a) investigated the anticoagulant compound of GAGs from *Donax fava* through FTIR analysis.

2.3. Antioxidant activity

A wide range of bioactive substances are being isolated and characterized from the food that is derived from the marine environment. Marine organisms are the resource of structurally novel and biologically active metabolites. Chemically exclusive compounds of marine origin with different biologically activity have been isolated and a number of them are under investigation and/or are being developed as new pharmaceuticals (Madhu et al., 2014). The marine animals
cyclic and linear peptides discovered have increased our acquaintance about new effective cytotoxic, antimicrobial, ion channels particular blockers, and many other properties with novel chemical structures associated to original mechanisms of pharmacological activity. There is an increasing curiosity in antioxidants particularly in those of free radicals in different diseases. In recent years, natural products from marine samples have a broad variety of biological activities and abundant therapeutic applications contain antiviral, antibacterial, anticancer and anti oxidant activity and very different kinds of substances have been obtained (Faulkner, 2000; Schwartsmann et al., 2001; Barvin vino, 2010).

Marine molluscs have pronounced antioxidant activity are attributed to the presence of polysaccharides particularly those that are sulfated glycosaminoglycan (Barvin vino, 2010). Heparins are strongly acidic GAG components of mast cell granules, from which they are released during inflammatory reactions to tissue damage, and they protect the tissue from radical damage. Heparin alleviates the effects of free radical production and enhances the in vivo activity of SOD (Flick et al., 1983). Maksimenko et al., (1999) studied the individual antithrombotic activities of superoxide dismutase (SOD) and sodium chondroitin sulfates (CHS) as well as the activities of covalent and noncovalent complexes of SOD with CHS were compared in a rat model of arterial thrombosis induced by ferrous chloride. Deepa and Varalakshmi (2003) investigated the salubrious effect of low molecular weight heparin on atherogenic diet-induced cardiac, hepatic and renal lipid peroxidation and collapse of antioxidant defenses. A model of human HaCaT keratinocytes overexpressing antioxidant enzyme genes was established to elucidate the mechanism of oxidative stress leading to the accumulation of HSPG and the role of its accumulation. Catalase overexpression induced an increase in anti-HS antibody (10En) epitope expression in these cells. Western blotting showed that the smeared bands of HSPG were obviously shifted to a higher molecular weight in the catalase transfectants due to glycosylation (Nakayama et al., 2008).

The DPP method was first reported by Blois (1958) who observed that the DPPH was reduced by thiol containing amino acid cysteine and other active
compounds. Anuoma et al. (1989) examined the ability of camosine, homocamosine and anserine to inhibit peroxidation of lipids, using liposomes and rat-liver microsomes as substrates for peroxidation. Later, Brand-Williams et al. (1995) revised the original method and the DPPH scavenging test became a popular method to estimate the antioxidant capacity. Oxidative free radicals and their oxidative action are considered dangerous for the human heart. Surprisingly, the paradoxical observation made by Trutto et al. (1997) reported that low doses of free radicals may have beneficial influences on several cellular functions in the absence of cell damages in the heart. In non-damaging doses, oxidative free radicals may behave as cardio protective constituent for the heart. Balu et al. (2000) investigated the possibility of local treatment of colitis with the adhesive antioxidant enzymes catalase and superoxide dismutase (SOD).

There are several studies available on extracts from plants (whole plant, stem, bark, root, etc.) for their antioxidant activity. Oral et al. (2004) studied the vasorelaxant activity, superoxide radicals (O$^{2-}$)-scavenging capacity and cyclic nucleotide phosphodiesterase (PDE)-inhibitory effects of hesperidin and hesperetin isolated from citrus fruits. Different molecular weight polysaccharides were extracted from Ulva pertusa by H$_2$O$_2$ degradation which are the group of sulfated heteropolysaccharides called the Ulvans and their antioxidant activities such as superoxide and hydroxyl radical scavenging activity, reducing power and metal chelating ability were performance (Qi et al., 2005). Antioxidant activity of defatted methanolic extracts of D. malabarica bark was studied for its free radical scavenging property on different in vitro models (DPPH, nitric acid, superoxide, hydroxyl radical and lipid peroxide) model (Mondal et al., 2006). Amornlerdpison et al. (2007) studied the antioxidant activity of seaweed (Padina minor) extracts using DPPH (1-diphenyl-2-picrylhydrazyl) radical scavenging and ABTS$^{+}$ (2,2'-azino-bis 3e--ethylbenzthiazoline -6-sulfonic acid cation) radical scavenging assays. Zubia et al. (2007) evaluated the antioxidative potential of 48 species of marine macro algae from the coasts of Yucatan and Quintana Roo (Mexico) by measuring the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and total content of phenolic compounds in alcoholic extracts. Antioxidant activity
of *Cyperus rotundus* rhizomes extracts (CRRE) was evaluated in a series of in vitro assay involving free radicals and reactive oxygen species and IC50 values were determined by Nagulendran *et al.* (2007).

The antioxidant activity of aqueous and ethanol extracts of iris (*Iris germanica*, family Iridaceae) has been evaluated *in vitro* using various antioxidant assays, including reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities (Nadaroglu *et al.*, 2007). Kaşpán *et al.* (2008) to evaluate the Antioxidant potential and the study the protective effect of aminothiazole derivative against *H₂O₂* induced oxidative damage on pBR322 DNA and RBC cellular membrane. Hazra *et al.* (2008) evaluated the antioxidant potential and free radical scavenging activity of a 70% methanol extract of *Spondias pinnata*. Mandal *et al.* (2009) evaluated the free radical scavenging properties like DPPH, nitric oxide radical, superoxide and anti-lipid peroxidation and to quantify phenolic constituents in methanol extracts from the leaves and stems of *D. diandra* plant. Methanol extracts of *Plumbago zeylanica* (Root), *Acorus calamus* (Rhizome), *Hemidesmus indicus* (Stem) and *Holarrhena antidysenterica* (Bark) were evaluated for their antioxidant activity by ferric thiocyanate (FTC) assay and compared with thiobarbituric acid (TBA) method (Zahir *et al.*, 2009). Huda-Faujan *et al.* (2009) evaluated the antioxidant activity of several Malaysian plants (*Cosmos caudatus*, *Polygonum minus*, *Oenanth javanica*, *Centella asiatica* and *Murraya koenigii*) using three different methods (ferric reducing power, ferric thiocyanate and thiobarbituric acid) and evaluated the relationship between the antioxidative activity and total phenolic content of the plants. Antioxidant potential and total phenolics content of 70% acetone extracts of the raw and processed seeds of *Bauhinia vahlii* were evaluated by Sowndharajan *et al.* (2010).

Xie *et al.* (2001) evaluated the water-soluble chitosan derivatives by graft copolymerization of maleic acid sodium onto hydroxypropyl chitosan and carboxymethyl chitosan sodium. Their scavenging activities against hydroxyl radical (OH) were investigated by chemiluminescence technique. Xing *et al.* (2005a) determined high molecular weight and high sulfate content chitosans by
using chitosan bought from Qingdao Lida Sea Biochem. Corp., Shandong, China and evaluated their antioxidant potential through scavenging of hydroxyl radicals, superoxide radicals, reducing power and ion chelating assay. Guo et al. (2006) synthesized two derivatives of chitosan namely N- substituted chitosan and quaternized chitosan and evaluated their antioxidant activity against hydroxyl radicals to see the difference related to the amide group present in them.

Li et al. (2007) synthesized the oligosaccharides using glucose as a reactant by microwave irradiation and evaluated there in vitro antioxidant activity through superoxide anion radical, DPPH radical and hydrogen peroxide radical scavenging assay. They also determined the endogenous lipid peroxidation and total antioxidant capacity in aged mice. Yen et al. (2007) concentrated the preparation of fungal chitosan by alkaline N-deacetylation of crude chitin for 60, 90 and 120 min, which was obtained from air-dried shiitake stipes (L. edodes) and also studied it’s in vitro antioxidant activity by conjugated diene method, reducing power, scavenging of DPPH radicals, hydroxyl radicals and chelating ability on ferrous ion. Feng et al. (2008) investigated the possibility of enhancing the antioxidant activity of irradiated chitosan through the irradiation of chitosan in acetic acid solution (1%) with different doses. Radical mediated lipid peroxidation inhibition, reducing power, superoxide anion radical and hydroxyl radical quenching assays were made for evaluating its antioxidant activity and its structure was characterized through GPC, FT-IR and 1H NMR spectroscopy. The antioxidant activity against DPPH and ABTS radicals was also studied apart from its structural characterization through FT-IR and NMR (Yu et al., 2011).

The antioxidant activities of mussels were determined using some scavenging methods (Winston et al., 1998; Regoli, 2000). Antioxidative defense was studied in 3 different size groups of White Sea (Russia) blue mussels Mytilus edulis (Buhringer and Danischewski, 2001). Fifteen specimens of adult mussels Perna perna (Wilhelm et al., 2001) were analyzed for antioxidant and detoxifying enzymes. The apparent lack of correlation between trace organic pollutants and some of the enzymatic antioxidants may be due to the inhibitory effects caused by these chemicals (Cheung et al., 2002). Kristinsson and Rasco (2002) have
described marine organisms, such as fish, molluscs etc., found to be good source for antioxidan
t peptides. Digestive glands of the mangrove mussel *Mytella guyanensis*, collected at one non-polluted site and two polluted sites, were analyzed for different antioxidant defenses (Torres *et al.*, 2002). Other analysis can be done on antioxidant defenses such as glutathione S-transferase and glutathione, overproduction of oxygenreactive substances and oxidative stress (Boelsterli, 2003). Prem Anand *et al.* (2010) studied biochemical composition and antioxidant activity of *Pleuroloca trapezium* meat. Barvin vino (2010) investigated the antioxidant activity of the GAGs from the cuttlefish *Sepia brevimana* was evaluated in terms of DPPH assay, total reducing power, total antioxidant activity, hydrogen peroxide scavenging and nitrous oxide scavenging activity.

Nazeer and Srividya (2011) studied antioxidant peptides from the protein hydrolysates of *C. betulinus* and purified peptides has more antioxidant activity that could reduce the excess free radicals in body in order to prevent free radical induced disease. Ramesh (2012) investigated the antioxidant activity of the conus venom from the cone snail of *Conus amadis* was evaluated in terms of DPPH assay, total reducing power, total antioxidant activity, hydrogen peroxide scavenging and nitrous oxide scavenging activity. Nutritional and antioxidant properties test shown *Discodorris* sp. from Buton Island Indonesia contained some chemical substance played a role as antioxidant (Nurjanah *et al.*, 2012). Ramasamy *et al.* (2012) investigated the extraction, characterization and *in vitro* antioxidative potential (superoxide radicals and hydroxyl radicals scavenging, reducing power and metal in chelating) of chitosan and sulfated chitosan prepared from the cuttlebone of *Sepia aculeata*. Subbhapradha *et al.* (2013) investigated the whole body tissue of *Bursa spinosa* and extracted secondary metabolites with methanol and its antioxidant activity through various in vitro assays such as DPPH radical, superoxide radical and hydroxyl radical. Sivaperumal *et al.* (2014) determined the antioxidant activity of the protein from the edible green mussel of *P. viridis* was evaluated in terms of DPPH assay, total reducing power, total antioxidant activity, hydrogen peroxide scavenging and nitrous oxide scavenging activity.
The water-soluble sulphated polysaccharide conjugates were obtained from abalone viscera (*Haliotis discus hannai*) by alkaline proteases extraction followed by ethanol precipitation. Their antioxidant activities were evaluated *in vitro* by hydroxyl radical scavenging activity, reducing power and metal chelating activity. Those various antioxidant activities were compared to standard such as ascorbic acid and EDTA (Zhu *et al.*, 2008).

### 2.4. Anticoagulant and anticancer activities

Heparin, the blood anticoagulant present circulatory tissue is now recognized to be important and chemically unique polysaccharide of considerable biological significance. Heparin’s discovery can be attributed to the research activities of two men, Jay Mc Lean and William Henry Howell (Linhardt, 1991). It was Holwell and Holt (1922) who coined the form ‘Heparin’ for this type of fat soluble anticoagulant. In 1918 in early 1920s, Howell isolated a water soluble polysaccharides anticoagulant also termed heparin although it was distinct from the phosphatide preparation previously isolated.

Between 1933 and 1936, Connaught Medical Research Laboratories then a part of University of Toronto perfected a technique for producing safe non toxic heparin that could be administered to patient in salt solution. The first human trial of heparin begins in May 1935 and by 1937 it was clear that coagulant heparin was a safe easily available and effective blood anticoagulant (Jordan and Marcum, 1986). Lane *et al.* (1984) studied the anticoagulant activity of heparin oligo nucleotides and their neutralization by platelet factor IV.

Tissues of the surf clam, *Spisula solidissima* contain a large amount of heparin like anti-coagulant material of a sulfated polysaccharide nature (Thomas, 1954). Dietrich *et al.* (1985) isolated and characterized the heparin with high anticoagulant activity from *Anomalocardia brasiliana*. The anticoagulantly active heparin from clam (*Mercenaria mercenaria*) was isolated and structure-activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III (AT III) and eliciting high anti-factor Xa activity Jordan and Marcum (1986). Heparin with high anticoagulant activity has been isolated from
the mollusca *Anomalocardia brasiliana* (Pejler et al., 1987). Dietrich et al. (1989) described the anticoagulant activity of heparin from *A. brasiliana*, *Donax striatus* and *Tivela mactroides*.

Research on the molecular weight-dependent-anticoagulation activity with various polysaccharide sulfates and a relationship between the molecular weight and anticoagulant activity has been reported (Karen et al., 1995). Paiva et al. (1995) investigated the comparative studies on the mechanism of the anticoagulant action of mollusc (*A. brasiliana*) and mammalian heparin. Chondroitin sulfate from bovine tracheal cartilage with the anticoagulant activity was reported by Tomohiro Maruyama et al. (1998). Song Ji Wu et al. (1998) observed the acharan sulfate is a good source of anticoagulant activity prepared from the giant African snail, *Achatina fulica*. The unusual structure of this GAG, being similar but structurally different from both heparin or heparan sulfate, suggests that it might be a good precursor for the preparation of semi-synthetic heparin or heparan sulfate analogs having important biological activities (Song Ji Wu et al., 1998).

Dietrich et al. (1999) isolated a natural low molecular weight heparin (1.5K Da) with an anticoagulant activity of 95 IU/mg to assay on from the shrimp *P. brasiliensis*. The results of the previous studies on heparin sulfate may be involved in defense mechanisms against bacteria and other foreign materials. All infection obtained this for suggest that there molecules perform the same functions in vertebrates and invertebrates (Nader et al., 1999). Dietrich et al. (1999) studied the anticoagulant activities of a novel low molecular weight heparin from the shrimp *Penaeus brasiliensis*.

Arumugam and Shanmugam (2004) studied the anticoagulant activity from the body tissues of the mesogastropad *T. attenuata*. In vitro assays with human plasma, the anticoagulant from *S. broughtonii*, prolonged the activated partial thromboplastin time (APTT) and inhibited the factor IX in the intrinsic pathway of the blood coagulation cascade (Won-Kyo Jung et al., 2001). Preeyanat Vongchan et al. (2002) studied the three sulfated chitosan preparations showed strong anticoagulant activities from crabs *Somanniathelphusa dugasti*. Barwin Vino
(2003) extracted the same from the body tissues of *L. duvauceli* and *D. sibogae* and also studied the anticoagulant activity of the polysaccharides extracted from intestinal shell (Gladius); whereas Mahalakshmi (2003) also investigated the same from studying the anticoagulant activity of the polysaccharides extracted from the cuttlebone of those two species of the cuttlefishes *S. aculeate* and *S. brevimana*. Ronghua *et al.* (2003) studied the preparation and *in vitro* anticoagulant activities of alginate sulfate and its quarterized derivates. Sakai *et al.* (2003) reported the anticoagulant activity of dermatan sulfate through binding to heparin cofactor II, which was found two times higher than that of dermatan sulfate from eel *Anguilla japonica*. Melo *et al.* (2004) investigated the mechanisms of anticoagulant activity mediated by sulfated polysaccharide galactans.


The sulfated galactans from *Gelidium crinale* showed a lower on a clotting assay when compared with polysaccharide from *Botryocladia occidentalis* when tested in assay using specific protease and coagulation inhibitors; these two galactans showed significant differences in their activity (Pereira *et al.*, 2005). To assessed the anticoagulant activity of cellulose sulfates (CS) with different intrinsic viscosities by Shaofei Shi *et al.* (2007). The cost of developing a novel anticoagulant is also given due consideration, whereby a cheaper yet effective
alternative would be of immense welcome (Spyropoulos, 2008). In addition, there is a pressing need for an orally available anticoagulant agent to replace warfarin. Vijayabakar (2008) determined the anticoagulant activity of heparin from bivalve *K. opima* and *D. cuneatus*.

Saravanan (2010) observed the anticoagulant activity of GAGs by APPT and PT methods was found in *A. pleuronectus*. Caroline Manicam *et al.* (2010) observed the aqueous leaf extract of *Melastoma malabathricum* possesses potent anticoagulant property by using methods activated partial thromboplastin time (APTT), prothrombin time (PT) thrombin time (TT). Shabeena Yousuf Naqash (2011) studied the anticoagulant activity of GAGs from the marine fishes *Nemipterus japonicas* and *Exocoetus volitans*. Poonguzali (2011) determined the anticoagulant activity GAGs from marine gastropod *Ficus gracilis*. Periyasamy *et al.* (2013a) studied the anticoagulant activity from the body tissues of the marine bivalve *Donax fava*. Subhapardha *et al.* (2013) evaluated the anticoagulant activity of sulfated chitosan from the shell of donacid clam *Donax scortum*.

Natural Products, especially plants, are used for the treatment of anticancer diseases for thousands of years. Jisaka *et al.* (1992) demonstrated that veronaldine and veronilde elicited antitumor activities in leukemia cells. Recently, Izevbogie (2003) reported that some peptides (edotides) from the aqueous extract of *V. amygdaлина* showed cell growth inhibitory effects in prostate cancer cell line (PC-3) but no effect on normal human peripheral blood mononuclear cells (PBMC). Antileukemia activity from root cultures of *Vernonia amygdaлина* reported by Khalafalla *et al.* (2009). Acharya *et al.* (2011) investigated the antileukemic properties of five fenugreek genotypes (L3068, L3375, Tristar, PI143504 and Amber) grown in western Canada for their potential use as nutraceuticals. Pawinwongchai and Chanprasert (2011) evaluated the *Houttuynia cordata* extract has anti-leukemic activity and suggest its use as an alternative medicinal plant in the treatment of leukemia. Kuan-Hung Lu, *et al.* (2012) reported the Synergistic Apoptosis-Inducing Antileukemic Effects of Arsenic Trioxide and *Mucuna macrocarpa* Stem Extract in Human Leukemic Cells via a Reactive Oxygen Species-Dependent Mechanism. Mustahil *et al.* (2013) studied the antileukemic
Activity of various parts from *Aegle marmelos* (leaves, stem bark and roots) have afforded eleven compounds.

In the early century of mankind, plant derived secondary metabolites have been used by humans to treat acute infections, health disorders and cancer diseases. Only during the last 100 years have natural products been largely replaced by synthetic drugs (Wink *et al.*, 2005). However, some important anticancer agents are still extracted from plants because they cannot be synthesized chemically on a commercial scale due to their complex structures that often contain several chiral centers. Further, some patients show resistance to some established treatments based on their genetics or repeated exposures (Mishra *et al.*, 2006). Therefore, new treatments with different modes of action are constantly sought.

Marine organisms represent a valuable source of new bioactive compounds. The biodiversity of the marine environment and the associated chemical diversity constitute a practically unlimited source of new active substances in the development of bioactive products (Malaker and Ahmad, 2013). The marine natural products are investigated predominantly for their anticancer, cytotoxic, antitumor and anti-leukemia properties (Kan *et al.*, 1999; Indap Pithare, 1998; Rinehart, *et al.*, 1981; Ruben *et al.*, 1989). *Lissoclinum patella* has furnished several cyclic peptides of these ulcyclamide, ulthiacyclamide, and patellamide A, B, and C exhibit antitumor activity against L1210 murine leukemia culture in vitro studies (Ireland, *et al.*, 1980; Pettit, *et al.*, 1981). Ptilocaulin and isoptilocaulin isolated from Ptilocaullistaff and *P. spiculifer* exhibit high order of antimicrobial activity against Grampositive and Gram-negative bacteria, and also inhibit cell growth against L 1210 leukemia cells (Harbour *et al.*, 1981; Ruben *et al.*, 1989). A range of bioactive compounds are found in about 11 sponge genera. Three of these genera (*Haliclona, Petrosia* and *Discodemia*) produce influential anticancer and anti-inflammatory agents (Blunt, *et al.*, 2004). Didemmin B is the first marine peptide to enter into clinical trial as a potent anticancer drug (Newman and Cragg, 2004). Soft corals are likely to be rich sources of biologically active secondary metabolites. Recent experiments show that methanol extracts of two *Nephthea* (*Alcyonacea, Nephtheidae*) species of soft coral have exhibited anticancer
properties (Januar et al., 2009). Zubia et al. (2009) performed antitumoral activities determined by cytotoxic assay with three different tumoral cells lines (Daudi, Jurkett and k562).

Marine microorganisms are a rich source of new genes, exploitation of which is likely to lead to the discovery of new anticancer drugs and therapeutic approaches. Secondary metabolites produced by marine bacteria have yielded pharmaceutical products (Boopathy et al., 2010) such as novel anticancer agents (e.g., Bryostatins, Discodermolide, Eleutherobin and Sarcodictyin). The Anthracenedione derivatives acting as the potent anticancer agents screened from the mangrove endophytic fungus Guignardia sp. for example, Cytarabine, an antileukemic drug and Trabectedin, an agent for treating soft tissue sarcoma are developed from marine fungi sources (Molinski, et al., 2009). L-asparaginase is the first enzyme with anti-leukemic activity to be intensively studied in human beings (Savitti et al., 2003). It is an enzyme drug of choice used in combination therapy for treating acute lymphoblastic leukemia in children (Cory and Cory, 2006; Verma et al., 2007). Arico et al. (2005), showed the results using a mouse leukemia cell line. Partial purification and anti-leukemic activity of L-asparaginase enzyme of the actinomycete strain LA-29 isolated from the estuarine fish, Mugil cephalus studied by Sahu et al. (2007).

Marine molluscs one of major group of invertebrates are not only highly delicious seafood because of their nutritive value but also they are very good source for bio-medically important bioactive compounds (Kamboj, 1999). Many studies on bioactive compounds from molluscs exhibiting antitumour, anticancer and antileukemic activities have been reported worldwide. Studies relating to presence of anticancer and antileukemic properties in molluscs have also been reported earlier. Few of the important reports are, identification and isolation of Aplysianin E from Aplysia species and peptides Dolastatin 10 and Dolastatin 15 from sea hares, Dolabella auricularia (Pettit et al., 1989), high molecular mass compounds such as aplysianins (Pettit et al., 1989). Kahlenalide-F is an US-NCI COMPARE negative compound that seems to have the lysosomes as the cellular target (Garcia-Rocha et al., 1996). Keivan Zandi et al. (2007) isolated of a 60 kDa

From the above review, it is clear that there is only minimum work has been done on estuarine clams and no attempt on blood clam. Hence, the present study is a preliminary attempt on blood clam, *A. granosa* to know their biochemical composition, isolation, purification & characterization of GAG, antioxidant, anticoagulant and anticancer activities.