# *Isolation and studies of marine algae lectins*

A Thesis submitted to Goa University for the Award of the Degree of

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***In***

***Biotechnology***


***By***

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# *STATEMENT*

*As required under the Goa University ordinance OB-9.9 (ii), I state that the present thesis entitled "**Isolation and Studies of Marine algae Lectins**" is my original contribution and that the same has not been submitted on any previous occasion for any degree. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities and suggestions have been availed of.*

*Place: - Goa, India*

*Date:- 29/04/2011*

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# ***List of abbreviations***

NaCl - Sodium chloride

PBS - Phosphate buffered saline

$Na_2HPO_4$ - di-Sodium hydrogen phosphate anhydrous

$KH_2PO_4$ - Potassium dihydrogen orthophosphate

TBS - Tris-buffered saline

Con A - Concanavalin A

d/w - Distilled water

BSA - Bovine serum albumin

g - Gram

µg - Micro gram

µl - Micro litre

L - Litre

RBC - Red blood cells

rpm - Revolutions per minute

M - Molar

mM - Milli molar

kDa - Kilo Dalton

w/v - Weight/volume

v/v - Volume/volume

v/w - Volume/weight

EDTA - Ethylenediamine tetraacetic acid

$CaCl_2$ - Calcium chloride

$MnCl_2$ - Manganese chloride

APS - Ammonium per-sulphate

TEMED - N,N,N',N'-tetramethylethylenediamine

PAGE - Poly-acrylamide gel electrophoresis

SDS - Sodium dodecyl sulphate

$Na_2Co_3$ - Sodium carbonate

NaOH - Sodium hydroxide

$CuSo_4$ - Cupper sulphate

$NaKC_4H_4O_6.4H_2O$

DMEM - Dulbecco's modified eagle medium

MEM -Minimum essential medium

Hanks F12-K

FBS - Foetal Bovine Serum

MTT - Tetrazolium derivative reduction

DPPH - 1, 1-diphenyl-2-picrylhydrazyl

# ***Preface***

This thesis has been compiled into six small chapters to enable clarity of presentation and to facilitate ease of comprehension to the reader. Each chapter from numbers 2 to 6 is presented with an abridged introduction relevant to the respective chapter, individual methodology followed and the results obtained.

The opening chapter of this thesis is Introduction and Review of literature.

Foremost objective of this thesis is to isolate an algal species that has the potential of agglutination. In order to fulfill the motive stated in “Aim”, the first steps undertaken in this study are detailed in chapters 2 and 3 wherein algae commonly found were collected and screened for agglutinins. Thus the work recorded in Chapters 2 and 3 fulfill the motives 1 to 3 in “Objectives”.

Chapter 4 covers the methodologies undertaken and results obtained during purification of agglutinins from *Ulva fasciata* and *Caulerpa sertularioides*.

Biochemical characterization of the purified products has been recorded in chapter 5.

Chapter 6 elucidates the various studies undertaken to fulfill the last objective and records the attempts to explore the Biotechnological application of these products.

All findings of this study have been discussed in relevance to reported work in this field, in chapter 7 and is termed “General Discussion”.

A brief summary of the work and conclusions inferred have been covered in “Summary and Conclusions”.

The loop holes of this study are finally projected as “Future Prospects”.

Literature that has been cited is indexed in “Bibliography”.

Appendix I-III covers the technical aspects of preparation of chemicals that were used in this work.

Shortcuts taken in terms have been indexed in “Abbreviations”

Last but not the least ….is attached the output of this work that has already been published and presented.

This chapter is initiated with a general introduction and further surveys reported literature on marine algae, lectins, and finally focusing more on lectins from marine algae as indicated below:

**1.1 General introduction**

**1.2 Marine macro algae**

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## 1.1 General introduction

Our earth has enormous resources of natural products which man has been harnessing since its evolution. Natural products, the secondary or non primary metabolites produced by terrestrial living organisms have been exploited by human beings for varied purpose including food, fragrances, pigments, insecticides and medicines.

Oceans constitute approximately 71 % of earth's surface. Yet as of today only a very small fraction of world's bioactive compound's supply, comes from the sea. The ocean offers abundant resources for research and development, yet the potential of this domain, as a field or new area of marine biotechnology, remains largely unexplored. However, with the increase in imbalance in human population the available proportion of land resources have started declining. Under these conditions, marine environment has evolved as a promising avenue.

Study of marine organisms for their bioactive potential, being an important part of marine ecosystem, has picked up rhythm in recent years with the growing recognition of their importance in human life. New trends in drug discovery from natural sources emphasize on investigation of the marine ecosystem to explore numerous, complex and novel chemical entities. The selection of samples for assays of biological activities useable in drug development is often based on ecological observations and includes specimens with unique (usually chemical) mechanisms for coping with environmental pressures (Haefner, 2003).

The importance of marine organisms as a source of novel bioactive substances is growing rapidly. Marine organisms represent a valuable source of new compounds. The biodiversity of the marine environment and the associated chemical diversity constitutes a practically unlimited resource of new active substances for the development of bioactive products. Marine organisms are rich sources of structurally diverse bioactive compounds with various biological activities. In their review, Aneiros and Garateix, (2004**)** postulated that with marine species comprising approximately one half of the total global biodiversity, the sea offers an enormous resource for novel compounds and can be classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity. Moreover, since marine organisms live in very extreme, competitive and aggressive surroundings, very different in many aspects from the terrestrial environment, very different kinds of substances can be identified because they are in a situation that demands the production of quite specific and potent active molecules. Marine environment serves thus as a source of functional materials, including polyunsaturated fatty acids (PUFA), polysaccharides, minerals and vitamins, antioxidants, enzymes and bioactive peptides (Kim and Wijesekara, 2010).

Seaweeds are macroscopic algae found attached to the bottom in relatively shallow coastal waters. They grow in the intertidal, shallow and deep-sea areas up to 180 meter depth and also in estuaries and back-waters on the solid substratum such as rocks, dead corals, pebbles, shells and other plant material (Smith, 1944).

Global utilization of macroalgae is a multi-billion dollar industry. Much of this is based on farming of edible species or in the production of agar, carrageen and alginate. Of all seaweed products, hydrocolloids have had the biggest influence on modern Western societies (Smit, 2004). They have attained commercial significance through their use in various industries which exploit their physical properties such as gelling, water-retention and their ability to emulsify (Renn, 1997). Little commercial exploitation of products extracted from seaweeds occurs outside the hydrocolloid industry. However, in recent years pharmaceutical firms have started looking towards marine organisms, including seaweeds, in their search for new drugs from natural products.

The discovery of the bio-regulatory role of different lectins in the marine algae, the understanding of the molecular mechanisms of action of some new lectins obtained from natural sources on specific cellular targets, contributes to consider algal lectins also as promising lead drug candidates.

Lectins are carbohydrate-binding proteins of non-immune origin which agglutinate cells or precipitate glycoconjugates (Goldstein et al., 1980). They are a very heterogeneous group of proteins, artificially classified together, solely on the basis of their capability to agglutinate cells. The first definition of lectins was based primarily on the sugar specificity and inhibition of the agglutination reaction. However, the definition appeared restrictive as it excluded some poorly agglutinating toxins such as Ricin, Abrin, Modeccin etc, which were known to contain lectin subunits. Moreover, some lectins contained a second type of binding site that interacted with non-carbohydrate ligands and lectins were

further re-defined as carbohydrate-binding proteins other than antibodies or enzymes. As a consequence, the presence of at least one non-catalytic domain, which binds reversibly to a specific carbohydrate, is considered to be the only criterion for a protein to be considered as a lectin (Peumans and Van Damme, 1995)

Lectins are widely distributed throughout the plant kingdom where they have been found in a variety of tissues of a large number of different plants as early as 19$^{th}$ century. Subsequently, lectins have been reported to be present in plant seeds particularly in cotyledons where they appear during the later stages of maturation of the seeds (Van damme et al., 1998c).

Many of the animal lectins, even in diverse sources, exhibit sequence similarity and common features, which serve as a basis for their classification into a number of families, the most prominent of which are galectins, C-type lectins and siglecs. Although a number of lectins of various molecular weights have been found in marine invertebrates, very limited information concerning their structures has thus far been obtained (Kilpatrick, 2000).

Boyd *et al*, (1966), first identified a hemagglutinin from marine algae. However, the first agglutinin to be characterized from marine algae was in 1977 by Rogers et al., and later several algal hemagglutinins have been isolated, to date. Unfortunately, the practical uses of marine algal hemagglutinins have been limited to research and their routine use for clinical purposes remains limited.

## 1.2 Marine macro algae

**1.2 a**. General characteristics of algae

Seaweeds or benthic marine algae are the group of plants that live either in marine or brackish water environment on solid substratum such as rocks, dead corals, pebbles, shells and other plant materials. Seaweeds are macroscopic algae found attached to the bottom in relatively shallow coastal waters. They grow in the inter-tidal, shallow and deep sea areas up to 180 meter depth where 0.01% photosynthetic light is available and also in estuaries and back waters (Smith, 1944).

"Seaweeds" refers to any large, marine benthic algae that are multi-cellular, macrothallic, and thus differentiated from most algae that are of microscopic size (Smith, 1944). Like land plants, seaweeds contain photosynthetic pigments and with the help of sunlight and nutrients in the sea water, they photosynthesize and produce food. Plant pigments, light exposure, depth, temperature, tides and shore characteristics combine to create different environments that determine the distribution and variety among seaweeds.

The important criteria used to distinguish the different algal groups based on the recent biochemical, physiological and electron microscopic studies are: photosynthetic pigments, storage food products, cell wall components, fine structure of cell and flagella (Smith, 1944) Accordingly, marine algae are classified into three main groups i.e. Chlorophyceae (green algae), Phaeophyceae (brown algae) and Rhodophyceae (red algae). Like land plants,

seaweeds contain photosynthetic pigments and with the help of sunlight and nutrients present in the seawater, they photosynthesize and produce food.

Seaweeds are similar, in form, with the higher vascular plants but the structure and function in part significantly differs from that of higher plants. Seaweeds do not have true roots, stem or leaves. Whole body of the plant is called thallus and consists of the hold fast, stipe and blade. The hold fast resembles the root to the higher plants but its function is for attachment and not for nutrient absorption. The hold fast may be discoidal, rhizoidal, bulbous or branched depending on the substratum it attaches. The most significant differences of seaweeds from the higher plant is that their sex organs and sporangia are usually one-celled or if multi-cellular, their gametes and spores are not enclosed (Prescott, 1984).

**1.2b** Localization of algae in India

Seaweeds grow abundantly along the coast of India. Rich growth of seaweeds is found around Tamil Nadu, Gujarat, Lakshwadweep, Andaman and Nicobar Islands, Mumbai, Ratnagiri, Goa, Karwar and Orissa. Survey carried out by the Central Salt and Marine and Chemical Research Institute (CSMCRI), Central Marine Fishery Resource Institute (CMFRI) and other research organizations have revealed abundant seaweed resources along the coastal belts of South India. On the west coast, especially in the state of Gujarat, huge seaweed resource is present on the inter-tidal and sub-tidal regions. These resources translate into great potential for the development of seaweed-based industries in India. (Dhargalkar and Pereira, 2005)

Although, India (08.04–37.06 N and 68.07–97.25 E), a tropical South Asian country has a stretch of about 8000 km coastline, excluding its island territories with 2 million $km^2$ Exclusive Economic Zone (EEZ) and nine maritime states ((Rao and Mantri, 2006), Indian seaweed industry suffers from absence of commercial cultivation practices, lack of infrastructure for commercial cultivation and absence of policy support. The seaweed flora of India is highly diversified and comprises mostly of tropical species, but boreal, temperate and subtropical elements have also been reported (Rao and Mantri 2006). In all, 271 genera and 1153 species of marine algae, including different forms and varieties have been enumerated till date from the Indian waters (Anon, 2005). Distribution of species of seaweed in India have been reported to be (Seaweed cultivation and utilization, Policy paper 22, NAAS) 202 in Gujarat; Maharashtra, 152 ; Goa, 75; Karnataka, 39; Kerala, 20; Lakshwadeep, 89; Tamil Nadu ,302; Andhra Pradesh 78; Orissa 1; West Bengal 6 and Andaman & Nicobar Islands 34. India presently harvests only about 22,000 tonnes of macro-algae annually, a mere 2.5 per cent, compared to a potential harvest of 870,000 tonnes.

**1.2.c**. Importance of seaweeds: uses and utilization

**1.2.c1**. Food:

People do not have very good impression of seaweeds. They think they are just some stinking, slimy nuisance that washes up on clean sandy beaches. Most people do not realize how important seaweeds are, both ecologically and commercially. In reality, seaweeds are crucial primary producers in oceanic food webs. They are also valuable sources of food, micronutrients, and raw

materials for the pharmaceutical industry. Seaweeds have plenty of essential nutrients, especially trace elements and several other bioactive substances and thus, today, seaweeds are considered as the food supplement for 21st century, and a rich source of proteins, lipids, polysaccharides, mineral, vitamins, and enzymes.

Products extracted from seaweeds are increasingly being used in medical and biochemical research. Prior to the 1950s, the medicinal properties of seaweeds were restricted to traditional and folk medicines (Lincoln et al., 1991). During the 1980s and 90s, compounds with biological activities or pharmacological properties (bioactivities) were discovered in marine algae (Mayer & Lehmann, 2000).

Seaweeds are the only source of phytochemicals namely agar-agar, carrageenan and algin, which are extensively used in various industries such as food, confectionary, textiles, pharmaceuticals, dairy and paper industries mostly as gelling, stabilizing and thickening agents.

Seaweeds are used in many maritime countries as a source of food, for industrial applications and as a fertilizer. The major utilization of these plants as food is in Asia, particularly Japan, Korea and China, where seaweed cultivation has become a major industry. In most Western countries, food and animal consumption of seaweeds is restricted and there has not been any major pressure to develop seaweed cultivation techniques, even through there is an increasing tendency to consume seaweeds as “heath food”.

**1.2c2**. Medicinal use of seaweeds

Algae have been the source of about 35% of the newly discovered chemicals between 1977-1987 followed by sponges (29%) and cnidarians (22%) with varied functions and applications in medicine, from therapy to research (Corgiat, 1993) as briefly described below. Several seaweeds from the marine ecosystem have also been reported to have bioactive compounds. For example, Caulerpa, Carollina, Hypnea, Padina and Sargassum have shown high level of anti-viral activity while maintaining low levels of cytotoxicity (Zhu et al., 2003). Depsipeptides extracted from Bryopsis sp. were active against Mycobacterium tuberculosis (Sayed et al., 2000). Many toxins with excitatory & cytotoxic activity have been reported from Digenea sp., Chondria sp., Amansia sp. and other macroalgae. Poly-halogenated monoterpenes, aplysiaterpenoid & relfairine isolated from Plocamium showed activity against Anopheles larvae and Culex larvae (Watanabe et al., 1990). Oxylipins isolated from alga had shown activity resembling eicosanoid hormones which help in innate immunity (Bouarab et al., 2004). Algal products such as algin, carrageenan, funoran, fucoidan, laminarin, porphyran and ulvan have been noted to produce hypocholesterolemic and hypolipidemic responses due to reduced cholesterol absorption in the gut. An antiplasmic inhibitor has been isolated from Ecklonia karome (Fakuyama et al., 1989).

Due to its immense potential, drug discovery has been widely encouraged by funding authorities and a large number of labs are actively in the process of screening large numbers of pure organic compounds or crude extracts to provide new leads. Marine algae have historically been an exceptionally rich source of

pharmacologically active metabolites with anti-neoplastic, antimicrobial and antiviral effects (Faulkner, 2000; Tziveleka et al., 2003). Random screenings were effective to have found marine algae with various biological activities (Harada et al., 1997), and many of these reports have been reviewed (Nekhoroshev, 1996). In addition, some natural products previously ascribed to marine invertebrate animals were proved to be secondary metabolites from algae (Scheuer, 1990).

Anti-tumor activity is one of the most important aspect in/of marine drugs, and there are many reports (Fuller et al., 1994; Harada et al., 2002; Mayer and Gustafson, 2003; Sheu et al., 1997) demonstrating potent cytotoxic functions of algae and their metabolites. These metabolites have played an important role in paving the path to new pharmaceutical compounds for anti-tumor drugs (Yoo et al., 2002). Several representative anti-tumor compounds from algae, such as Halomon, had been developed upto the clinical phase (Egorin et al., 1996). The accumulated metabolites have shown various potential biological activities including antibacterial, antioxidant and $\alpha$-glucosidase inhibitor activities (Choi et al., 2000).

### 1.3 Lectins

Definition of Lectins

Lectins were first described in 1888 by Stillmark, working with castor bean extracts. Since lectins were originally isolated only from plant extracts and were used for agglutination of red blood cells, they were also called as ‘phytohemagglutinins’. Later it was reported that they could also be obtained

from animal organs, especially those of invertebrates and did not all bind to erythrocytes. So, in 1954, the term ‘lectin’ was coined (Boyd & Shapleigh, 1959).

International Union of Biochemistry Nomenclature (Dixon, 1981) defined “lectin” as carbohydrate-binding protein of non-immune origin that agglutinates cells and precipitates polysaccharides. The emphasis on non-immune origin of lectins is to distinguish them from carbohydrate-specific antibodies. To stress that lectins are different from carbohydrate-specific enzymes, such as kinases, glycosidases, transferases and transporters, which in rare cases agglutinate cells, another definition has been proposed. By this definition lectins are carbohydrate-binding proteins that do not modify the carbohydrates to which they bind (Kocoureck & Horejsi, 1981). It should be noted that in addition to carbohydrate-binding sites, lectins may contain one or more sites that interact with non-carbohydrate ligands (Barondes; 1988).

Carbohydrate-binding proteins include enzymes that act on sugars as substrates, carbohydrate-specific antibodies, membrane transport proteins involved in sugar transport and chemotactic detection. The distinct function served by these proteins is reflected in differences in their intrinsic affinities for monosacharides, which are often much higher than that of lectins and in their relative importance of oligosaccharides as ligands, which form the primary high affinity ligands for lectins.

Of the hundreds of monosaccharides found in nature, large majority of lectins recognize just a few, primarily mannose, glucose, galactose, N-

acetylglucosamine, N-acetylgalactoseamine, fucose and N-acetylneuraminic acid; in addition, they combine specifically with large numbers of oligosaccharides composed of these monosaccharides. A striking feature of the above monosaccharides is that they are typical constituents of animal glycoconjugates and are present on the surface of cells, including erythrocytes. Lectins specific for other sugars have very rarely been encountered. This may be a reflection of the method routinely employed for the detection of lectins, namely agglutination of erythrocytes, or hemagglutination in brief (Ahmed, 2005).

Lectins differ from antibodies in several different aspects. Many lectins are found in plants, microorganisms and viruses that are not capable of an immune response. Another marked difference between the two classes of protein is that antibodies are structurally similar whereas lectins are structurally diverse. In general, lectins are oligomeric proteins composed of subunits, one or more of which carries a sugar-binding site. They vary, however, in size, amino acid composition, metal requirement, domain organization, subunit number and assembly, as well as their three dimensional structure and in the constitution of their binding sites. In their structural diversity, lectins are akin to enzymes, although they are devoid of catalytic activity. In spite of this variation, they can be grouped in families of homologous proteins, the largest and best characterized of which is that of legume lectins (Ahmed, H.; 2005).

**1.3a**. History of lectins

Lectins were first designated as “hemagglutinins”, or more commonly as “phytohemagglutinins”, because they were detected by agglutination of

erythrocytes and were found almost exclusively in plants. The first report on the occurrence in plants of such proteins appeared in 1888 in the doctoral thesis of Hermann Stillmark, a student of Robert Kobert at the University of Dorpat (now Tartu) in Estonia. Sillmark was studying the toxicity of the beans of the castor tree (*Ricinus communis*). Mixing an extract of beans with blood, he made the starting observation that erythrocytes were agglutinated. Using the very primitive methods available at the time, designated by him as "the way of pharamacological isolation", namely salt extraction of the beans, precipitation with magnesium sulphate and ammonium sulphate and dialysis, he obtained "an odorless, snow-white powder", which was hemagglutinating (Franz, 1988). It took however, more than half a century before it was definitely demonstrated that Stillmark's "Ricin" was a mixture of a weakly agglutinating protein toxin and the nontoxic agglutinin (*Ricinus communis* agglutinin, or RCA). Ricin came to the attention of the general public in 1978, following its use as a weapon in the notorious, politically motivated "umbrella murder" (Knight,B. 1979).

Shortly after Stillmark presented his thesis, H. Hellin, another student of Kobert, discovered that the toxic extract of the Jequirity bean (*Abrus precatorious*) also caused the red cells to clump. The new agglutinin was named "abrin**".**

Already the early results obtained by Stillmark indicated some selectivity in the lectin-mediated agglutination of red blood cells from different animals. This observation was corroborated and further extended by Karl Landsteiner to his discovery in 1900 of the human A, B and O blood groups. In 1908, Karl Landsteiner& H. Raubitschek demonstrated that the relative hemagglutinating

activities of various seed extracts were quite different when tested with red blood cells from different animals.

From the commencement, research of lectins has covered/crossed many milestones as condensed by Sharon and Lis, (2003) in Table 1.1.

**Table 1.1- Milestones of lectin research**

| *Year* | *Scientist(s)* | *Discovery/Event* |
|---|---|---|
| 1888 | P.H. Stillmark | Hemagglutinating activity of castor bean extracts |
| 1908 | K. Landsteiner | Species specificity of plant hemagglutinins |
| 1919 | J.B. Sumner | Isolation and crystallization of concanavalin A |
| 1936 | J.B. Sumner & S.F. Howell | Sugar specificity of concanavalin A |
| 1948-1949 | W.C. Boyd; K.O. Renkonen | Blood type specificity of plant hemagglutinins |
| 1952 | W.M.Watkins & W.T.J. Morgan | Use of lectins for identification of cell surface sugars |
| 1954 | W.C. Boyd & E. Shapley | Blood antigen specific agglutinins named 'lectins' |
| 1960 | P.C. Nowell | Mitogenic activity of phytohemagglutinin |
| 1963 | J.C. Aub | Preferential agglutination of cancer cells by wheat germ agglutinin |
| 1965 | I.J. Goldstein | Affinity purification of concanavalin A & its use for structural studies of carbohydrates |
| 1970 | J. Porath | Use of lectins for affinity purification of glycoproteins |
| 1972 | G.M. Edelman; K.O. Hartman & C.F. Ainsworth | Primary and 3-D structure of concanavalin A |
| 1974 | G. Ashwell & A. Morell | Animal lectins function in endocytosis of glycoproteins |
| 1974 | S. Kornfeld; R.C. Hughes; P. Stanley | Lectin-resistant cell mutants |
| 1976 | Y. Reisner & N. Sharon | Use of lectins for fractionation of lymphocytes |
| 1977 | I. Ofek, D.Mirelman & N. Sharon | Role of bacterial lectins in infectious disease |
| 1981 | Y. Reisner & N. Sharon | Use of soybean agglutinin in purging of human bone marrow for transplantation |
| 1983 | E.C. Butcher & I. Weissman; S.D. Rosen | Lectins in lymphocyte homing |
| 1988 | K. Drickamer | Identification of carbohydrate recognition domains in animal lectins |
| 1989 | Various | Selectins and their function in inflammation |

**Source: - Sharon and Lis, 2003, Lectins Second Eds**

For the first seven or eight decades from the description at the turn of the 19th century, lectins were hardly of any interest and just a few, almost all from plant, were investigated in detail. The importance of this early work has only recently become appreciated, as is clear that studies of plant lectins have helped to catapult the field of glycobiology in modern era and have made an enormous contribution to modern biochemistry (Varki et al 1999).

**1.3 b.** Sources of lectins

Lectins are ubiquitous in nature, and are found in all classes of organisms and families of organisms although not necessary in every genus or species (Sharon and Lis, 2003) .They are easy to detect and often to isolate. In addition, some are available from commercial suppliers. Their tissue and cellular distribution is variable, and it may be affected by miscellaneous factors, such as development stage, age and pathological conditions.

**1.3b1**. Lectins from microorganisms

Almost all microorganisms express surface-exposed carbohydrates. The carbohydrates may be covalently bound, as in glycosylated teichoic acids to peptidoglycan, or non-covalently bound, as in capsular polysaccharides. Every surface-exposed polysaccharide is a potential lectin-reactive site. The ability of lectins to complex with microbial glyco-conjugates has made it possible to employ the proteins as probes and sorbents for whole cells, mutants and numerous cellular constituents and metabolites. Microbial receptors for lectins consist of several unique chemical structures. For example, secreted and often cell adherent dextrans produced by members of the genera Leuconostoc and

Streptococcus are neutral polysaccharides capable of interacting with Concanvalin A (Con A). Another Con A receptor found on many Gram-positive bacterial surfaces is glycosylated teichoic acid, a polyelectrolyte.

Many bacterial species and genera express lectins, frequently of more than one type and with distinct specificity (Ofek & Doyle, 1994; Sharon & Lis, 1997). It is not known, however, whether individual cells co-express multiple lectins or if each lectin is confined to a distinct cell population. In Gram negative bacteria (such as *E.coli, K. pneumoniae* and *Salmonellae* spp.), lectins are often in the form of sub-microscopic hair-like appendages, known as fimbriae (or pili), that protrude from the surface of the cells. Fimbrial surface lectins are also produced by Gram positive bacteria, among them the oral *Actinomyces naeslundii* and *Actinomyces viscosus*. Non-fimbrial lectins associated with the bacterial surface have been purified from *Rhizobium lupinii*, and *Agrobacterium tumefaciens*, also a member of the Rhizobia family (Sharon and Lis, 2003).

Marine surfaces are colonized by a diversity of microorganisms and sessile marine organisms are collectively known as biofouling communities. **B**iofouling process is initiated by the attachment of bacteria to a surface followed by the settlement and adherence of diatoms, free-swimming algal spores and invertebrate larvae (Bryers & Characklis; 1982). Some sessile higher organisms employ chemical defenses against biofouling through the production of secondary metabolites that inhibit the development and formation of a biofouling community (Harrison; 1992). For example, furanones produced by the red alga *Delisea pulchra* have been reported to inhibit the settlement of

common fouling organisms. For marine organisms without intrinsic defense mechanisms, it has been proposed that protection against fouling is maintained by the secondary metabolites produced by surface-associated bacteria (Egan et al., 2001).

A lectin has also been identified in protozoa, (Ward, 1997) a surface protein, in the pathogenic ameba, *Entamoeba histolytica* having specificity for Gal/GalNAc (Petri & Schnaar, 1995). Two lectins, one specific for N-acetylneuramic acid, the other for N-acetylglucosamine, were isolated from merozoides of the human malarial parasite *Plasmodium flaciparum* (Ward, 1997).

**1.3b2.** Viral lectins

Viruses contain sugar-specific surface proteins or glycoproteins that act as hemagglutinants and therefore classified as lectins (Sharon & Lis, 1997). Much information is available on the influenza and polyoma viruses, belonging to the orthomyxoviruses and papoviruses, respectively. Similar lectins that are less well defined are found in myxoviruses, such as those of Newcastle disease, Sendai and rotaviruses. Other viral lectins include those of HIV (Haidar et al., 1992) and foot-and-mouth diseases (Fry et al., 1999).

**1.3b3.** Lectins from Fungi

The first lectin to be purified from these sources was from the fruiting bodies of the meadow mushroom, *Agaricus campestris,* and the common (commercial) mushroom, *Agaricus bisporus* (Goldstein & Poretz, 1986) and now, many other fungal lectins are known (Guillot & Konska, 1997). Lectins have also been

found in phytopathogenic fungi, such as *Botrytis cinerea* (Kallens et al.1992), *Pleurotus ostreatus* (Chattopadhyay et al. 1999; Wang et al. 2000), *Rhizoctonia solani* (Candy et al. 2001) in different members of the *Sclerotiniaceae* (Goldstein, 1990; Inbar & Chet, 1994) and in the nematode-trapping fungus *Arthrobotrys oligospora* (Rosen et al. 1996). Couple of years ago, a lectin with unique carbohydrate-binding properties, including blood group B specificity, and high affinity for Galα3Gal and Galα3Galβ4GlcNAc has been isolated from the mushroom *Marasmius oreades* (Winter et al. 2002).

Lectins have been isolated from a few yeast species, namely a galactose-specific one from a fatty acid auxotroph of *Saccharomyces cerevisiae* (Kundu et al., 1987) and two from the culture medium of *Kluyveromyces bulgaricus*, one specific for galactose and the other for N-acetylglucosamine (Al-Mahmood et al. 1991).

There are few reviews about fungal and mushroom lectins (Singh et al., 2010; Khan and Khan, 2011). In the last few years, mushroom and other fungal lectins have attracted wide attention due to their biomedical applications. Singh et al., (2011) have recently purified a mucin-specific lectin from *Aspergillus nidulans*. Lectins from fungi have previously been reported by Bhowal et al., (2005), Thakur et al., (2007) Khan et al (2007) and Singh et al., (2010b).

**1.3b4.** Lectins in plants

Lectins have been detected in over a thousand species of plants and several hundreds have been isolated (Van Damme et al. 1998a). Some of the better

characterized plant lectins and their specificities are listed by Sharon and Lis, (2003) in Table 1.2. The majority of plant lectins have been isolated from seeds, especially those of the dicotyledonous legumes, where they accumulate during maturation and disappear upon germination (Sharon and Lis, 2003). Their location within the seeds however differs among various plant families (Rudigers, 1998).

**Table 1.2**- Characterized plant lectins and their Specificity

| Family and species | Name/ abbreviation | Location in plant | Specificity |
|---|---|---|---|
| **Monocotyledons** | | | |
| Amaryllidaceae | | | |
| *Galanthus nivalis* (snowdrop) | GNA | Bulb | Man |
| *Narcissus pseudonarcissus* (daffodil) | NPL | Bulb | Man |
| Gramineae | | | |
| *Oryza sativa* (rice) | | Seed | GlcNac |
| *Salt-stresses Oryza sativa* (rice) | | Seed | Man |
| *Triticum aestivum* (bread wheat) | WGA | Germ | GlcNac & NAc & Man |
| Iridaceae | | | |
| *Iris hollandica* (Dutch iris) | | Bulb | Gal/GalNAc &Man |
| Liliaceae | | | |
| *Allium sativum* (Garlic) | ASA | Bulb | Man |

| | | | |
|---|---|---|---|
| *Scilla campanulata* | SCA | Bulb | Man |
| **Dicotyledons** | | | |
| Caprifoliaceae<br>*Sambucus nigra* (elderberry) | SNA | Bark | Neu5Ac-OS |
| Compositae<br>*Helianthus tuberosus* (Jerusalem) | HTL | Tuber | Man |
| Convolvulaceae<br>*Calystegia sepium* (hedge bindweed) | Calsepa | Rhizome | Man & maltose |
| Cucurbitaceae<br>*Momordica charanita* (bitter gourd) | | Seed | Gal/GalNAc |
| Euphorbiaceae | | | |
| *Ricinus communis* (castor bean) | RCA | Seed | Gal/GalNAc |
| | Ricin | Seed | Gal/GalNAc |
| Leguminosae | | | |
| *Abrus precatotorius* (jequirity bean) | Abrin | Seed | Gal/GalNAc |
| *Arachis hypogaea* (peanut) | PNA | Seed | Gal/GalNAc |
| *Canavalia ensiformis* (jack bean) | Con A | Seed | Man/Glc |
| *Dolichos lablab* (lablab purpureum) | FRIL | Seed | Man |
| *Glycine max* (soyabean) | SBA | Seed | Gal/GalNAc |
| *Lens culinaris* (lentil) | LCL | Seed | Man/Glc |
| *Phaseolus vulgaris* (red kidney bean) | PHA | Seed | Gal/GalNAc |
| *Phaseolus lunatus* (lima bean) | LBA | Seed | Gal/GalNAc |
| *Pisum sativum* (pea) | PSL | Seed | Man/Glc |
| *Viscum album* (mistletoe) | Viscumin | Green | Gal/GalNAc |

| | | | |
|---|---|---|---|
| | | tissue | |
| *Phytolacca Americana* (pokeweed) | PWM | Root | (GlcNAc) |
| *Lycopersicon esculentum* (tomato) | | Fruit | (GlcNAc) |
| *Solanum tuberosum* (potato) | STL | Tuber | (GlcNAc) |

(Source:- Sharon and Lis, 2003,Lectins Second edition)

Besides seeds, lectins have been found in all kinds of vegetative tissue. The level of lectins in these tissues is variable, and exhibits seasonal changes. It is usually lower than in seeds, but can be as high as 30% of the total tissue proteins, e.g., the bulb lectins of garlic and ransom, or as low as 0.01% in leaves of the leek (Peumans et al.2000). Most plant tissues contain a single lectin, although occasionally two (or more) lectins differing in their sugar specificities and other properties are found in the same tissue (Peumans et al.2000). The most extensively studied plant with respect to the distribution of lectins in various tissues is *Dolichos biflorus*, wherein the leaves contain a lectin (DB58) homologous to the seed lectin (DBL), but with some differences in the fine specificity (Etzler, 1997). In addition, a root lectin (LNP) has been found in the same plant that is distinct from the lectin in its seed both in amino acid composition, molecular weight, isoelectric point and specificity (Etlzer et al. 1999).

Lectins isolated in India from plants

There are several reports of lectins isolated from plants in India, some of which have been purified, characterized and their properties have been assessed. (Islam et al,2009). D-galactose-binding lectin has been identified from the seeds of the

Indian coral tree *Erythrina variegata* having a leucoagglutinating property (Datta and Bashu 1981). Soon after in 1985, Khan et al purified a basic lectin from the seeds of winged bean *Psophocarpus tetragonolobus and termed it WBA l.* Using affinity chromatography followed by gel filteration, Dam et al (1997) purified two mannose-binding lectins. Since these were extracted from garlic bulbs, they were termed *Allium sativum* agglutinin, ASAI (25kDa) and ASAIII (48kDa). Kaur et al (2005) purified an N-acetyl-D-lactosamine-specific (LacNAc) lectin that was extracted from tubers of *Alocasia cucullata* and further purified by affinity chromatography. This lectin was reported to be a potent mitogen for human mononuclear cells at low concentrations but had growth-inhibition potential towards cancer cell lines at higher doses. A glucose-specific lectin was isolated from the roots of *Sesbania aculeate (*Biswa et al., 2009). This lectin bound with lipopolysaccharides isolated from different rhizobial strains indicating the plant's interaction with multiple rhizobial species.

**1.3b5**. Lectins identified from Animals

Practically all classes and subclasses of invertebrates examined have lectins. These includes crabs, snails, worms (helminths) (Greenhalgh et al., 1999; Hirabayashi et al., 1998), insects (Ingram & Molyneux, 1991; Kubo et al., 2001), mollusks and sponges (Muller et al. 1997). Lectins in invertebrates are present mainly in the hemolymph and sexual organs (Vasta, 1992). The best known invertebrate lectins are from the garden snail, *Helix pomatia*, from the body wall of the slug, *Limax flavus,* and from the serum of the horseshoe crab, *Limulus polyphemus* (Kilpatrick, 2000).

Many invertebrates contain multiple lectins, several of which have been purified and characterized. Examples are the four and three lectins, respectively, from cockroaches, *Periplaneta Americana* and *Blaberus discoidalis*, two from sea urchin, and three of sea cucumber (*Cucumaria echinata*) (Kilpatrick, 2000)

Although lectins from various plant and invertebrate sources have been known for many years, their presence in vertebrate tissues has been investigated first by Ashwell and Morell, (1977). They have described a hepatic-binding protein which has been implicated in the clearance of glycoproteins from plasma; in mammals, this binding protein is a β-galactoside-specific, integral membrane protein of large molecular weight, which can be solubilized by detergents but not by hapten saccharides and which requires divalent cations for binding activity. Subsequently, hepatic and reticuloendothelial cell-binding proteins which recognize mannose, N-acetylglucosamine, and fucose have been detected (Briles et al, 1979). A "lectin" from platelet plasma membranes has been described by Gartner et al. (1986) which is inhibited by free amino sugars and amino acids; the large external transformation-sensitive (LETS) protein or fibronectin also agglutinates erythrocytes and is inhibited by amines (Yamada, 1975). In addition to these membrane-bound lectins, several soluble lectins have been identified in vertebrate tissues. Mir-Lechaire and Barondes (1978) have reported a lectin from chick embryo muscle which is specific for N-acetylgalactosamine. The most widely occurring family of vertebrate lectins is that of the galectins, so called because they are glactose specific lectins. Many mammalian galectins have been described, as well as many additional ones from

other species, including birds, lower vertebrates, worms and sponges (Leffler, 2001; Rabinovich et al., 2002).

Lectins isolated from marine organisms in India

Dam et al., (1994) isolated a heparin-binding lectin, Anadarin MS, from the plasma of the marine clam *Anadara granosa*. This lectin agglutinated infective promastigotes of *Leishmania donovani* suggesting its role as a novel biochemical surface marker for the parasite. Devaraj et al (1995), isolated and characterized a high molecular weight glycoprotein from embryonated eggs of the mole crab, *Emerita asiatica.* A natural agglutinin was further purified and characterised from the serum of the hermit crab *Diogenes affinis* (Murali et al., 1999). The HA activity of *D. affinis* agglutinin was susceptible to inhibition by lipopolysaccharides from diverse Gram-negative bacteria, indicating a possible *In-vivo* role of this humoral agglutinin in the host immune response against bacterial infections. In addition to tissues, a novel lectin was purified from the coelomic fluid of the sea cucumber *Holothuria scabra* (HSL) and from the foot muscle of bivalve *Macoma birmanica*.

1.3c.Characteristics of lectins

Lectins have accordingly been defined as sugar-binding proteins of non-immune origin that agglutinate cells and precipitate polysaccharides or glycoproteins (Goldstein et al., 1980). The carbohydrate specificity of lectins has made them attractive proteins. This property has enabled them to become useful tools for various scientific purposes including detection and identification of blood groups and microorganisms, mitogenic stimulation of immune cells,

determination of carbohydrates in solutions, on macromolecules and cells, purification of glycoproteins and cell fractionation and as a tool for taxonomy. They have also been used as molecular probes for histochemical studies. Griffin et al. (1995) first demonstrated that *Codium fragile* lectin could be conjugated to gold particles and could be used as a histochemical reagent.

Lectins are multivalent i.e. they posses at least two sugar recognition sites which enable them to agglutinate animal and plant cells and/or to precipitate polysaccharides, glycoproteins, teichoic acids, glycolipids etc. (Leiner *et al*., 1986).

Lectins differ markedly in their sugar-binding specificity. A sequence participating in carbohydrate binding site of Concanavalin A, for instance, is poorly conserved in other lectins. However, though termed anti-carbohydrate antibodies, there are differences such as:

a) Antibody synthesis is inducible whereas lectin synthesis is not.

b) Antibodies can be produced against every determinant, lectins only against a defined set of sugar molecules.

c) All antibodies are a single class of protein family whereas lectins belong to different protein families.

d) Some lectins require metal ions as chelating agents while antibodies do not.

e) Antibodies are structurally similar, whereas lectins are subunits, one or more of which carries a sugar-binding sites.

Studies on marine algal lectins reveal a proteinaceous nature similar to those from land plants, but different in some of their properties. They have generally lower molecular masses than most land plant lectins and are more specific for complex oligosaccharides or glycoproteins.

The agglutinating and precipitating activities of lectins are similar to those of antibodies. They can likewise be specifically inhibited by low molecular weight compounds (haptens), which in case of lectins are sugars or sugar-containing ones. Many lectins are found in plants, microorganisms and viruses, which are not capable of an immune response. Being specific for certain blood groups, lectins can also prove useful in blood typing, since there is natural non-availability of anti-O antibodies (Kocourek, 1986). They vary, however, in size, amino acid composition, metal requirement, domain organization, subunit number and assembly, as well as in their three dimensional structure and in the constitution of their combining sites. In their structure diversity, lectins are akin to enzymes, although they are devoid of catalytic activity (Sharon and Lis, 1990). In spite of this variation, they can be grouped in families of homologous proteins, the largest and best characterized of which is that of the legume lectins (Van Damme et al., 1998).

**1.3.d**. Biological properties of lectins

Some important biological properties of lectins are as follows:-

I. Agglutination of cells is the easiest way to detect lectins. The ability of lectins ability to agglutinate cells distinguishes them from other sugar

binding macromolecules such as glycoside & glycotransferases (Goldstein et al., 1980).

II. Lectins trigger quiescent, non-dividing cells into a state of proliferation (Trevin et al., 1986).

III. Mitogenic activity of lectins was first reported from red kidney beans *Phaseolus vulgaris* (Nowell, 1960).

IV. Lectins co-stimulate T-cell proliferation along with cytokines (Gollob et al., 1995).

V. Wheat gram lectin (Kurisu et al., 1980) & few others like *Griffonia simplicifilia* (Maddox et al., 1982) posess the ability to mediate carbohydrate-specific binding of mouse macrophages and tumor cells and to induce killing of tumor cells by macrophages.

VI. Lectins mediate binding and phagocytosis of target cells (Sharon; 1984). Thus the binding of Con A to the surface of macrophages mediates the attachment of bacteria to the macrophages, although no phagocytosis of bacteria was observed (Allen et al., 1974).

VII. Con A, a wheat gram lectin and some other lectins mimic the effect of insulin on adipocytes such as stimulation of lipogenesis, transport, oxidation and inhibition of lypolysis (Shechter et al., 1981). Insulinomimetic activities were also observed *in vitro* (Margaret et al., 2000).

VIII. Cytotoxic lectins have been isolated from an extract of *Viscum album*. Tumor cells that was treated with this lectin showed typical apoptotic cell death, with apparent DNA fragmentation (Ichiro Azuma *et al*., 1998).

IX. Apoptosis activity was shown to be blocked by the addition of $Zn^{++}$ or inhibition by $Ca^{++}/Mg^{++}$ dependent endonucleases in a dose dependent manner (Yoon et al., 1998).

X. The lectin designated Hypnin A, from red alga *Hypnea japonica* inhibited platelet aggregation in a dose dependent manner (Matsubara *et al*., 1996).

Lectins can bind reversibly with free sugars or with sugar residues of polysaccharides, glycoproteins, or glycolipids (Goldstein and Poretz, 1986). Both the lectin and its ligand can be free or bound. Lectins can bind to glycoprotein receptors on cell membranes and this binding is necessary for cell agglutination.

### 1.4 Lectins from Marine algae

Marine natural products have attracted the attention of biologists and chemists, the world over, for the past few decades. As a potential source for new drug discovery, marine natural products have attracted scientists from different disciplines such as different branches of chemistry, pharmacology, biology and ecology. This interest has led to the discovery of almost 8,500 marine natural products to date and many of the compounds have shown very promising biological activity (Faulkner et al., 2000). The ocean is also considered to be a great source of potential drugs (Bhakuni and Ravat, 2005).

The increasing interest in marine natural product's chemistry has led to the discovery of new biologically-active compounds and marine algae have been

subjected to increasing study for this purpose (Amico, 1995). They have been reported to contain high amounts of water-soluble macromolecules such as polysaccharides, proteins, glycoproteins and other less polar compounds of low molecular weight, some of them exhibiting particular biological properties *in vitro*.

Though lectins have been isolated and characterized from various biological sources, mainly land plants, there is a limited amount of information available about algal lectins in comparison with those from higher plants and invertebrates. However, considering their particular characteristics, marine algal lectins appear to be a potential tool for biochemical and biomedical applications.

Biochemical experiments based on agglutinating tests have revealed the presence of hemagglutinating activity in many algal extracts against erythrocytes from several animal species. In most studies this hemagglutinating activity is referred to the presence of proteins or glycoproteins having specificities for carbohydrate structures binding selectively to red blood cells and microorganisms. Compared with lectins from land plants, discovered as way back as in 1888, the occurrence of lectins from marine algae, however, was first reported in 1966 by Boyd exhibiting the protein in the sap of some marine algae (Boyd et al., 1966). Following this pioneering work, other workers have demonstrated that lectins are present in many algal species (Blunden et al., 1975; Rogers et al., 1980; Munoz et al., 1987; Hori et al., 1981 & 1988 Dalton et al., 1995; Freitas et al.,1997; Kakita et al., 1999; Calvete et al., 2000; Benevides et al., 2001; Sampaio et al., 2002; Wang et al., 2004; Nagano et al., 2005; Kim et al., 2006; Yoon et al., 2008; Dinh et al., 2009; Han et al., 2010;

Molchanova et al., 2010; Jung et al., 2010; Sato et al., 2011). Later workers have extended this information by studying a wide range of species across the globe. Marine algae have been screened in surveys at/in Puerto Rico (Boyd et al., 1966), England (Blunden et al., 1975, 1978; Rogers et al., 1980), Japan (Hori et al., 1981, 1988), Spain (Fábregas et al., 1985, 1992), United States (Chiles and Bird, 1989; Bird et al., 1993), Brazil (Ainouz and Sampaio, 1991; Ainouz et al., 1992; Freitas et al., 1997), Vietnam (Dinh et al., 2009) and Korea (Kim et al., 2006; Han et al., 2010, Jung et al., 2010). More than 200 algal species have so far been reported to contain hemagglutinins. The first agglutinin to be isolated and characterized from marine algae was by Rogers et al., (1977).

Further, most of marine algal lectins do not require divalent cations for their biological activity (Rogers and Hori, 1993). They occur mainly in monomeric form and have a high content of acidic amino acids with isoelectric points from 4 to 6. Although several studies on lectins from marine algae have been reported (Fabregas, 1998; Oliveira et al., 2002), few lectins from algae have been characterized in detail.

Lectins show differential agglutination property with RBCs isolated from different sources as well as with differential processing of RBCs. Based on the hemagglutination properties of lectins, Boyd et al., (1996) and Blunden et al*.,* (1975) assayed blood group -specific lectins. In 1994, Dalton *et al.* investigated protein extracts from 9 species from green & red marine macro algae for their ability to agglutinate human blood groups A, B, O, sheep and rabbit erythrocytes. Chiles and bird (1989) investigated 15 species of algae, of which

all showed agglutination against rabbit erythrocytes but only 7 showed agglutination activity of human erythrocytes. Hori (1990) isolated 12 different lectins from four species of algae. Of these, four were observed to agglutinate trypsinized rabbit erythrocytes strongly and their activity was inhibited by glycoproteins. All specimen studied by Bird et al. (1993) were found to agglutinate either sheep or rabbit erythrocytes. Trypsinization and $Ca^{++}$ enhancement of agglutinating lectin activity were shown by Aleli et al. (2000).

The progress in developing applications for algal lectins has been limited mainly due to three factors. First, very few scientists have been focused on studying algal lectins. This has led to a paucity of information concerning characteristics which contribute to their properties *In vitro* and their possible functions *In vivo*. Secondly, marine macro-algae for lectin research are collected from their natural habitat and thus species which contain interesting lectins may occur in remote geographical areas or may be relatively rare plants, both of which contribute to collection difficulties apart from ecological and conservation concerns. Third, algal extracts have low concentrations of lectins (Rogers et al., 1986), making detection and production of sufficient purified lectin for detailed biochemical characterization a difficult endeavor.

The first two of these problems are now being resolved. Many researchers have made important contributions by their observations on lectins from different marine algae. The availability of marine algal species known to contain interesting lectins may be improved by cultivation of relevant species (Rogers et al., 1982). Also cloning the lectin genes may results in the production of recombinant lectins.

It is in the third area i.e. marine algal lectin detection, purification and characterization, that most progress has been made (Rogers and Hori, 1993). Among them, hemagglutinins (lectins) have been isolated from about 50 species. Characterization studies reveal that many algal lectins, especially from red algae, share the common characteristics of low-molecular size, monomeric form, having no affinity for monosaccharides and being more specific for complex oligosaccharides or glycoproteins and thermo stable. Further, most of marine algal lectins do not require divalent cations for their biological activity (Hori et al., 1990), indicating that algae are also a good source of new lectins. They occur mainly in monomeric form and have a high content of acidic amino acid, with isoelectric points from 4 to 6 (Costa et al., 1999).

The first amino acid sequence of a lectin from marine algae was reported by Calvete et al., (2000). To date, amino acid sequences of only five marine algae lectins have been reported. These are from red alga *Bryothamnion triquetrum* (Calvete et al., 2000), *Hypnea japonica* (Hori et al., 2000), *Ulva pertusa* (Wang et al., 2004) and *Hypnea cervicornis (*HCA) and *Hypnea musciformis* (HML) (Nagano et al., 2005). Characterization of these lectin genes may help researchers to further understand the difference between terrestrial plant and marine algal lectins.

### 1.5. Applications of lectins

Lectins are widely employed in research for diverse purposes, primarily those in which detection, identification and functional evaluations of carbohydrates is

needed, and are also making a mark on medicine (Gabious et al., 1993; Goldstein et al., 1997; Rhodes et al., 1998). They offer many advantages, including ready availability, distinct specificity, and high solubility. Thus, lectin binding has frequently been used to demonstrate that the membrane receptors for many hormones, growth factors, neurotransmitters and toxins are glycoconjugates. Studies with lectins have been largely responsible for the realization that carbohydrates play a key role in cell-recognition (Sharon and Lis, 1989; Sharon, 1993), and for expanding the understanding of tissue-bound carbohydrates in histology and histopathology (Ewen et al., 1998). A new and promising application is lectin replacement therapy for the treatment of patients suffering from a lectin deficiency disease (Valdimarsson et al., 1998; Kilapatrick, 2002b)

In a nut-shell, the varied and major applications of lectins have been summarized by Sharon and Lis, (2003) as below:

- Cell identification and separation (Yarema and Bertozzi, 2001)
- Detection, isolation and structural studies of glycoproteins
- Investigation of carbohydrates on cells and sub-cellular organelles; histochemistry and cytochemistry. Selection of lectin-resistant mutants
- Studies of glycoprotein biosynthesis
- Diagnosis and targeting

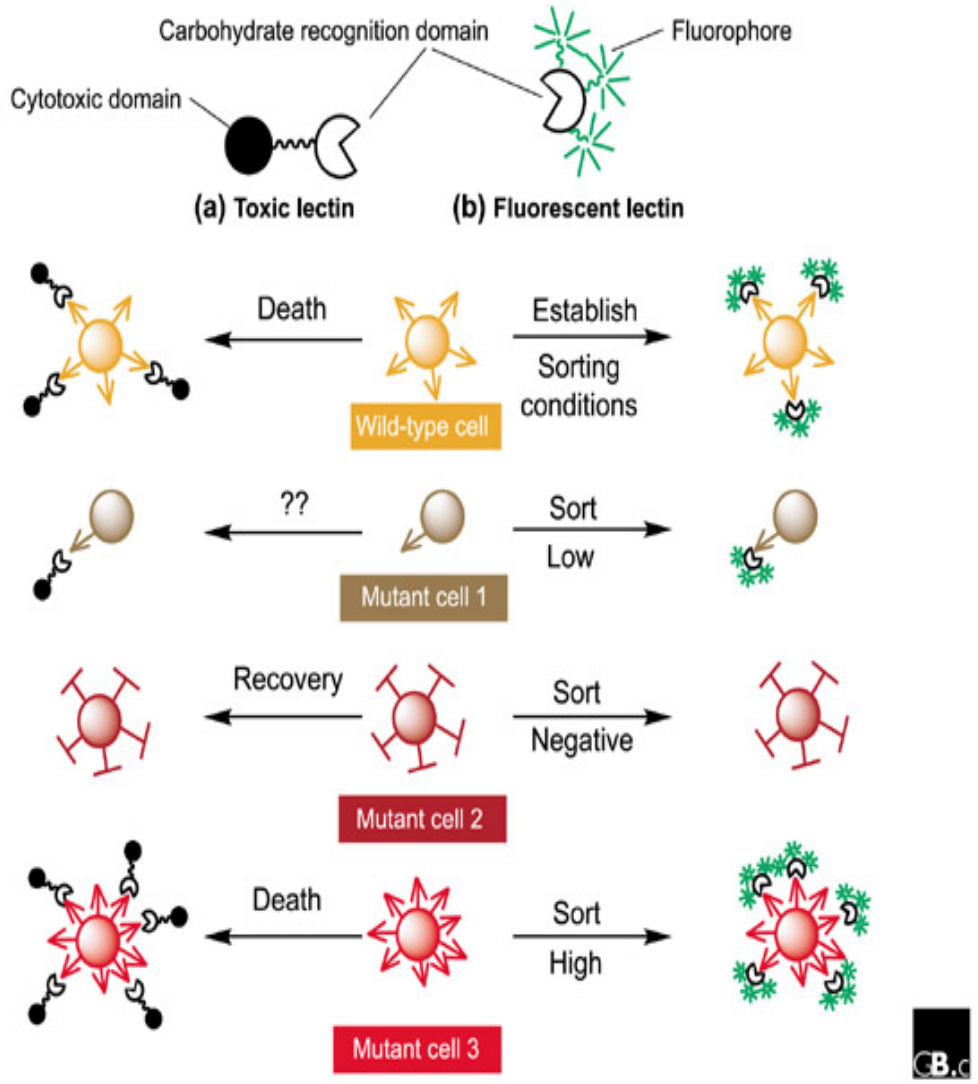


Fig 1.1:- Lectin-based cell-selection strategies ((Yarema and Bertozzi *Genome Biology* 2001).

- Mitogenic stimulation of lymphocytes (Kilpartrick, 1998.) Mitogenic lectins mimic the action of antigens on lymphocytes, except that they activate a large proportion (as much as 70-80%) of the cells, whereas antigens stimulate only specific clones. Because of their ability to stimulate multiple lymphocyte clones, lectins are classified as polyclonal mitogens.
- Evaluation of immunocompetence

Of the many mitogenic lectins, only Concanavalin-A, PHA and PWM are employed in clinical laboratories as an easy and simple means to assess the immunocompetence of patients suffering from a diversity of diseases and to monitor the effects of various immunosuppressive and immunotherapeutic manipulations (Di Sabato, 1987; Kilpatrick, 1998).

- Karyotyping
- Bone marrow transplantation
- Enzyme replacement therapy
- Construction of Immunotoxins

Attempts are being made to take advantage of the toxicity of ricin for therapeutic purpose, through the construction of immunotoxins (Ghetie et al., 2001). These are hybrid molecules, made by covalently linking a toxin (usually ricin) to monoclonal antibodies against the cells that one wishes to kill. The antibodies guide the immunotoxin to the target cells, which are then eliminated by action of the toxin.

Algal lectins also have been studied for their properties..from various biological functions such as anti-tumor, mitogenic, and anti-virus activities (Hori et al., 2007). As a practical example, strong anti- HIV lectins have recently been isolated ((Boyd et al., 1997; Botos et al., 2002; Bewley et al., 2004) and characterized from blue-green algae (cyanobacteria); Sato et al., 2007) and a red alga (Mori et al., 2005). Studies performed with extracts of several marine algae demonstrated that these extracts possess antinociceptive activity (Vieira et al., 2004; Neves et al., 2007, Figueiredo et al., 2010). Lectins from the *Eucheuma serra*

algae are generally obtained in the high yields. ESA-2 shows various biological activities such as mitogenic activity for mouse and human lymphocytes (Kawakubo et al., 1997), in vitro growth inhibition of tumor cells (Suzuki et al., 2000) and antibacterial activity (Liao et al., 2003). Algal lectins play a pivotal role in cell-cell recognition and thus consequently find application in the fields of immunology and wound healing process (Kim et al., 2001, 2006, and 2007)

Although considerable progress has been made in understanding the biochemical character of lectins, little is known about their biological role in nature. Hori et al. (1988) suggested that lectins may play a common, but as yet unknown, physiological function in marine algae.

Thus, lectins serve as useful tools and markers in biological research with unlimited use. Thus, algal lectins are interesting targets for basic research into lectins and their applications.

## Aim

The main objective of the present study was to isolate lectins from untapped marine algae along the coast of Goa, India. Collection and identification of the marine algae were the preliminary objective of the present study. Followed by isolation of collected marine algae and confirmation of hemagglutination activity were the core design of this study. Selection of species for further purification, biochemical characterizations of these lectins were studied. This work include the biotechnological potential of the purified lectins towards cancer cell lines, antimicrobial and antioxidant activity. This work, therefore, been done with the following objectives in mind.

## Objectives of the study

The present study was initiated with the following objectives:

1) To collect and identify marine macro algae commonly found along the shores of Goa.
2) To screen for prospective hemagglutinins and selection of two species that show highest activity in terms of agglutination.
3) Extraction of a hemagglutinin from each of the algae selected.
4) Purification of lectin from both the species.
5) Biochemical Characterization of both the lectins.
6) Exploring the potential biotechnological application of the lectin.

This work carried out and presented in this chapter meets objective number one of the entire plan of study. This chapter describes the collection and identification of marine macroalgae that were collected from the shores of Goa. Since identification was done on the basis of references, only this chapter is appended with a bibliography.

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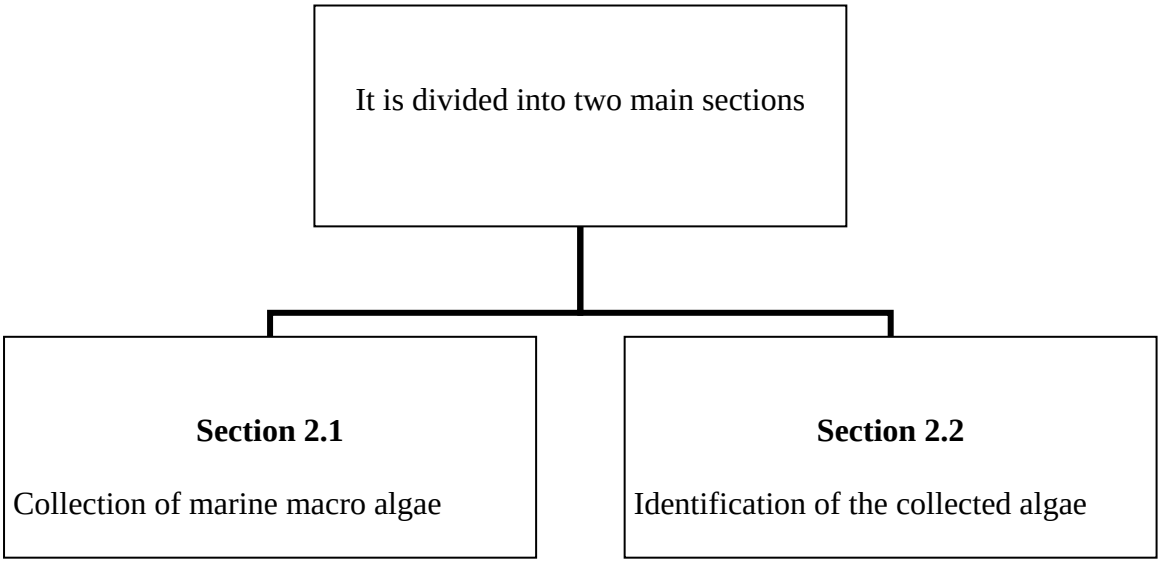



## Section 2.1:- Collection of marine macro algae

### 2.1a. Introduction

Seaweeds are macroscopic algae found attached to the bottom in relatively shallow coastal waters. They grow in the inter-tidal, shallow and deep sea areas up to 180 meter depth and also in estuaries and back waters on solid substratum such as rocks, dead corals, pebbles, shells and other plant material (Smith, 1944). Benthic marine algae are the group of plants that live either in marine or brackish water environment. Like the land plants, seaweeds contain photosynthetic pigments and with the help of sunlight and nutrients present in

the seawater, they photosynthesize and produce food. Seaweeds are found in the coastal region between high tide to low tide and in the sub-tidal region up to a depth where 0.01 % photosynthetic light is available (Dhargalkar & Kavlekar, 2004). Plant pigments, light, exposure, depth, temperature, tides and the shore characteristics combine to create different environments that determine the distribution and variety among seaweeds**.**

Seventy percent of the Earth's surface is covered by oceans and seas. Marine environment, which occupies 70% of the earth's surface, is a rich natural resource and its products can be utilized by mankind. Seaweeds form one of the important living resources grouped under three divisions namely, Chlorophyceae (green algae), Phaeophyceae (brown algae) and Rhodophyceae (red algae). About 624 species have been reported in India with a potential of 77,000 tons (wet weight) per annum. The red seaweeds contribute 27.0%, brown 0.2 % and others 72.8 % (Rao and Mantri 2006).

Marine benthic flora of the Goa coast comprises of 31 families, 53 genera and 84 species. Otherwise considered as the paradise of the east, Goa has a coast line of 120 km long. There are seven estuaries along the Goa coast that open into the Arabian Sea. The coastal region of Goa harbors a variety of ecologically and economically important marine macro algae (Untawale et al, 1983).

In collecting marine organisms, it is important that certain criteria have to be taken into consideration before collection of the samples, such as:

- Depth of the ocean where the sample may be exposed and distributed.

- The tide level for access to sample collection if source is from inter-tidal zone (as with marine algae).

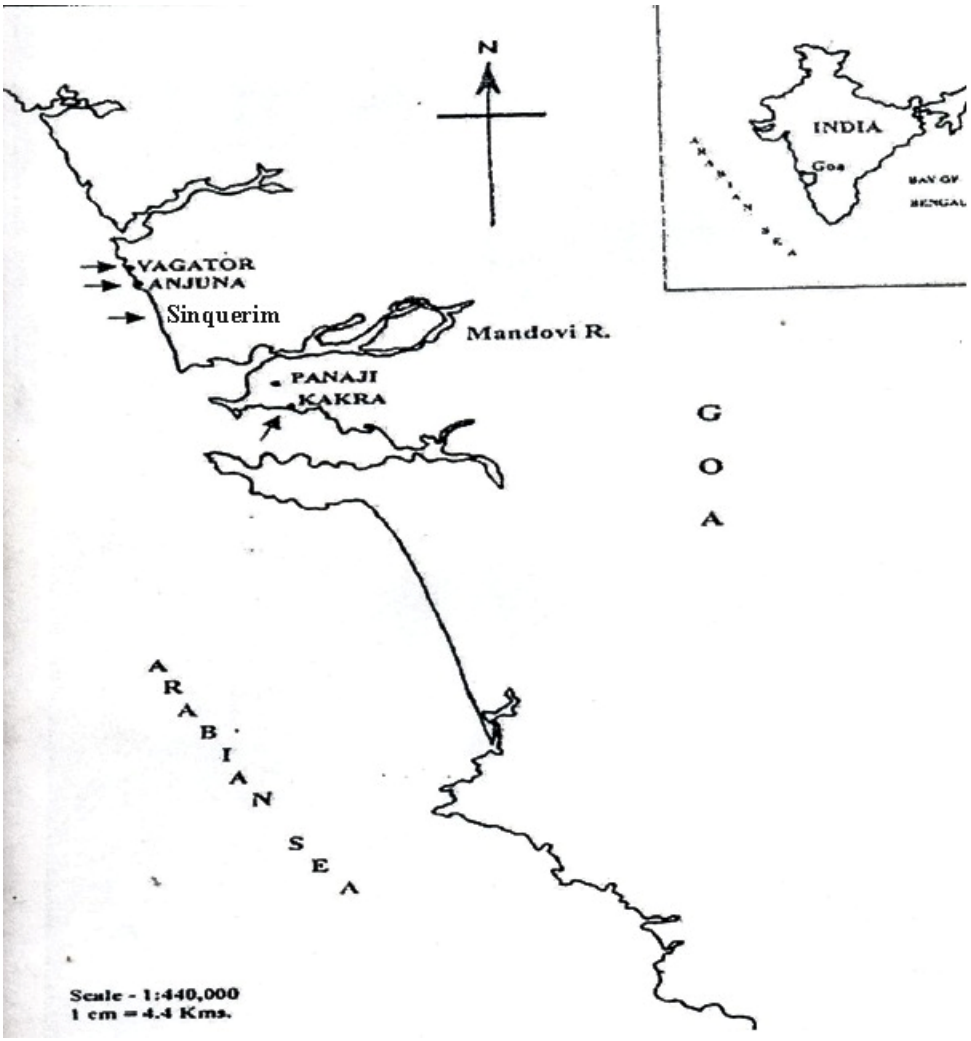



Fig: 2.1- An outline map of Goa showing the sites of sample collection.

- Rocky beaches are preferred for collecting samples of the inter-tidal region due to accessibility of the substratum on which the sample may be anchored.
- Lowest tide levels facilitate collection of samples wherein the inter-tidal organisms are well exposed and easier to collect.
- In order to extract sufficient amount of product of interest, large conspicuous and abundant species may be preferred to small inconspicuous and rare ones.

- Near- shore species are generally preferred to those occurring offshore for economical reasons as well as the ease of collection.
- It may be preferred to work or study organisms that are easily available through the proposed period of study.

**2.1b. Materials and Methods:-**

Glass double distilled was taken from our laboratory, zipper bags were obtained from market.

2.1b (I) Collection of marine macro algae

Since marine algae are in bloom mainly through the monsoon period, samples were collected during the lowest tide levels, so that submerged species were exposed. The dates and time of collection of samples are detailed in **Table 2.1** and samples were collected referring to the tide table obtained from the National Institute of Oceanography, Dona Paula, Goa.

The samples, which were distinctly exposed on rocky surfaces, (Figs 2.3a, b, c & d), were collected from inter-tidal zone along the beaches of Kakara, Sinquerim, Vagator and Anjuna (Fig 2.1) along the Coast of Goa (West coast of India) during the monsoon and post monsoon period. If attached to rocky substratum, (Fig 2.2), a scraper was used else it was plucked out.

After washing off all associated sediments in sea water, the collected samples were transferred into polythene bags containing seawater. On returning to the laboratory, collected samples were rinsed under running tap water to wash off

any epiphytes that may be present. After a thorough wash in distilled water, the algae were placed in zipper polythene bags and stored at -20°C until further use.

Fig 2.2:- Carpet of algae on a rocky substratum

a)Sinquerim

b) Anjuna

c) Kakara

d) Vagator

Figs: 2.3a, b, c & d- Collection of marine algae from rocky shores

**2.1c. Observations and Identification of marine macro algae**

Altogether, twenty one species of marine algae were collected from the various beaches of Goa. Seven were green (**X3, X5- X8, X16** and **X17**), nine were red (**X4, X10, X12- X14, X18- X21**) and five were brown (**X1, X2, X9, X11** and **X15**) algae.

Samples were collected mainly during monsoon and post monsoon period based on observations of rich blooms during this period. Thus as detailed in table 2.1, collection was done from the month of September when there was a respite in the monsoon up to February. The sites of collection were restricted to within 32 km road distance of Goa University.

It was observed that the blooms of algae were seasonal in that with the onset of monsoons, green algal blooms dominated the shores of the sampling sites in reference. These were basically blooms of Enteromorpha and Chaetomorpha genus except for one kind of Porphyra.

As the monsoons progressed i.e. into the month of August, Ulva and Caulerpa species were observed to be growing and these two species were seen to persist until November and March, respectively.

After the monsoon receded, shores were observed to sprout Red and brown algae. Growth of Red algae dominated and was luscious in November and December. This was followed by an outburst of growth of brown algae.

**Table 2.1:- Details of sample collection**

| **Site of collection** | **Date of collection** | **Time of collection (hrs)** | **Tide level (mts)** | **Samples collected** |
|---|---|---|---|---|
| Kakara | 2/09/2005 | 16.45 | 0.86 | ***X3, X5- X8, X10, X11& X14*** |
| Anjuna | 1/11/2005 | 16.00 | 0.37 | ***X1-X5, X9 & X15*** |
| Anjuna | 26/11/2005 | 16.00 | 0.37 | ***X9, X15 - X21*** |
| Vagator | 26/11/2005 | 16.30 | 0.37 | ***X12, X20 & X21*** |
| Anjuna | 7/9/2006 | 16.25 | 0.57 | ***X3, X5,-X8 & X14*** |
| Kakara | 8/9/2006 | 15.00 | 0.32 | ***X10-X12 & X14*** |
| Vagator | 22/09/2006 | 17.02 | 0.59 | ***X3, X5, X19 & X21*** |
| Sinquerim | 22/09/2006 | 17.30 | 0.59 | ***X7 & X13*** |
| Anjuna | 19/1/2007 | 16.20 | 0.52 | ***X1, X2, X9, X15-X18*** |
| Anjuna | 22/09/2007 | 17.30 | 0.59 | ***X4-X6, X8, X9 & X12*** |
| Anjuna | 12/10/2007 | 16.30 | 0.11 | ***X1, X2, X5, X8, X9 & X15*** |
| Vagator | 12/10/2007 | 17.00 | 0.11 | ***X18, X20 & X21*** |
| Anjuna | 9/1/2008 | 16.25 | 0.29 | ***X16-X18 & X20*** |
| Vagator | 9/1/2008 | 17.05 | 0.29 | ***X16*** |
| Kakara | 23/01/2008 | 17.26 | 0.18 | ***X16*** |
| Anjuna | 12/09/2009 | 13.05 | 0.27 | ***X5*** |
| Anjuna | 21/12/2009 | 14.35 | 0.39 | ***X16*** |
| Anjuna | 09/01/2010 | 17.05 | 0.41 | ***X16*** |
| Anjuna | 17/2/2010 | 16.35 | 0.87 | ***X16*** |

Fig 2.4a: ***Chaetomorpha media***

.

Fig 2.4b: ***Ulva fasciata***

**Section 2.2 Identification of the collected marine algae**

Identification was done on the basis of i) morphological and macroscopic observations ranging from occurrence and colour ii) observations recorded on algae database (http://www.niobioinformatics.in/seaweeds/system) and referring to standard, recommended literature indexed in Bibliography. The identification was confirmed from a resource faculty in the Dept. of Botany, Goa University.

Salient observations and identification is reported below:

**GREEN ALGAE**

**1)** ***Chaetomorpha media*** (Fig 2.4a)

**X3** (fig 2.4a): - The algae were found attached to the hard rocky substratum by a basal holdfast. It was dark green in colour. The plant was tufted, bush-like, filamentous and un-branched. It was erect, stiff and rigid below and flexuous above. The plant was about 10 cm in height. This specimen was identified as ***Chaetomorpha media.***

Locality: - It was found in abundant bloom during the months of August to October at Kakara, Anjuna and Vagator beaches.

**2)** ***Ulva fasciata*** **(**fig 2.4b**)**

**X5** (fig 2.4b): - This alga was found attached to rocky substratum in the littoral region. It was bright green and its thallus had undulated margins, lobes were linear and fronds delicate. Basal part of the frond narrowed into a short stalk, expanding into an attaching disc. On the basis of above morphological characters and reference, the specimen was identified as ***Ulva fasciata***.

Locality: - It was observed to be in bloom through the months of August to October at Anjuna, Vagator and Kakara beaches.

2.4c: ***Enteromorpha flexuosa***

2.4d: ***Enteromorpha Compressa***

**3)** ***Enteromorpha flexuosa*** (Fig 2.4c)

**X7** (Fig 2.4c):- Plant was found attached to the rocky substratum near the littoral region. It was yellowish-green, up to 10 cm in length and attached to the substratum by a small round basal disc. The thalli were grass-green colour consisting of erect branches. The thallus was strap-shaped and filaments were branched. This specimen was identified as ***Enteromorpha flexuosa.***

Locality**: -** It was found in abundance from the months of June to September at Kakara, Anjuna and Sinquerim beaches.

**4)** ***Enteromorpha compressa*** (Fig 2.4d):-

**X6** (Fig 2.3d):- Plant was found attached to rocky substratum near high-tide region. It was dark green in colour. The plant body was tubular, more or less compressed and constricted. Thallus was simple, attached to the substratum with the help of primary attaching cell and cells of thallus were small and round. Filaments were narrow and tubular at base, compressed above. Several branches arose from the base with the branches being similar to the main fronds and upto 10.5 cm long. On the basis of above mentioned morphological characters and reference, specimen was identified as ***Enteromorpha compressa***.

Locality: - This alga was found in abundance through the months of June to September at Kakara and Anjuna beaches.

2.4e: ***Enteromorpha intestinalis***

2.4f: ***Caulerpa peltata***

**5)** ***Enteromorpha intestinalis*** (Fig 2.4e)

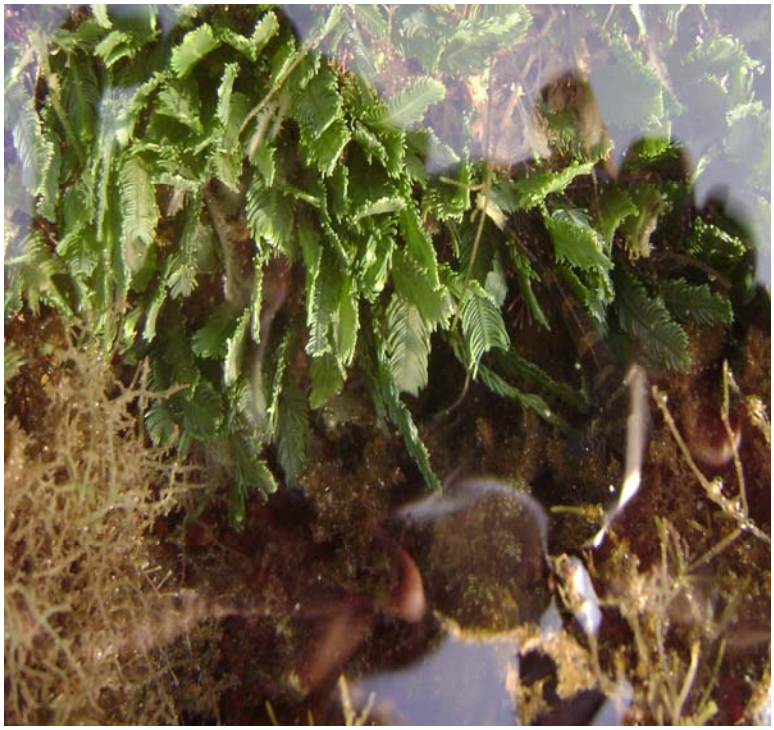
**X8** (Fig 2.4e):- This plant was found attached to the rocky substratum near high-tide region. It was light green to dark green in colour. The thalli were up to 17 cm long, bright green when fresh and whitish when dry. It was made up of many erect, thin tubular, hollow branches proliferating from a common slender stripe attached to the substratum by a small and discoid hold-fast. The branches were tapered below, inflated above, irregularly twisted and length up to 30-35 cm. On the basis of above mentioned morphological characters and reference, specimen was identified as ***Enteromorpha intestinalis*** .

Locality: - This alga was found in abundance through the months of June to September at Kakara and Anjuna beaches.

**6)** ***Caulerpa peltata*** (Fig 2.4f)

**X17** (Fig 2.4f):- It was located in rock puddles in the mid-littoral zone. It was light green to yellowish green in colour. Miniature, round, grape-like clusters were observed on the branchlets that stemmed upright from the rhizome. It was flattened on the tip, assimilators were erect, simple or branched and 4-6 cm high. On the basis of above mentioned morphological characters and reference, specimen was identified as ***Caulerpa peltata***.

Locality: - This specimen was observed to be rare being localised only at one of the sites i.e. Anjuna shores. Moreover, it was in bloom in the cooler months of November to February.



2.4g: ***Caulerpa sertularioides***

2.4h: ***Gracilaria corticata***

**7)** ***Caulerpa sertularioides*** (Fig 2.4g)

**X16** (Fig 2.4g):- It was light green to yellowish-green in colour. The thalli were grass-green in colour consisting of erect, feather-like branches, which were about 4.5 cm long, branched, cylindrical rhizome-like stolons. Branches were creeping on substrata and anchored by well branched rhizoids. This specimen was identified as ***Caulerpa sertularioides***.

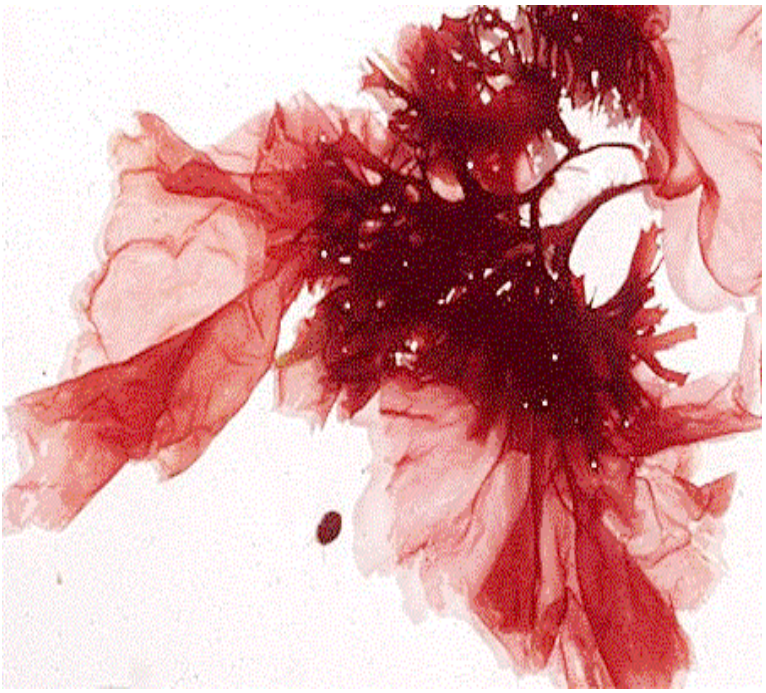
Locality: - This alga was in bloom from the months of November to February at Anjuna, Vagator and Kakara beaches. It grew in rock puddles in the mid-littoral zone.

***RED ALGAE***

**1)** ***Gracilaria corticata*** (Fig 2.4h)

**X4** (Fig 2.4h):- it was growing in the mid-littoral region and pale red in colour. The thallus was somewhat thickened and cartilaginous with entire margins compressed and flat. Plants were erect and showed dichotomous branching. Thallus was growing in tufts of flattened fronds, 2-3 cm in height, rigid and cartilaginous. Fronds were repeatedly dichotomously branched with narrow segments. This specimen was identified as ***Gracilaria corticata***.

Locality: - This alga was located at Anjuna beach through the months of August to November.



2.4i: ***Porphyra vietnamensis***

2.4j: ***Gelidium pusillum***

**2)** ***Porphyra vietnamensis*** (Fig 2.4i)

**X14** (Fig 2.4i):- It was growing attached to rocky substratum and was reddish-purple to pinkish-purple in colour. Blades were monostomatic, more or less lacerated from a common base into several bladelets. Marginal portion was undulated, edge dentate and length up to 30 cm. This specimen was identified as ***Porphyra vietnamensis***.

Locality: - This alga was in abundance in the months of June to September at Kakara and Anjuna beaches.

**3)** ***Gelidium pusillum*** (Fig 2.4j)

**X19** (Fig 2.4j):- It was growing attached to rocky substratum and was pale reddish in colour. It was observed to have a small plant, solitary or forming loose tufts, creeping below and giving rise to erect blades. Plants were 5-15 mm long lengthwise from the axis. This specimen was identified as ***Gelidium pusillum***.

Locality: - This sample was found in abundance from the months of September to December at Anjuna and Vagator beaches.

2.4k: ***Acanthophora specifera***

2.4l: ***Hypnea musciformis***

**4)** ***Acanthophora specifera*** (Fig 2.4k)

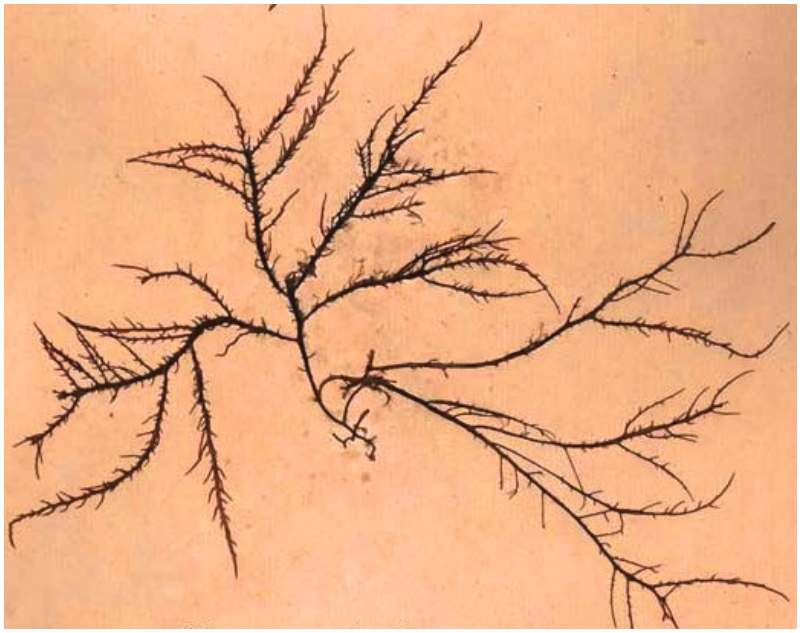
**X18** (Fig 2.4k):- It was found attached to rocky substratum. It was red to deep maroon in colour. The plants were bushy and attached to the substratum by means of a small disc-shaped hold-fast. Its branches were spirally arranged along the main axes. The branchlets were spinous and irregular. This red alga was identified as ***Acanthophora specifera.***

Locality: - It was in bloom from October to December at Anjuna and Vagator beaches.

**5)** ***Hypnea musciformis*** (Fig 2.4l)

**X20** (Fig 2.4l):- It was growing attached to rocky substratum. This alga was greenish when young and short, but purplish- brown when long. A common epiphytes was easily recognized on the inflated and hooked tips of the main branched and the dense clothing with simple and slender branchlets. Length of the whole plant was about 10 cm. This specimen was identified as ***Hypnea musciformis***.

Locality: - It was found in abundance from the months of October to December at Anjuna and Vagator beaches.



2.4m: ***Hypnea valentiae***

2.4n: ***Grateloupia lithophila***

**6)** ***Hypnea valentiae*** (Fig 2.4m)

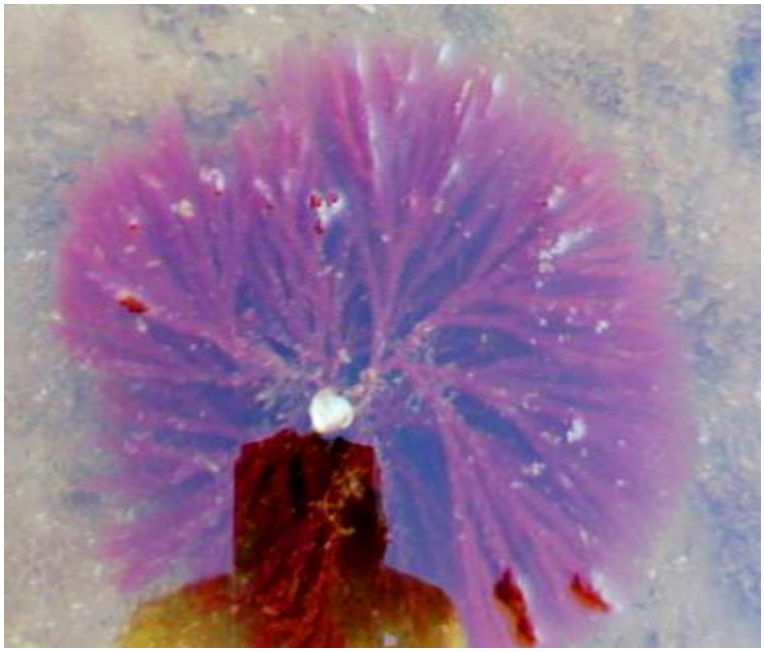
**X21** (Fig 2.4m):- It was growing in the mid-littoral region and was dark brown to black in colour. The plants were bushy, robust and erect with their main axis cylindrical and thick, arising from a callus, disc-like structure. The branches were irregularly arranged around the main axis. Branches were simple and fusiform (thread-like) but occasionally forked and were distinctly oriented at right angles to the axis. These algae were up to 35 cm long and gradually tapering to an acute tip. This brown alga was identified as ***Hypnea valentiae***.

Locality: - It was growing in full bloom from the months of October to December on Anjuna and Vagator beaches.

**7)** ***Grateloupia lithophila*:-**

**X13** (Fig 2.4n):- It was growing in the mid-littoral region. This alga was dark purple to pale reddish in colour. It was observed to have spine-like leaf, tough, often ramified and hard. Plants occurred as dense tufts on hard substratum. Thallus was flat, simple or irregularly divided and above 10-15 cm long. Erect thallus was 15-75 cm in height. This red alga was identified as ***Grateloupia lithophila***.

Locality: - This alga was not commonly found and was located only at Sinquerim beach from September to November. This was indeed a rare alga.



2.4o: ***Scinaia hatei***

2.4p: ***Amphiroa fragillissima***

**8)** ***Scinaia hatei*** (Fig 2.4o)

**X10** (Fig 2.4o):- It was growing in the inter-tidal and sub-tidal region and was dark purple and reddish in colour. It was observed to have risen from discoid holdfast and dichotomously branched. On the basis of above mentioned morphological characters and reference, specimen was identified as ***Scinaia hatei***.

Locality: - This alga was not commonly found along the coast line/examined except at Kakara during the months of August to October.

**9)** ***Amphiroa fragillissima*** (Fig 2.4p)

**X12** (Fig 2.4p):- It was growing in the mid-littoral region. This alga was light purple in colour. The thalli were generally cream in colour, forming thick hemispherical clumps on the substratum and always intermingled with other seaweeds. Branching was dichotomous to trichotomous, divaricates forming acute angles. This specimen was identified as ***Amphiroa fragillissima.***

Locality: - Rich blooms were found at at Kakara and Anjuna beaches from September to November.

2.4q: ***Dictyota dichotoma***

2.4r: ***Padina tetrastromatica***

**BROWN ALAGE**

**1)** ***Dictyota dichotoma*** (Fig 2.4q)

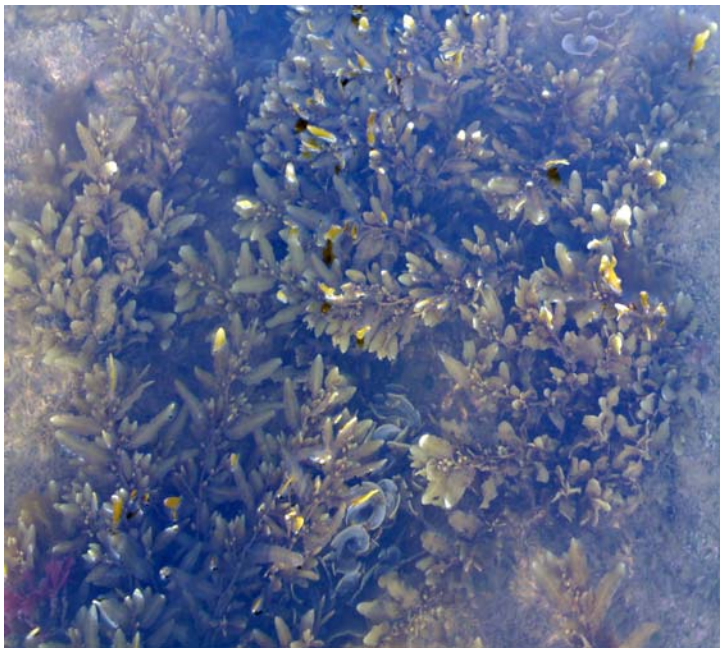
**X1** (Fig 2.4q):- It was attached to rocky puddles in the mid-littoral region. This alga was yellowish brown in colour. It was observed to have ribbon-like basal portion forming a small cuneate disc and upper portion regularly branched dichotomously. The plant was flat, branched and up to 6-12 cm long. This specimen was identified as ***Dictyota dichotoma.***

Locality: - It was found in abundance from the months of November to February at Anjuna beach.

**2)** ***Padina tetrastromatica*** (Fig 2.4r)

**X2** (Fig 2.4r):- It was growing in rock-puddles of the upper and mid-littoral regions. This alga was brown in colour. Plants were erect in several clumps and several blades arising from the same stupors and fronds stalked. The algae were varying in size, numerous, fan-shaped, flat, much lobed, up to 10-15 cm in height. On the basis of above mentioned morphological characters and reference, the specimen was identified as ***Padina tetrastromatica***.

Locality: - It was found in abundant bloom during the months of November to February at Anjuna beach.



2.4s: ***Sargassum cinereum***


2.4t: ***Sargassum tenerrimum***

**3)** ***Sargassum cinereum*** (Fig 2.4s)

**X9** (Fig 2.4s):- This alga was growing in the mid-littoral region. It was dark brown in colour. The plants were with well marked discoidal holdfast and an upper portion richly branched sometimes radically organized. The plant axes were cylindrical glabrous and leaves alternately arranged. The margins of the leaves were serrated, apex obtuse and cuneate at the base. On the basis of above mentioned morphological characters and reference, specimen was identified as ***Sargassum cinereum***.

Locality: - It was observed to be in bloom in the months of November to February at Anjuna and Vagator beaches.

**4)** ***Sargassum tenerrimum*** (Fig 2.4t)

**X15** (Fig 2.4t):- It was growing in the mid-littoral region and was yellowish-brown in colour. The plants were pyramidal in form, delicate and with a disc-shaped holdfast. The plant axis was glabrous and rounded, ultimate branchlets modified into vesicles and receptacles. The algae leaves were thin, alternately arranged. On the basis of above mentioned morphological characters and reference, specimen was identified as ***Sargassum tenerrimum***.

Locality: - It was found in abundance during the months of November to February at Anjuna beach.

**5)** ***Colpomenia sinuosa*** (Fig 2.4u)

**X11** (Fig 2.4u):- It was growing on rocky substratum in well-exposed places of the mid littoral region. It was light green to yellowish green in colour. The plants were forming ball-shaped vesicles and older thalli becoming convoluted with corrugated surface. Thallus was composed of a single-layered epidermis consisting of short rectangular cells with round corners. On the basis of above mentioned morphological characters and reference, specimen was identified as ***Colpomenia sinuosa*.**

Locality: - This alga was rarely seen along the coast except for Kakara beach during the period between Augusts to October.

2.4u: ***Colpomenia sinuosa***

**Conclusion:-**

Identification of the marine macro algal species is known to be difficult, time consuming and tedious. The identification is often not only based on simple morphological criteria but also on reproductive structures, type of life history, cross sectional anatomical details, type of growth, cytology and ultra-structural criteria and increasingly molecular evidence.

However, in this study, all samples were identified by referring to (i) standard texts, (appended below) (ii) algae data base and (iii) by consulting experts in the field. The identification was an interesting, challenging, frustrating and humbling experience.

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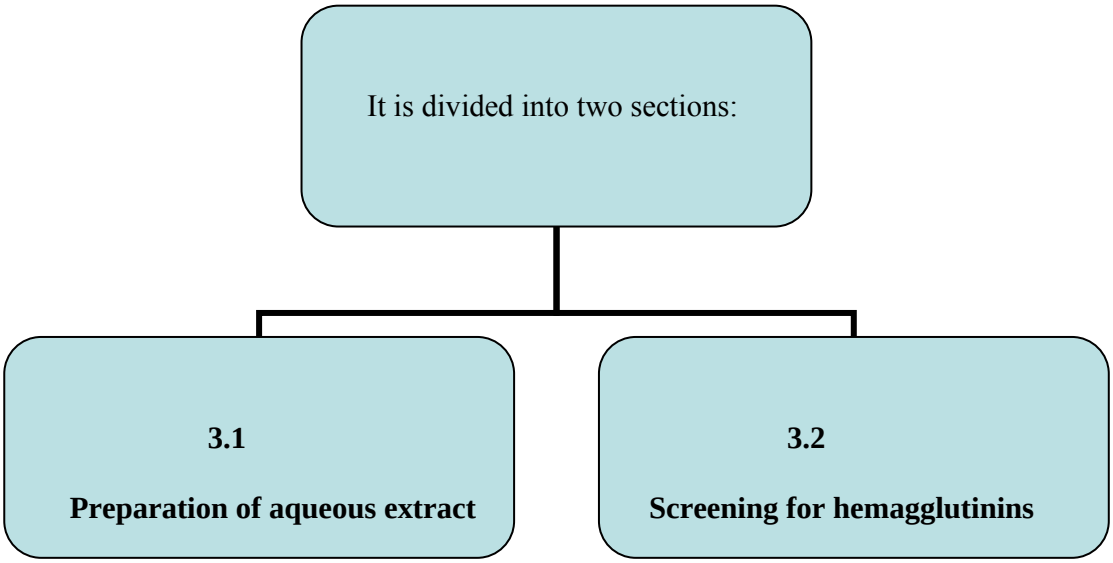
This chapter describes the preparation of aqueous extracts from the collected marine algae (obtained by different methods) and screening them for the presence of hemagglutinins.




**3.1 Preparation of aqueous extract:** It is divided into the following subsections.

3.1a. Introduction

3.1b. Materials and Methods

Results of both sections are noted together at the end of this chapter

### 3.1 a. Introduction

The isolation of any bio-molecule from a cell is a difficult task because a typical cell contains thousands of different substances which resemble other cellular constituents, in their physical and chemical properties. Furthermore, the material of interest may be unstable and exist in vanishingly small amounts; however proteins constitute the major fraction of all organisms (Walker, 2005).

The initial steps of any purification procedure must of course be to disrupt the source tissue to release proteins from within the cells. The means of disrupting the tissue will depend on the cell type, but thought must first be given to the composition of the buffer used to extract the proteins.

The composition of the extraction buffer is an important consideration. For soluble proteins, a first approximation would be buffer consisting of an ionic strength (0.1- 0.2M) and pH (7.0-8.0) that is considered to be compatible with that found inside the cell. Tris and phosphate buffers are most commonly used (Doonan, 1996). However, in addition, a range of other reagents may be included in the buffer for specific purposes. The pH of the extraction buffer is a critical parameter, and the optimal pH should be determined initially. Careful consideration should be given to the type of buffer used. A paramount consideration in choosing a buffer is that the effective buffering range overlaps the desired pH. Therefore, it is advisable to use a buffer in the middle of its effective buffering range. The solubility of the protein depends on ionic strength and the optimal ionic strength varies greatly among proteins.

Membrane-bound proteins (normally glycoproteins) require special conditions for the extraction as they are not released by simple cell disruption procedures alone.

Two classes of membrane proteins have been identified:-

1) Extrinsic (or peripheral) membrane proteins are bound only to the surface of the cells, normally via electrostatic and hydrogen bonds.

These proteins are predominantly hydrophobic in nature and are relatively easily extracted either by raising the ionic concentration of the extraction buffer or by changes of pH.

2) Intrinsic membrane proteins are those that are embedded in the membrane (integrated membrane protein). These invariably have significant regions of hydrophobic amino acids and have low solubility in aqueous buffer.

Many proteins within cells occur physically associated with membranes. Such associations range in strength from the relatively weak i.e., mainly electrostatic interactions of peripheral proteins to the much stronger hydrophobic interactions of internal membrane proteins. Peripheral proteins can usually be extracted by increasing ionic strength. However, extraction of integral proteins usually requires treatment with organic solvents (e.g. Butanol), or some other chemicals (e.g. Urea or Sodium hypochlorite) or detergent (e.g. Triton X-100) often in conjugation with enzymes such as phospholipases and proteinases, would help disrupt the membrane structure (Tanford *et al.,* 1976; Helenius *et al.,* 1975; Lenstra *et al.*, 1983; Garavito *et al.*, 2001).

Extraction of crude extract from tissue depends on a number of factors, to name a few being:-

- Economical feasibility of extraction procedure.
- Ease of availability of samples.
- Plant tissues pose a number of difficulties as far as the preparation of extract is concerned.

- Moderately harsh methods are required to break the cellulose cell wall.
- Disruption of vacuoles could lead to the release of proteases and lowering of pH of the extract if there is inadequate buffering.
- In the presence of oxygen, the phenolic compounds present in the cells are converted by the action of phenol oxidases to polymorphic pigments which can adsorb and damage protein in the extract. To avoid these problems, reducing agents can be added to inhibit the phenol oxidases.

General Extraction of Lectins**,** isolation of a lectin begins commonly with its extraction from the tissue or organs in which it is present. This is fairly simple in the case of plants especially their seeds whereas isolation from other parts may not be easy (Goldstein & Poretz, 1986).

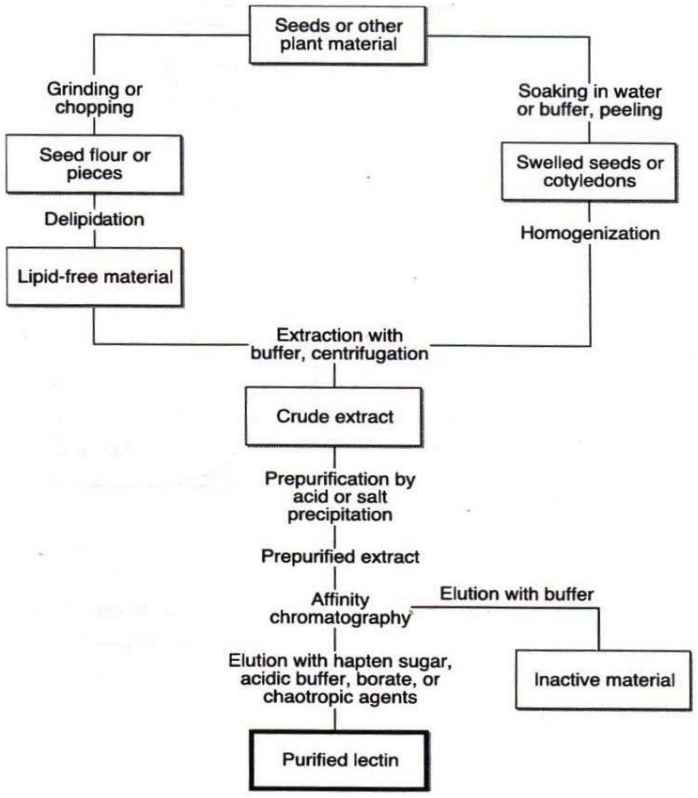



Fig 3.1:- Flowchart of general procedure for extraction of lectins (Sharon and Lis, 2003).

The seeds are grounded and the meal obtained is extracted with a neutral buffer. Often it is preferred to pre-extract the dry meal with an organic solvent, to remove colored material derived from the seed coat and lipids that may be present in large amounts (Sharon and Lis, 2003) and is charted in Fig (3.1)

Animal tissue is extracted first with acetone to remove water and lipids. The extraction buffer should preferably contain protease inhibitors to prevent degradation of the lectin during purification, and, in the case of membrane bound lectins, a detergent as well.

Preliminary fractionation of the crude extract then by ammonium sulfate precipitation is often done to obtain a protein fraction devoid of other constituents followed by Ion-exchange chromatography or affinity chromatography on suitable adsorbent.

Extraction of lectin from marine algae

The extraction of hemagglutinins from marine macroalgae is often more challenging than from other or bigger plants mainly because marine macroalgae are often rich in phenolic compounds and other molecules, which interfere with stability, separation and analysis. Furthermore, marine macroalgae contain relatively low concentration of hemagglutinins.

The ideal extraction protocol would attempt to remove, reproducibly, full component of hemagglutinins in a given marine macroalgae, with low contamination by other molecules. In general, hemagglutinins derived from

plant homogenates require the removal of particulate contamination. Simple filtration or centrifugation effectively removes insoluble components. After cell disruption, the extract is clarified by removing insoluble material by centrifugation. Liquid is trapped within the precipitated residue, and there will be a small loss related to the proportion of residue volume to total volume (Scopes, 1993). Generally, half the volume of the residue is trapped in liquid. To realize more recovery, the volume of the supernatant should be about more than twice the volume of the residue. Therefore it is advisable to make an extract, three volumes of extraction buffer. It is necessary that although the amount of material is slightly increased by using more extraction buffer, the extract will be in a larger volume and more dilute. The volume of the extraction buffer will thus be a compromise between maximum extraction and minimal volume of the extract.

Isolation of a lectin begins commonly with the extraction of the tissue or organ in which it is present. In different organisms, lectins may be present in the soluble form or may be found integrated into membrane (Brondes, 1986). Isolation of lectins generally begins with saline extraction of finely ground source. Pre-extraction with organic solvents helps to remove lipids or other interfering substances (Summer and Howell, 1976; Hayer and Goldstein, 1974; Shaper *et al.*, 1973). Ammonium sulfate or alcohol fractionation, centrifugation and dissolution of precipitate yields supernatant containing lectins (Tavasoliam and Kharrazy, 1978). Lectins are purified employing affinity chromatography for specific sugar-binding capacity of lectin (Lic *et al.*, 1974; Liz and Sharon, 1981).

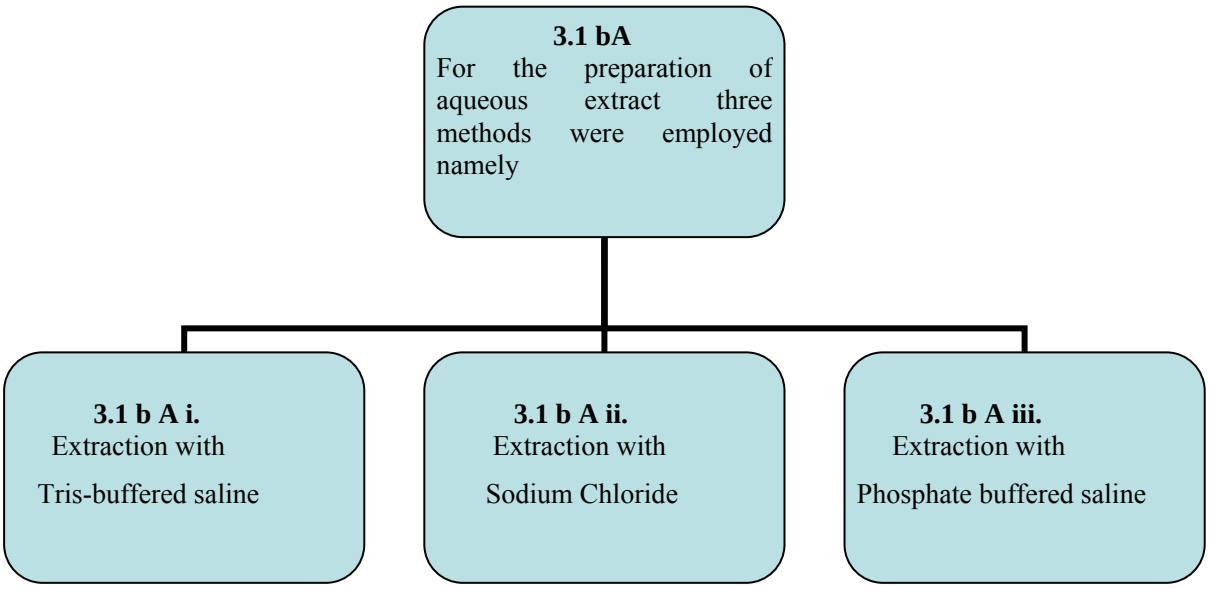
As lectins generally were to be extracted from seeds and plants, the general scheme for extraction and purification of lectin is described by Franz *et al.* (1998), as follows.

- Starting material (seeds, leaves, roots etc)
- Grinding of material (dry or soaked in buffer)
- Extraction with buffer or organic solvent.
- Centrifugation to remove cellular debris to get crude extract from supernatant.
- Salt or acid precipitation/fractionation to get purified extract
- Activity of each sample is checked and sample containing desired lectin is collected.

### 3.1 b. Materials and Methods

Based on reported literature, attempts were made to obtain an aqueous extract using three different buffers namely i) Tris-buffered saline (TBS) ii) 0.85% Sodium Chloride (NaCl) and iii) Phosphate buffered saline (PBS). Lectins from marine algae have been extracted inTris –buffered saline by Chiles and Bird (1989). There have been reports of extraction using Sodium Chloride (Freitas *et. al,* 1997) and Phosphate buffered saline by Sampaio *et al.*, (2002). The rationale behind using three different buffers was to compare for achieving and retaining highest activity of the extract. All steps of the extraction were carried out at 4°C unless mentioned otherwise. Extraction was done at 4°C to maintain the stability of the protein and also to inhibit protease activity, if any.

The chemicals used in lectin extraction were Di-Sodium Hydrogen Phosphate, Sodium Hydrogen Phosphate, Sodium Chloride and Tris Chloride. All chemicals were obtained from Himedia Labs, Mumbai, India.




**3.1 b A.** Extraction with Tris-buffered saline

The method used was a modification of Chiles and Bird, (1989).

The algal samples, which were kept frozen at -20$^{o}$C, were allowed to thaw at room temperature. A semi-dry weight of 30g of each sample was homogenized /macerated in cold Tris-buffered saline (TBS) at pH 7.4, over ice, with the help of a mortar & pestle. Care was taken to prevent foaming of the protein extract. The homogenate obtained was transferred to a clean beaker with a magnetic bar in it, suspended in (1:3 w/v) of TBS, and it was placed on a magnetic stirrer in the cold room and allowed to stir at 4$^{o}$C, for 18 hours.

The next day, the homogenate was filtered through a nylon mesh. Insoluble material and debris was discarded and the filtrate was transferred to clean, autoclaved centrifuge tubes. The tubes were centrifuged at 12,000 rpm for 30 minutes, at $4^0$C to pellet down all cellular debris. The clear supernatant (extract) after being checked for agglutination activity was aliquoted into clean vials and stored at -20$^o$C until further use. This extract was named as "TBS extract".

**3.1 b B.** Extraction with 0.85% Sodium Chloride

The protocol reported by Freitas *et.al,* (1997) was modified as under:

Frozen algae, which were kept at -20$^o$C, were allowed to thaw at room temperature. A semi-dry weight of 30g of each sample was homogenized in cold 0.85% sodium chloride (pH 7.4) by grinding in a mortar and pestle over ice. The homogenate obtained was re-suspended in three volumes of NaCl buffer and stirred for 12 hrs at 4°C. The homogenate was filtered through muslin cloth and centrifuged at 12,000 rpm for 30 minutes at 4°C after discarding insoluble material and debris. The clear supernatant was checked for agglutination and the balance was stored at -20°C until use and termed as "NaCl extract".

**3.1 b C.** Extraction with Phosphate buffered saline

The protocol followed was a modification of the technique reported by Sampaio *et al.,* (2002). Liquid nitrogen was not used in this study.

The algal samples, which were kept frozen at -20$^o$C, were allowed to thaw at room temperature. A semi-dry weight of 30g of each sample was homogenized /macerated in cold PBS at pH 7.4, over ice with the help of a mortar & pestle.

Care was taken to prevent foaming of the protein extract. The homogenate obtained was transferred to a clean beaker with a magnetic bar in it and it was placed on a magnetic stirrer in the cold room and allowed to stir at $4^{o}C$, for 18hrs. The next day, the homogenate was filtered through a muslin cloth and the filtrate was transferred to clean, autoclaved centrifuge tubes. The tubes were centrifuged at 12,000 rpm for 30 minutes, at $4^{0}C$ to pellet down all cellular debris. The clear supernatant (extract) was aspirated into clean vials and stored at $-20^{o}C$ for further use after checking activity by HA assay. This extract was called "the PBS extract".

**3.2 Screening for hemagglutinins: -** It is divided into the following subsections.

3.2A. Introduction

3.2B. Materials and Methods

3.2C. Results of both section 3.1 and 3.2

### 3.2 A. Introduction

The classical and still the simplest way to detect the presence of a lectin in a biological material is to prepare an extract from the material and examine its availability to agglutinate erythrocytes (Rudiger, 1993). According to the blood grouping system, different surface-oligosaccharides are responsible for hemagglutinin activities. In hemagglutination assay, non-agglutinated cells settle to the bottom of the microtiter plate as dots, while the agglutinated cells are observed as a pink-red diffused mat at the bottom (Talwar, 1983).

Lectins bind with RBCs and agglutinate them by forming non-covalent reversible interactions with cell-surface carbohydrates and glycoproteins. The ability to agglutinate erythrocytes distinguishes lectins from other sugar-binding proteins (Goldstein et al., 1980). The occurrence in plant extracts of proteins that possess the remarkable ability to agglutinate erythrocytes has been known since the turn of the century (Sharon et al., 1972). Because of the wide use of the agglutination reaction, Lis & Sharon, (1986) have stated that" for agglutination to occur, the lectin must bind to the cells and form cross- bridges between them". There is however no simple relation between the amount of lectin bound and agglutination. When agglutination does occur and when it is inhibited by mono-or oligosaccharides, it serves as an indication that carbohydrate structures for which the lectin is specific are present on the surface of the cell.

Since Boyd *et al*. (1966) first identified hemagglutinins from marine algae, many researchers have investigated or screened the hemagglutinating capacity of marine algal extracts as has been reviewed by Rogers and Fish (1991). A British survey of over 100 species of seaweed extracts revealed that only 19 species agglutinated human erythrocytes (Blunden et al., 1975). Hori et al (1981) in a Japanese survey reported that only 9 of over 53 species agglutinated tested human erythrocytes whereas seven out of fifteen were observed to be agglutinins among the algae screened by Chiles and Bird (1989). A survey of Vietnamese marine algae of over 44 species of extracts revealed hemagglutination activity in 22 species against tested human erythrocytes (Dinh et al., 2009).

Agglutination is affected by many factors such as the molecular properties of the lectin for example saccharide- binding sites, molecular size, cell surface properties, number and accessibility of receptors sites, membrane fluidity and metabolic state of cells (Nicolson, 1974). Therefore, for agglutination to occur the bound lectin must form cross bridges between opposing cells (Sharon et al., 1972).

There are no rapid screening methods for all lectins. Because most lectins tend to be multivalent, they generally have the ability to aggregate cells, such as erythrocytes. In examining biological specimens or their extracts for lectins, it must be considered that frequently lectins exhibit a narrow specificity. One kind of red cell may be agglutinated by a lectin, or only the red cells of one or a few species may be susceptible to aggregation. This is because different red cells have unique glycoconjugate compositions and unique distribution of lectin receptors. Nevertheless, hemagglutination is the most reliable and direct means of screening for lectins (Doyle and Malcolm, 1994). A more refined screening procedure is based on the ability of these proteins to precipitate polysaccharides or glycoproteins (Goldstein, 1976). If a positive result is obtained, it is essential to show that the agglutination or precipitation is specifically inhibited by mono or oligosaccharides, i.e, it is sugar specific (Ahmed, 2005).

### 3.2 B. Materials and Methods

The 96 well V-bottomed microtiter plates were obtained from Tarsons, (Kolkata). Standard antisera (antisera A and antisera B) antiserum kit ERYSCREEN®, obtained from Tulip Diagnostics (P) Ltd. Goa

**3.2 B.a**. Collection of Blood

Approximately 1ml of each blood group $RhA^+$, $RhB^+$ and $RhO^+$ was withdrawn from volunteers in the Department of Biotechnology at the clinic of Goa University or collected from the Blood Bank, Goa Medical College

Blood samples were collected in 3ml EDTA vials using disposable syringes and immediately capped, mixed well and stored at $4^oC$ until required for use.

**3.2 B.b.** Preparation of 2% RBC Suspension

About 150µl of each blood sample was taken in ependorff tubes and centrifuged at 3000 rpm at room temperature for 5 minutes. The supernatant of serum was discarded and the pellet of erythrocytes was washed with cold PBS (pH 7.4) 3-4 times by repeated centrifugation at 3000 rpm at room temperature. Finally, the pellet of RBCs was suspended in cold PBS to make a suspension of 2% (v/v) and used for the hemagglutination assay.

**3.2 B.c**. Assay for hemagglutinating activity

Respective antisera were used as positive control and PBS or NaCl was used as the negative control.

The assay for hemagglutination was performed in V-shaped micro-titre plates.

The hemagglutination assay was performed as follows:

- 75µl of the aqueous extract obtained of each of the alga (in 3 different buffers) was aliquoted in each well of the microtitre plate.
- Antisera Anti-A, Anti-B and Anti-D were used as positive controls for blood groups A, B and O respectively (The assay was performed with

an antiserum kit ERYSCREEN®, obtained from Tulip Diagnostics (P) Ltd. Goa).

- PBS or NaCl (pH 7.4,) were used as negative controls for all three blood groups.
- Finally, 75µl of the 2% RBC suspension was added to each of the wells containing the aqueous extract/ antisera or PBS/NaCl.
- The plate was swirled gently and incubated in a moist chamber for one hour at room temperature. The plates were swirled intermittently.
- Observations were recorded after one hour.
- Hemagglutination was observed with the naked eye and compared with agglutination of positive and negative controls.

**3.2 c. Results**

Three different buffers (sec 3.1b) at the same pH of 7.4 were used to extract agglutinins. Positive controls containing the antisera with the respective blood groups showed a positive hemagglutination reaction (Fig 3.1: lane 1).Negative controls containing buffer with the three blood groups showed a negative hemagglutination reaction (Fig.3.1: lane 11). Thus, the results indicate that the hemagglutination assay was performed properly and details are discussed below:

Results of Screening with TBS extract.

The results may be seen in Fig 3.1. Of the twenty one algae that were screened, strong agglutination was observed in six (X5, X12, X13, X16, X17 and X21) extracts of *Ulva fasciata*, *Amphiroa fragilissima, Grateloupia lithophila, Caulerpa sertularioides, Caulerpa peltata* and *Hypnea valentiae.* Overall

comparison with the three different blood groups yielded no specificity as can be seen in the results tabulated in 3.1. Though seven extracts showed hints of agglutination, eight extracts could be termed as non agglutinins.

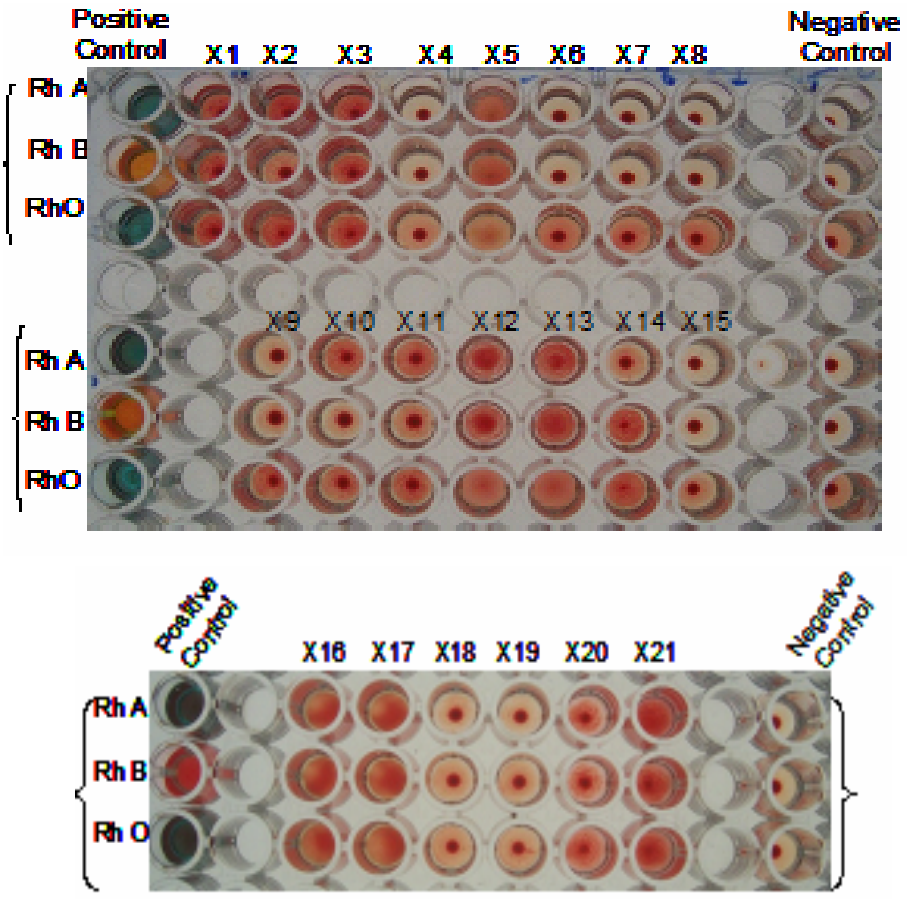



Fig: 3.1 - Hemagglutination assay using TBS extract

Table: 3.1:- Agglutination results from TBS extract

| **Extracted marine algae/quote** | **Blood group A+** | **Blood group B+** | **Blood group O+** |
|---|---|---|---|
| *Dictyota dichtoma* (X1) | +/- | +/- | +/- |
| *Padina tetrastomatica* (X2) | +/- | +/- | + |
| *Chaetomorpha media.* (X3) | +/- | +/- | +/- |
| *Gracilaria corticata* (X4) | - | - | - |
| *Ulva fasciata* (X5) | + | + | + |
| *Enteromorpha compressa* (X6) | - | - | - |
| *Enteromorpha flexuos* (X7) | - | - | - |
| *Enteromorpha intestinalis* (X8) | - | - | - |
| *Sargassum cinereum* (X9) | - | - | +/- |
| *Scinaia hatei* (X10) | +/- | - | +/- |
| *Colpomenia sinuosa* (X11) | +/- | +/- | +/- |
| *Amphiroa fragilissima* (X12) | + | + | + |
| *Grateloupia lithophila (*X13) | + | + | + |
| *Porphyra vietnamensis* (X14) | +/- | +/- | +/- |
| *Sargassum tenerrimum* (X15) | - | - | - |
| *Caulerpa sertularioides* (X16) | + | + | + |
| *Caulerpa peltata* (X17) | + | + | + |
| *Acanthophora specifera* (X18) | - | - | - |
| *Gelidium pusillum* (X19) | - | - | - |
| *Hypnea musciformis* (X20) | +/- | +/- | +/- |
| *Hypnea valentiae* (X21) | + | + | + |

**Key:** + Strong hemagglutination

+/- Partial hemagglutination

- No agglutination

Results of Screening with NaCl extract

The observations (Fig 3.2) indicate that the NaCl extract was more potent in agglutinating cells. As observed from the plate, it can be seen that eight (X5, X10, X12,X13, X16, X17, X20 and X21) out of twenty extracts agglutinated RBCs strongly. These were extracts from (*Ulva fasciata*, *Scinaia hatei, Amphiroa fragilissima, Grateloupia lithophila,, Caulerpa sertularioides, Caulerpa peltata, Hypnea musciformis, Hypnea valentiae* ). Five extracts caused partial agglutination and eight were negative.

As seen from Table 3.2 none of these extracts showed specificity towards the blood groups tested

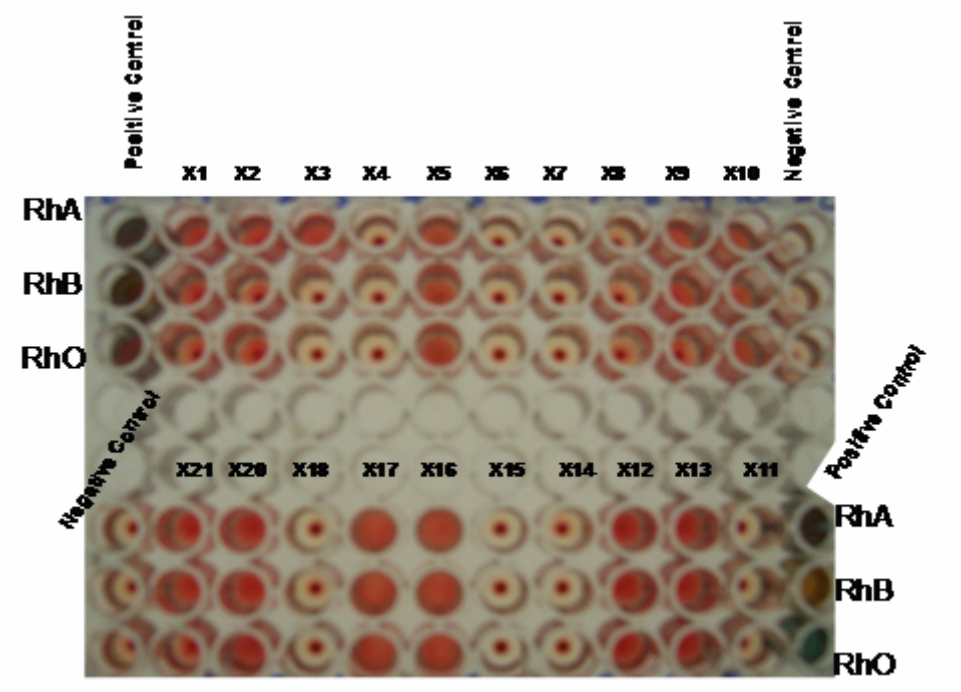



Fig 3.2 Hemagglutination assay of NaCl extracts

Table: 3.2:- Agglutination results from NaCl extract

| **Extracted marine algae/quote** | **Blood group A+** | **Blood group B+** | **Blood group O+** |
|---|---|---|---|
| *Dictyota dichtoma* (X1) | +/- | +/- | +/- |
| *Padina tetrastomatica* (X2) | +/- | +/- | + |
| *Chaetomorpha media.* (X3) | +/- | - | - |
| *Gracilaria corticata* (X4) | - | - | - |
| *Ulva fasciata* (X5) | + | + | + |
| *Enteromorpha compressa* (X6) | - | - | - |
| *Enteromorpha flexuos* (X7) | - | - | - |
| *Enteromorpha intestinalis* (X8) | - | - | - |
| *Sargassum cinereum* (X9) | +/- | +/- | +/- |
| *Scinaia hatei* (X10) | + | + | + |
| *Colpomenia sinuosa* (X11) | +/- | +/- | +/- |
| *Amphiroa fragilissima* (X12) | + | + | + |
| *Grateloupia lithophila (*X13) | + | + | + |
| *Porphyra vietnamensis* (X14) | +/- | +/- | +/- |
| *Sargassum tenerrimum* (X15) | - | - | - |
| *Caulerpa sertularioides* (X16) | + | + | + |
| *Caulerpa peltata* (X17) | + | + | + |
| *Acanthophora specifera* (X18) | +/- | +/- | +/- |
| *Gelidium pusillum* (X19) | +/- | +/- | +/- |
| *Hypnea musciformis* (X20) | + | + | + |
| *Hypnea valentiae* (X21) | + | + | + |

**Key:** + Strong hemagglutination

+/- Partial agglutination

- no agglutination

Results of Screening with PBS extract

Six of the twenty one algal extracts that were soluble in PBS agglutinated human erythrocytes strongly as observed in plate Fig 3.3 (X5, X12, X13, X16, X17,X21) .These agglutinins were from extracts of (*Ulva fasciata, Amphiroa fragilissima, Grateloupia lithophila, Caulerpa sertularioides, Caulerpa peltata and Hypnea valentiae*). Eight extracts did not show any agglutination whereas partial agglutination was observed in seven extracts

It appears that none of these extracts have specificity for the human blood groups that were assayed, as shown in Table 3.3.

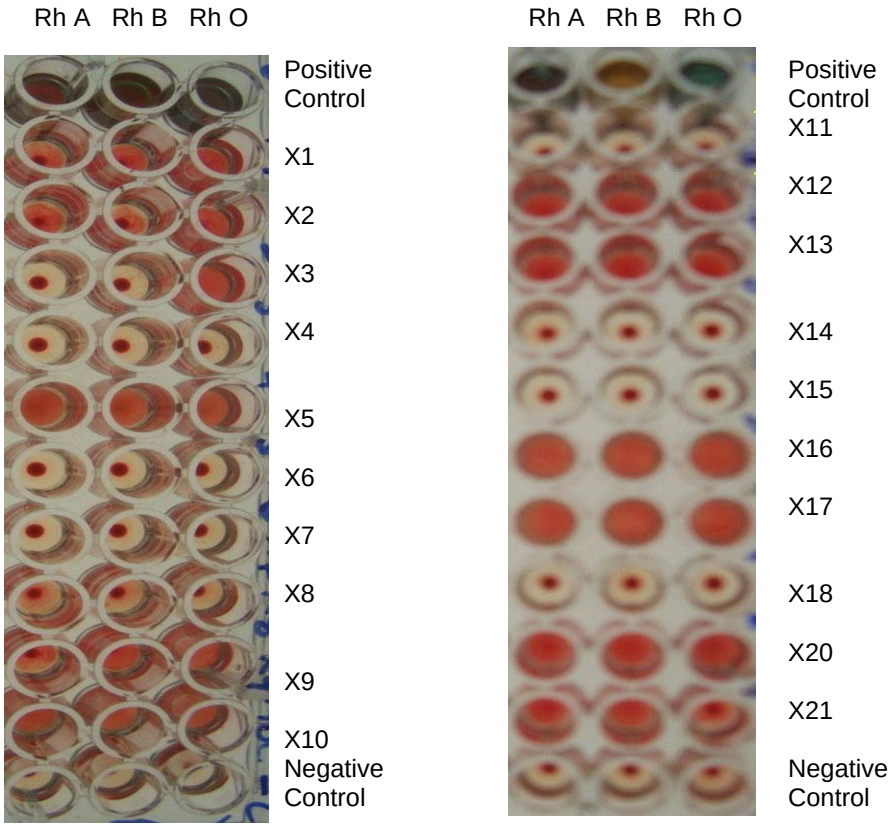



Fig 3.3:- Hemagglutination assay of PBS extract

Table 3.3.:-Results of Hemagglutination assay of PBS extracts

| **Extracted marine algae/quote** | **Blood group A+** | **Blood group B+** | **Blood group O+** |
|---|---|---|---|
| *Dictyota dichtoma* (X1) | +/- | +/- | +/- |
| *Padina tetrastomatica* (X2) | +/- | +/- | + |
| *Chaetomorpha media* (X3) | +/- | - | - |
| *Gracilaria corticata* (X4) | - | - | - |
| *Ulva fasciata* (X5) | + | + | + |
| *Enteromorpha compressa* (X6) | - | - | - |
| *Enteromorpha flexuosa* (X7) | - | - | - |
| *Enteromorpha intestinalis* (X8) | - | - | - |
| *Sargassum cinereum* (X9) | + | + | + |
| *Scinaia hatei* (X10) | +/- | +/- | +/- |
| *Colpomenia sinuosa* (X11) | +/- | +/- | +/- |
| *Amphiroa fragilissima* (X12) | + | + | + |
| *Grateloupia lithophila (*X13) | + | + | + |
| *Porphyra vietnamensis* (X14) | +/- | +/- | +/- |
| *Sargassum tenerrimum* (X15) | - | - | - |
| *Caulerpa sertularioides* (X16) | + | + | + |
| *Caulerpa peltata* (X17) | + | + | + |
| *Acanthophora specifera* (X18) | +/- | +/- | +/- |
| *Gelidium pusillum* (X19) | +/- | +/- | +/- |
| *Hypnea musciformis* (X20) | + | + | + |
| *Hypnea valentiae* (X21) | + | + | + |

**Key: +** Strong hemagglutination

**+/-** Partial agglutination

**-** no agglutination

On comparing the entire data obtained in this chapter, maximum agglutination was observed in the extract that was soluble in PBS and thus all further work was carried out using PBS extraction. It was observed Table 3.4 that extract from red algae seemed to contain and gave higher incidence of agglutination among all three groups. However, the potency of agglutination was higher in green algae. Minimal agglutination was observed in brown algae.

Further it was observed that irrespective of buffers and blood group, extracts from six out of twenty one algae that were screened, gave consistent and strong agglutination activity. Thus on screening twenty one algae, six of these namely *Ulva fasciata*, *Amphiroa fragilissima, Grateloupia lithophila, Caulerpa sertularioides, Caulerpa peltata* and *Hypnea valentiae* exhibited the presence of a potential lectin.

All three buffers used for extraction were pH 7.4. However, each of the buffers resulted in differential activity albeit to small degree of the same extract. It is interesting to determine the role played by the buffer in enhancing the hemagglutination activity.

Table 3.4: - Comparative hemagglutination of twenty one extracts

| **Extracted marine algae /quote** | Agglutination activity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PBS extract | | | TBS extract | | | NaCl extract | | |
| | A+ | B+ | O+ | A+ | B+ | O+ | A+ | B+ | O+ |
| Chlorophyta | | | | | | | | | |
| *Chaetomorpha media* (X3) | +/- | - | - | +/- | +/- | +/- | +/- | - | - |
| *Ulva fasciata* (X5) | + | + | + | + | + | + | + | + | + |
| *Enteromorpha compressa* (X6) | - | - | - | - | - | - | - | - | - |
| *Enteromorpha flexuosa* (X7) | - | - | - | - | - | - | - | - | - |
| *Enteromorpha intestinalis* (X8) | - | - | - | - | - | - | - | - | - |
| *Caulerpa sertularioides* (X16) | + | + | + | + | + | + | + | + | + |
| *Caulerpa peltata* (X17) | + | + | + | + | + | + | + | + | + |
| Rhodophyta | | | | | | | | | |
| *Gracilaria corticata* (X4) | - | - | - | - | - | - | - | - | - |
| *Scinaia hatei* (X10) | +/- | +/- | +/- | +/- | - | +/- | + | + | + |
| *Amphiroa fragilissima* (X12) | + | + | + | + | + | + | + | + | + |
| *Grateloupia lithophila* (X13) | + | + | + | + | + | + | + | + | + |
| *Porphyra vietnamensis* (X14) | +/- | +/- | +/- | +/- | +/- | +/- | - | - | - |
| *Gelidium pusillum* (X19) | +/- | +/- | +/- | - | - | - | +/- | +/- | +/- |
| *Hypnea musciformis* (X20) | + | + | + | +/- | +/- | +/- | + | + | + |
| *Hypnea valentiae* (X21) | + | + | + | + | + | + | + | + | + |
| Phaeophyta | | | | | | | | | |
| *Dictyota dichtoma* (X1) | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- |
| *Padina tetrastomatica* (X2) | +/- | +/- | + | +/- | +/- | + | +/- | +/- | + |
| *Sargassum cinereum* (X9) | + | + | + | - | - | +/- | +/- | +/- | +/- |
| *Colpomenia sinuosa* (X11) | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- |
| *Sargassum tenerrimum* (X15) | - | - | - | - | - | - | - | - | - |
| *Acanthophora specifera* (X18) | - | - | - | - | - | - | +/- | +/- | +/- |

+ Positive, +/- Uncertain, - Negative

This chapter details the step-wise methodology used to fractionate the aqueous extracts sequentially by ammonium sulfate precipitation. Aqueous extracts from six species extracted by all three buffers that showed hemagglutination activity were subjected to fractionation in three stages: 0 to 30%, 30 to 60%, and 60 to 90% ammonium sulfate saturation. Further, since two extracts gave highest activity, these were processed for purification. Methodology employed for purification of lectin from both species was common.

This chapter is divided into following section and subsections

**4.1 Introduction**

**4.2 Materials and Methods**

4.2 I. Ammonium sulfate fractionation

4.2 II. Dialysis

4.2 III. Ion Exchange chromatography

4.2 IV. Estimation of protein content

4.2 V. Determination of Hemagglutination titer

**4.3 Results**

**4.3.A** Purification of agglutinin from *Ulva fasciata*

**4.3.B** Purification of agglutinin from *Caulerpa sertularioides*

## 4.1 Introduction

Purification of proteins is vital in the study of their function and expression. It is generally, a multi-step process exploiting a wide range of biochemical and biophysical characteristics of the target protein, such as its source, relative concentration, solubility, charge and its hydrophobic nature. The ideal purification strives to obtain the maximum recovery of the desired protein, with minimal loss of the activity, combined with the maximum removal of other contaminating proteins that may be present in the mixture. These contaminants may be other proteins or completely different molecules, altogether.

Proteins are fragile molecules that denature readily at extremes of temperature and pH. Each proteins offer its own unique set of physicochemical characteristics. The methods used for protein purification therefore should be mild, to preserve the native confirmation of the molecule and its bioactivity. In most cases, having a reliable assay to be used as the means of following the target protein is essential (Wilson and Walker, 2005).

Since the reason for purifying a protein is normally to provide material for structural or functional studies, the final degree of purity depends on the purposes for which the protein will be used. Prior to extraction, it is important that information about the localization of the protein in the cell is known and on this basis, to develop the appropriate strategy for its extraction (Walker, J.M.; 2005).

Proteins are all made up of amino acids of various lengths. The amino acids can be positioned in any order in the sequence giving an almost infinite number of proteins that can be manufactured. However, the sequence of amino acids alters the properties of the protein molecule in the form of charge, hydrophobicity, geometry, and solubility. Different proteins can have similar shapes or isolelectric points. For this reason no one technique can be used to fully purify a mixture of proteins, therefore a sequence of physical and chemical tests need to be carried out (Voet and Voet, 2004).

**4.1A**. Ammonium sulfate precipitation

Once the extract or organelle preparation is ready, various methods are available for purifying one or more of the proteins it contains. Commonly, the extract is subjected to treatments that separate the proteins into different fractions based on a property such as size or charge, a process referred to as fractionation**.** Early fractionation steps in purification utilize differences in protein solubility, which is a complex function of pH, temperature, salt concentration, and other factors. The solubility of proteins is generally lowered at high salt concentrations, an effect called "salting out." The addition of a salt in the right amount can selectively precipitate some proteins, while others remain in solution. Ammonium sulfate is often used for this purpose because of its high solubility in water.

Ammonium Sulfate is the most common salt used for precipitating proteins due to its stability in cold solutions. Ammonium Sulfate fractionation is widely used in the first stage of protein purification to remove non protein molecules.

Fractionation of proteins by precipitation with ammonium sulphate is the most commonly used method to enrich /purify a particular protein. Proteins of high molecular weight usually precipitate below 25% ammonium sulphate saturation. Crude /aqueous extracts may be subjected to fractionation in three stages: 0 to 30%, 30 to 60%, and 60 to 90% ammonium sulphate saturation. Precipitated proteins from each stage plus the 90% supernatant are then tested for activity (Rhodes, J.M. and Milton, J.D.; 1998).

Proteins are soluble in aqueous media because they have hydrophilic amino acid side-chains facing outwards that can interact with water. Any compound that interferes with these interactions between amino acid side-chains and water, by reducing the available water, will reduce the solubility of the protein. As interactions with water become less marked, so protein-protein interactions become more important, and the protein will aggregate and come out of solution provided that the temperature is maintained low enough (around $4^0$C). In this method, the protein is not irreversibly denatured and the precipitate can be redissolved in buffer. Ammonium sulfate is highly hydrated, and a concentrated ammonium sulfate solution reduces the available water very considerably.

Precipitation of proteins by water-miscible organic solvents such as alcohol or acetone is another technique, but is not commonly used, probably because of its tendency to denature protein at room temperature. Precipitation with polyethylene glycol (PEG) has been used with considerable success for several proteins. A disadvantage of using PEG is that it is not easily removed from protein solutions (Rhodes, J.M. and Milton, J.D.; 1998).

Initially one should probably use a number of wide ranges of ammonium sulfate saturation, say 0 - 50% and see whether or not the desired protein is precipitated. If it is, then one can further refine the range until maximum recovery of the protein and maximum removal of interfering proteins is achieved.

**4.1B**. Dialysis

Having precipitated a protein fraction and redissolved it in buffer, it is necessary to remove the ammonium sulfate before one can proceed to subsequent steps in the purification process. The simplest way to achieve this is to dialyze the solution. Dialysis is a procedure that separates proteins from solvents by taking advantage of the proteins' larger size. Dialysis increases the volume of the protein solution, because of the initial osmotic effect of the ammonium sulfate (this is why it is important to leave an air gap at the top of the membrane tube, to prevent from bursting).

The partially purified extract obtained from ammonium sulfate fractionation is placed in a bag or tube made of a semi- permeable membrane. When this is suspended in a much larger volume of buffered solution of appropriate ionic strength, the membrane allows the exchange of salt and buffer but not proteins. Thus dialysis retains large proteins within the membranous bag or tube while allowing the concentration of other solutes in the protein preparation to change until they come into equilibrium with the solution outside the membrane.

Dialysis might be used, for example, to remove ammonium sulfate from the protein preparation.

**4.1C**. Chromatography

The most powerful methods for fractionating proteins, use of column chromatography**,** which takes advantage of differences in protein charge, size, binding affinity, and other properties. The term "chromatography" applies to a wide range of separation techniques that are based on the differential interaction of molecules between a moving phase and a stationary phase (Walker, J.M.; 2005).

A porous solid material with appropriate chemical properties (the stationary phase) is held in a column, and a buffered solution (the mobile phase) percolates through it. The protein-containing solution, layered on the top of the column, percolates through the solid matrix as an ever-expanding band within the larger mobile phase. Individual proteins migrate faster or more slowly through the column depending on their properties. For example, in cation-exchange chromatography, the solid matrix has negatively charged groups. In the mobile phase, proteins with a net positive charge migrate through the matrix more slowly than those with a net negative charge, because the migration of the former is retarded more by interaction with the stationary phase. The two types of protein can separate into two distinct bands. The expansion of the protein band in the mobile phase (the protein solution) is caused both by separation of proteins with different properties and by diffusional spreading. As the length of the column increases, the resolution of two types of protein with different net

charges generally improves. However, the rate at which the protein solution can flow through the column usually decreases with column length. And as the length of time spent on the column increases, the resolution can decline as a result of diffusional spreading within each protein band (Janson and Ryden, 1989).

The interaction of molecules can be based on any of the protein properties; charge, hydrophobicity, affinity, solubility or molecular weight (size). As the name indicates, column chromatography is performed with the stationary phase packed in a cylindrical container, the column. The mobile phase passes through the column driven by gravity or by a pump. Fractions of the mobile phase are collected as they leave the column. Each fraction can then be assayed to determine the location of the target protein. Column chromatography is a commonly used technique for protein purification. Column chromatographic methods are generally more complicated to set up than batch methods such as ammonium sulfate precipitation; however, they are capable of high resolution separation and are usually necessary for a successful purification. Like other separation techniques, different column chromatography methods exploit different properties of the protein. Although the focus of this chapter is on ion exchange chromatography, it being used in the present study.

**4.1CI.** Ion-Exchange chromatography

Ion exchange chromatography (IEC) is a high resolution technique for separating proteins according to their charge. It is the most commonly used chromatographic method of protein separation due to its ease of use and scale-

up capabilities. Large volumes of protein solution can be applied to ion-exchange columns, often much greater than the volume of the column itself (Bonnerjea et al., 1986)

In, Ion exchange chromatography, the packing material (resin) of the column has many charged molecules that are securely bound to it by covalent bonds. Manufacturers make and sell many different types of packing materials. Some have negatively charged groups and some have positively charged groups. When the packing material is suspended in buffer, the charged groups become loosely associated with ions of the opposite charge. Since the buffer contains NaCl that dissociates into Na+ and $Cl^-$, the loosely bound ions are called mobile counter-ions ((Walker, J.M.; 2005)

Ion exchange chromatography separates molecules based on differences between the overall charges of the protein. It is usually used for protein purification but may be used for purification of oligonucleotides, peptides, or other charged molecules. The protein of interest must have a charge opposite that of the functional group attached to the resin in order to bind. For example, immunoglobulins, which generally have an overall positive charge, will bind well to cation exchangers, which contain negatively charged functional groups. Because this interaction is ionic, binding must take place under low ionic conditions (Janon and Ryden, 1989). Elution is achieved by increasing the ionic strength to break up the ionic interaction, or by changing the pH of the protein.

The first step in the process is to “equilibrate” the column. Since the packing material of the column contains charged molecules, these will interact with the

ions in the buffer. Just as the pH of a solution changes when charged molecules are added to it, the pH of the column-buffer environment will change as buffer is added to the column. This process is stabilized before the protein mixture is applied to the column. When the pH of the buffer coming out of the column is the same as the pH going into the column, the column is said to be equilibrated (Scoopes, 1993).

As the liquid exits the column, absorbance characteristics for protein the $A_{280}$ is read by a UV monitor and these readings give an indication of the location of protein in the eluent. All the material that elutes from the column is collected in a numbered series of test tubes (1 ml each). These 1 ml aliquots are called "fractions". Although convenient and expedient, instead of automated collector and readers, fractions can be collected manually and their absorbance read on a UV spectrophotometer and plotting the results on graph paper (Doonan, 1996).

**4.1D.** Estimation of Protein Concentration

The Folin-Lowry assay method for determination of protein concentration is one of the most viable and widely-used protein assays. The Lowry method was first described in 1951 by Lowry et al. (Lowry et al., 1951).

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen(s) with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteau phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids (Dunn, 1992). The Lowry method is sensitive to low concentrations of protein such as concentrations ranging from 0.10 – 2 mg of protein per ml (Dunn, 1992) and even

concentrations of 0.005 – 0.10 mg of protein per ml (Price, 1996). The major disadvantage of the Lowry method is the narrow pH range within which it is accurate. However, using very small volume of sample has little or no effect on the pH of the mixture.

A variety of compounds could possibly interfere with the Lowry procedure. These include some amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids and sulphydryl reagents (Dunn, 1992). Price (1996) reported that ammonium ions, zwitterionic buffers, nonionic buffers and thiol compounds may also interfere with the Lowry reaction and thus these substances should be removed or diluted before running Lowry assay.

**4.1E**. Protein yields

The need to determine protein concentration in a solution is a routine requirement during each step of the isolation. Most of these are colorimetric methods, where a portion of the protein solution is reacted with a reagent that produces a colored product. The amount of this coloured product is then measured spectrophotometrically and the amount of colour related to the amount of protein present by appropriate calibration (Wilson and Walker, 2005).

Monitoring each step of fractionation is a prerequisite for successful protein purification. Most important is the establishment of a reliable assay of the biological activity. In addition one needs to determine the protein content in order to be able to assess the efficacy of the different steps of protein isolation.

A successful fractionation step is recognized by an increase in the specific activity of the proteins, where the specific activity of the protein relates its total activity to the total amount of protein present in the preparation. For lectins, the amount of hemagglutinins present in a particular fraction is expressed as HU (Hemagglutination Unit). For a purification step to be successful, therefore, the specific activity of the proteins must be greater after the purification step than it was before. This increase is best represented as the fold purification. A significant increase in specific activity is clearly necessary for a successful purification step. However, another important factor is the yield of the protein in each of the fractions (Doonan, 1996).

**4.1F.** Hemagglutination Titer

The highest dilution of lectin, resulting in agglutination, is termed as hemagglutination titer. The reciprocal of the highest dilution of extract, exhibiting positive hemagglutination is expressed as the "hemagglutination titer". It is also referred to as "the minimal hemagglutinating capacity" (MHC) .i.e. the minimum amount of protein-extract tested, that produces agglutination The higher the hemagglutination titer, the greater is the lectin affinity for carbohydrates of the RBC membrane or the higher is the density of carbohydrate receptors on the surface of the corresponding RBC. Within a single population of RBC of one animal species or human blood group this value is sufficiently stable, and may serve as an important feature of lectins. The value of hemagglutination titers may be used for the evaluation of the activity and quantitative assay of the lectin content in the solution. It should be borne in mind that this requirement is met only by providing RBC of one and the same

species or human blood group (it is preferable for them to be collected from one and the same donor). Besides, the result of determining lectin concentration using hemagglutination titer may contain the error of determination of upto 50%. This is understandable since practical analysis involves dilutions in geometric progression, e.g. 1:2, 1:4, 1:8, etc. Besides, the yield is assessed visually, i.e. subjectively (Grischenko, V.I.; 1994). Hemagglutination is commonly assayed by the serial dilution technique using erythrocytes from humans or rabbits. Occasionally, erythrocytes that have been treated with trypsin or sialidase are employed. Since such cells are often more sensitive to agglutination than untreated cells. Hemagglutination also serves to monitor and quantify the activity of lectins in the course of purification (Ahmed, H.; 2005).

**4.2 Materials and Methods:-**

The methods used sequentially for purification of aqueous extract were i) Ammonium Sulfate Precipitation, ii) dialysis and iii) Ion-exchange chromatography on DEAE-Cellulose columns.

All steps were carried out at 4ºC to maintain the stability of the isolated products unless mentioned otherwise.

The chemicals and dialysis membrane matrix used for the partial purification were, ammonium Sulfate, Disodium Hydrogen Phosphate, Sodium Hydrogen Phosphate, Sodium Chloride, dialysis membrane cut of 12 kDa and DEAE-Cellulose. All were obtained from Himedia Labs, Mumbai, India.

**4.2 I**. Ammonium sulfate fractionation

Aqueous extracts of the selected six species were precipitated with solid ammonium sulfate ($NH_2SO_4$) to isolate the proteins into different fractions, using varying degrees of saturation. Increasing degrees of saturation with ammonium sulfate were used. Saturation ranges of 0-30%, 30-60%, and 60-90% were initially used for all six extracts.

Solid Ammonium sulfate (Appendix 5) was added slowly to aqueous extract, placed in a beaker with a magnetic stirrer at $4^0$C, with constant stirring. The beaker was then left for 4 hours on the stirrer to precipitate the proteins. This solution with was then transferred to clean, autoclaved centrifuge tubes. Precipitated fraction was centrifuged at 12000 rpm at 4°C for 30 minutes. The pellet obtained was re-dissolved in minimal amount of PBS (Appendix 1) and stored as the 0-30% fraction at 4°C.

The supernatant obtained above was carried forward for higher saturation 30-60% (Appendix 5), following the same procedure except that the solution was stirred for 12 hrs. The pellet obtained, was stored as the 30-60% fraction and the supernatant was transferred to a beaker.

Supernatant was treated similarly as above to obtain 60-90% saturation (Appendix 5). The precipitate was re-suspended in PBS and stored as the 60-90% fraction at 4°C.

**4.2 II**. Dialysis

The re-suspended fractions 0-30%, 30-60% and 60-90% were extensively dialyzed (dialysis bag cut-off 12 kDa) against three changes of PBS (2 liters).

During dialysis, the buffers were refreshed once every 6 hrs. The dialyzed samples were then checked for activity by performing a hemagglutination assay.

**4.2 III**. Ion Exchange chromatography

As maximum activity was observed in the 60-90% fraction obtained from sequential ammonium sulfate precipitation, this fraction was subjected to ion-exchange chromatography on a DEAE-Cellulose column equilibrated with 0.15 M PBS. The resin was treated and column was packed as per the manufacturer's instructions.

- 5gm dry resin was suspended in 25 ml of distilled water and left at room temperature for 30-45 minutes to settle down the matrix. The settled volume of the resin was measured and this was termed as Column volume (CV) and used as a measure for washing.
- Further the filter resin was suspended in 2 CV of 0.15 M NaOH containing 0.5 M NaCl for 10 minutes, and washing was continued for 5 CV of 0.15 M NaOH containing 0.5 M NaCl.
- The resin was further washed with continuous 5 CV of 0.5 M NaCl, followed by 5 CV of 0.1 M HCl containing 0.5 M NaCl.
- Column was then washed with 10 CV of distilled water until the effluent attained a pH of 5 or more.
- Then the resin was suspended in 2 CV of 1 M NaCl and pH of the slurry was adjusted to 7-8 with NaOH.
- Resin was further suspended in 2 CV of 10X PBS and the resin suspension was filtered.
- Further washing was continued using 5 CV of 1X PBS.

- Resin was then considered to be ready for use.
- Resin was equilibrated in 0.15 M PBS at pH 7.4 until absorbance was reduced to zero. Precipitated protein 60-90% was loaded on equilibrated column and washing was continuous until absorbance was reduced to zero. The bound protein was eluted by gradual increase in sodium chloride concentrations from 0.5 M up to 1.5 M at a flow rate of 200 µl/ min. Absorbance was recorded at 280 nm. The active fractions were pooled and extensively dialyzed against distilled water. The active elutes were tested for titer activity and stored at -20°C until required for further use and considered to be purified hemagglutinins.

**4.2 IV**. Estimation of protein content

The protein concentrations of crude, ammonium sulfate precipitate and purified extract were determined by the Folin-Lowry assay (Lowry et al., 1951) using Bovine serum albumin (BSA) as a standard. The absorbance at 540 nm was used to estimate protein content in aqueous, saturated fractions and purified extract. Preparations of chemicals are mentioned in appendix 6.

**4.2 V**. Determination of Hemagglutination titer

Lectin-mediated agglutination of red blood cells was determined by the procedure previously reported by Hori *et al*. (1981). 2% erythrocyte suspension was prepared as detailed in section 3.2

The Hemagglutination titer assay was performed in 96 well V-bottomed microtitre plate as follows:

The test was performed in the same way as the hemagglutination assay.

The Antisera Anti-A, Anti-B and Anti-D were used as positive controls for the respective blood groups. PBS was used as the negative control for all three blood groups.

- The first well was maintained as 'neat' in which the algal extract was not diluted, but mixed in a 1:1 ratio with the RBC suspension. 75µl of the extract and RBC suspension was used.
- 75µl of plain PBS was added in all subsequent wells for dilution.
- 75 µl from the 'neat' well was aspirated and added to the next well containing 75µl of PBS, thus obtaining a dilution of 1:2.
- The same procedure was repeated for all the following wells until a dilution of 1:64 was obtained (75µl was discarded from the last well).
- 75µl of RBC suspension was further added to each of the wells and the plate was incubated in a moist chamber for one hour.
- The quantitative observations were recorded after one hour.

## 4.3 . Results

### 4.3.A Purification of agglutinins from *U. fasciata*

Ammonium sulfate precipitation

The first step in purification was ammonium sulfate precipitation. Extracts from six (Table 3.4 in section 3) samples that showed promising potential of agglutination were selected for purification. The extracts obtained by each of the three buffers were precipitated using ammonium sulfate and fractions were collected with 0-30%, 30-60% and 60-90% saturation respectively. Before proceeding further for purification these fractions were checked for agglutination potential to determine hemagglutination titer.

The fractions were checked for activity by determination of hemagglutination titer. The results are summarized in Table 4.1. Highest hemagglutination titer of 1:16 was observed in *Ulva fasciata* in the 60-90% fraction of the PBS extract as seen in fig 4.1. A similar titer was obtained from *Caulerpa sertularioides* in the 60-90% fraction of the NaCl extract.

These two fractions of the respective algae were dialyzed to remove ammonium sulfate and loaded on DEAE-Cellulose column for purification

Table 4.1:- Determination of hemagglutination titer

| Selected species | Blood Cells | PBS extract | | | NaCl extract | | | TBS extract | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0-30% | 30-60% | 60-90% | 0-30% | 30-60% | 60-90% | 0-30% | 30-60% | 60-90% |
| *Ulva fasciata* | A | 1:2 | 1:1 | **1:8** | 1:2 | 1:2 | 1:2 | 1:4 | 1:4 | 1:2 |
| | B | 1:2 | 1:1 | **1:8** | 1:4 | 1:2 | 1:4 | 1:4 | 1:2 | 1:4 |
| | O | 1:2 | 1:2 | **1:16** | 1:4 | 1:2 | 1:4 | 1:4 | 1:2 | 1:2 |
| | | | | | | | | | | |
| *Grateloupia lithophila* | A | 1:2 | 1:1 | **1:8** | 1:2 | - | 1:2 | 1:4 | 1:1 | 1:2 |
| | B | 1:2 | 1:1 | **1:2** | 1:2 | - | 1:2 | 1:2 | 1:1 | 1:1 |
| | O | 1:2 | 1:1 | **1:4** | 1:2 | - | 1:2 | 1:2 | 1:2 | 1:4 |
| | | | | | | | | | | |
| *Caulerpa sertularioides* | A | 1:4 | 1:2 | 1:4 | 1:4 | 1:1 | **1:8** | 1:4 | 1:2 | 1:2 |
| | B | 1:2 | 1:1 | 1:4 | 1:4 | 1:2 | **1:8** | 1:2 | 1:2 | 1:4 |
| | O | 1:4 | 1:2 | 1:4 | 1:4 | 1:2 | **1:16** | 1:4 | 1:2 | 1:4 |
| | | | | | | | | | | |
| *Caulerpa peltata* | A | 1:2 | 1:1 | 1:2 | 1:2 | 1:1 | 1:2 | **1:2** | **1:2** | **1:2** |
| | B | 1:2 | 1:1 | 1:2 | 1:2 | - | 1:2 | **1:2** | **1:2** | **1:2** |
| | O | 1:2 | 1:1 | 1:2 | 1:2 | - | 1:2 | **1:2** | **1:2** | **1:2** |
| | | | | | | | | | | |
| *Hypnea musciformis* | A | **1:4** | 1:4 | 1:1 | 1:2 | 1:1 | 1:2 | 1:2 | 1:1 | 1:2 |
| | B | **1:4** | 1:4 | 1:1 | 1:2 | 1:1 | 1:2 | 1:1 | 1:1 | 1:2 |
| | O | **1:4** | 1:4 | 1:1 | 1:2 | 1:1 | 1:2 | 1:1 | - | 1:2 |
| | | | | | | | | | | |
| *Hypnea valentiae* | A | **1:4** | - | 1:2 | 1:1 | 1:1 | 1:2 | 1:2 | - | 1:2 |
| | B | **1:4** | - | 1:2 | 1:1 | 1:1 | 1:2 | 1:2 | - | 1:1 |
| | O | **1:4** | - | 1:2 | 1:1 | 1:1 | 1:2 | 1:2 | - | 1:2 |

The profile of hemagglutination titer of *Ulva fasciata* as seen in Fig 4.1 indicated higher agglutination titer with human O blood group as seen in row 12 of the plate in fig 4.1

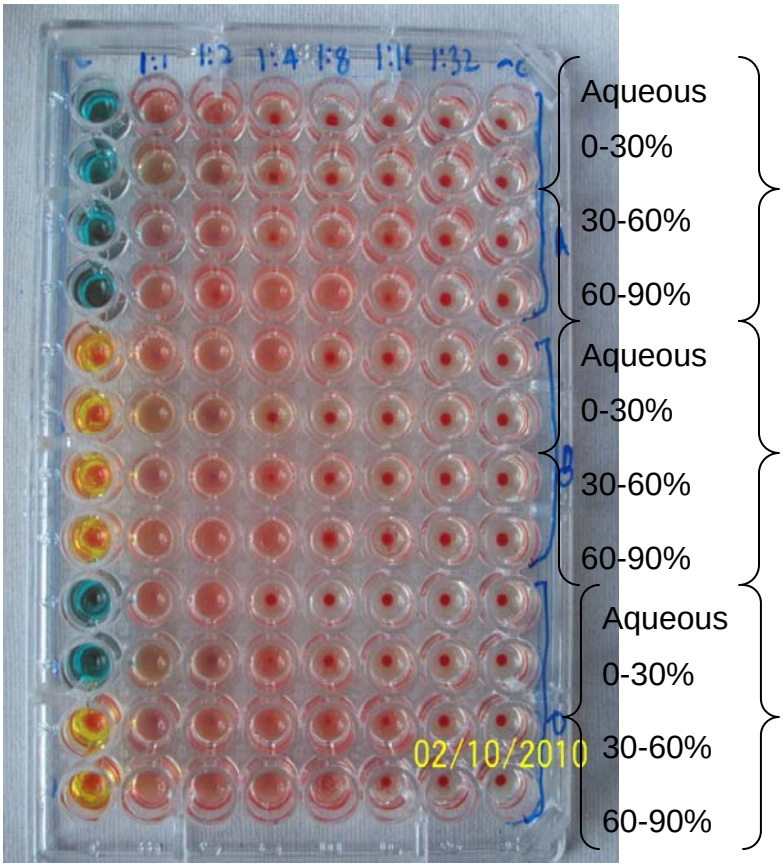



Fig 4.1 Hemagglutination titer of different fractions isolated from *U. fasciata*

The profile of hemagglutination titer of *Caulerpa sertularioides* as seen in Fig 4.2 indicated higher agglutination titer with human O blood group as seen in row 12 of the plate in fig 4.2

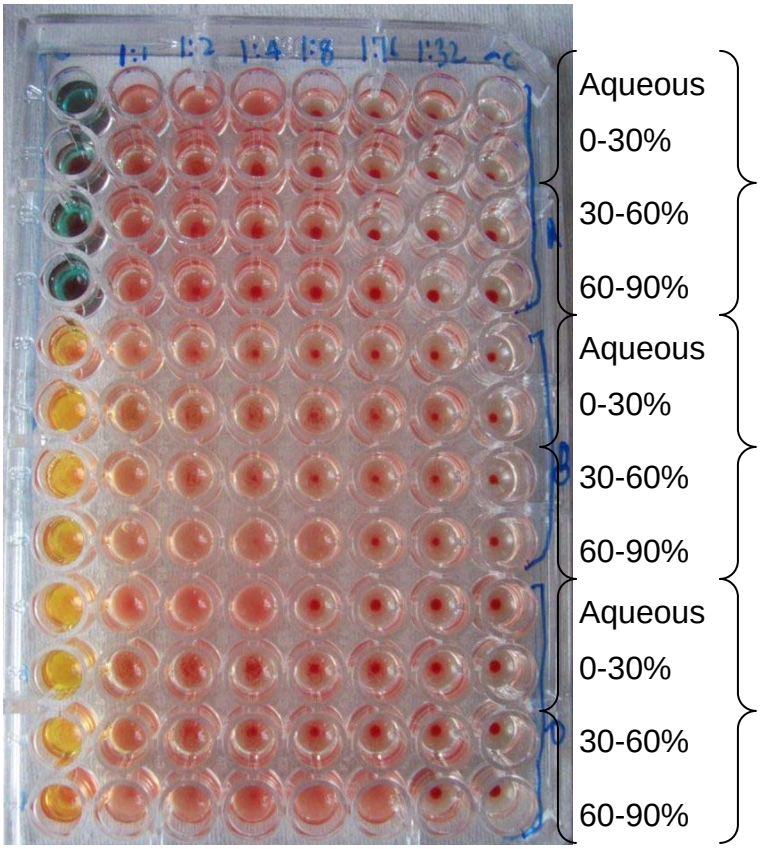



Fig 4.2:- Hemagglutination assay of different fractions isolated from *C. sertularioides.*

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Purification using DEAE-Cellulose column

The 60-90% ammonium sulfate fraction of *Ulva fasciata* was subjected to Ion-Exchange column chromatography on a DEAE-Cellulose column. Resin was equilibrated in 0.15 M PBS at pH 7.4 until absorbance was reduced to zero. Precipitated protein 60-90% was loaded on equilibrated column and washing was continued until absorbance was reduced to zero. Elution was carried out at 200µl/min and bound protein was eluted by linear NaCl gradient 0.5M-1.5M. 2ml fractions were collected.

Elution profile of agglutinin from DEAE-Cellulose column is shown in Fig 4.3. and protein was estimated by absorbance. Two peaks were observed. The first peak denotes the proteins which failed to bind to column and was eluted in the flow through. The bound proteins were eluted in one peak. Peak of the protein was eluted approximately when the salt concentration was 0.7M. Agglutinating activity was also detected in this single peak and amounted to seven individual fractions. The resolved fractions were tested for both absorbance and activity. These fractions were pooled and stored at -20 C for further characterization after determining protein concentration and checking for hemagglutination activity.

Assay for agglutination of the column fraction gave results very similar to the positive control as seen in fig 4.4.

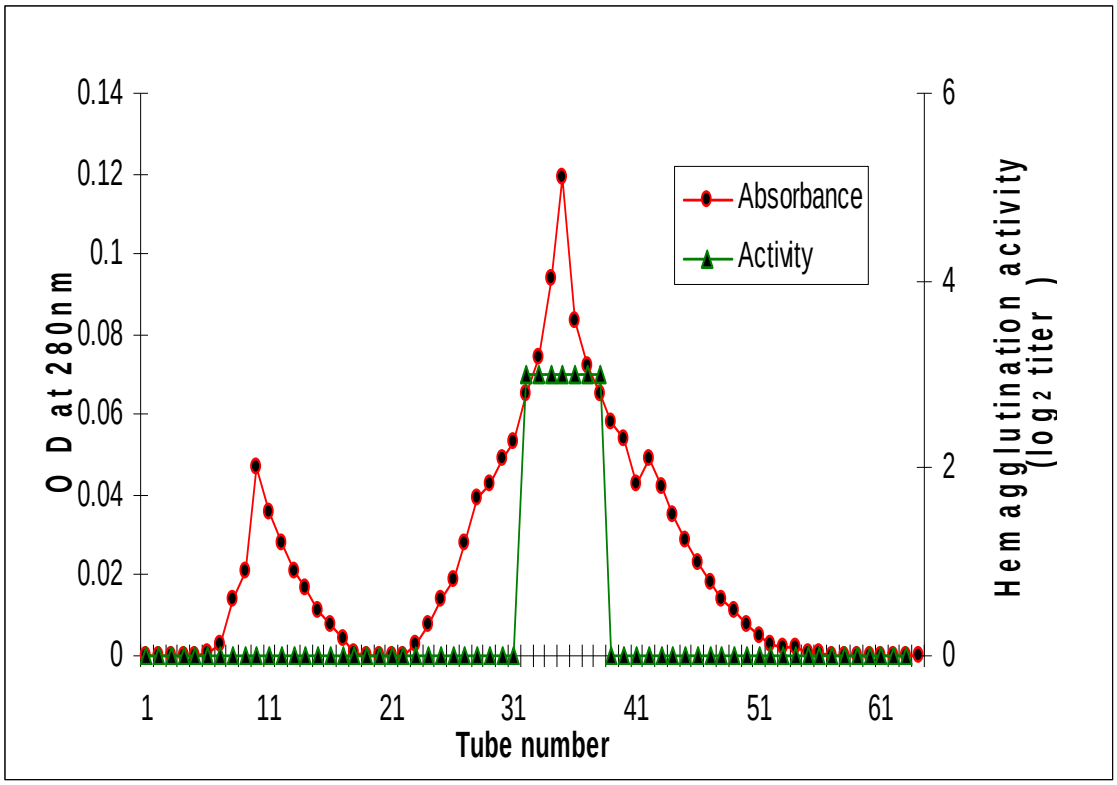


Fig 4.3:- DEAE -cellulose column chromatography of X5 60-90 % saturated ammonium sulfate precipitate. ●—● absorbance at 280 nm; hemagglutination activity against human blood cells ⟷

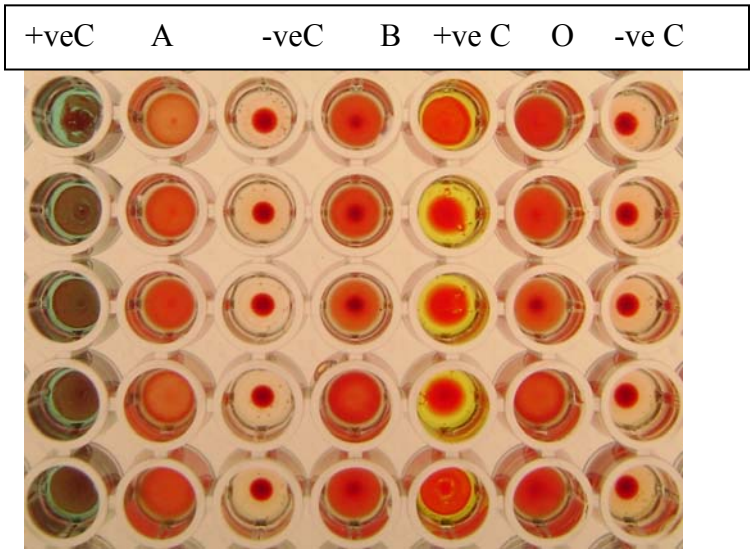


Fig 4.4:- Hemagglutination activity of column fractions *UFH*

The concentration of protein recovered and the corresponding agglutinating activity during the sequential steps of purification is summarized in fig 4.5.

Determination of protein and yields

Protein extracts from *Ulva fasciata* were precipitated with increasing quantities of ammonium sulphate and purified using column chromatography. During ammonium sulphate precipitation, highest hemagglutinating activity was observed in fractions precipitated with 60-90% ammonium sulphate whereas the 0-30% fractions showed moderate activity and minimal activity was observed in the 30-60% fractions.

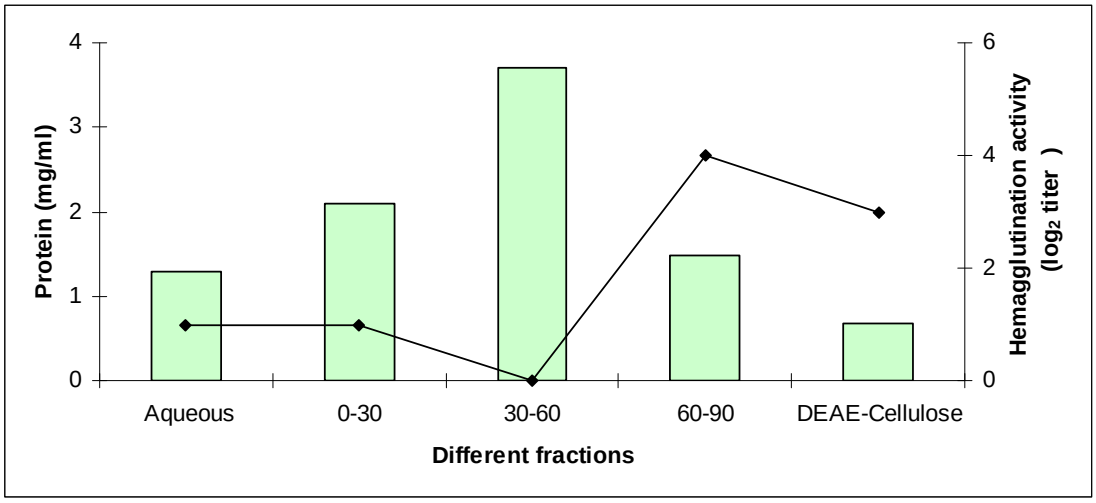


Fig 4.5:- Aqueous extract, ammonium sulfate precipitation and purification steps of hemagglutinins from *U. fasciata*. Protein concentration (bars) and hemagglutination activity (circles) were detected from various steps.

Estimation of protein concentration was done using standard graph of BSA. The concentration of protein and yield is summarized in Table 4. 2.

Table 4.2:- Protein yields of UFH from *U. fasciata*

| Fraction | Total Protein (mg) | Activity (HU) | Specific activity ($HUmg^{-1}$) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Aqueous extract | 39 | 60 | 1.538 | 1 | 100 |
| 60-90% of $(NH_4)_2SO_4$ | 5.9 | 64 | 10.84 | 7.1 | 106 |
| DEAE-Cellulose | 2.7 | 32 | 11.85 | 7.7 | 53.3 |

HU-Hemagglutination Unit

### 4.3B. Purification of *C. sertularioides*

Purification using DEAE-Cellulose

The 60-90% ammonium sulfate fraction of *C. sertularioides* was subjected to Ion-Exchange column chromatography on a DEAE-Cellulose column. Resin was equilibrated in 0.15 M PBS at pH 7.4 until absorbance was reduced to zero. Precipitated protein 60-90% was loaded on equilibrated column and washing was continued until absorbance was reduced to zero and bound protein was eluted by increasing NaCl gradient. Elution was carried out at 200µl/min and bound protein was eluted by linear NaCl gradient 0.5M-1.5M. 2ml fractions were collected. Elution profile of agglutinin from DEAE-Cellulose column is shown in Fig 4.6.

Two peaks were observed. The first peak denotes the proteins which failed to bind to column and were eluted in the flow through. The bound proteins were

eluted in one peak. The peak of the protein was eluted approximately when the salt concentration was 0.8M. Agglutinating activity was also detected in this single peak and amounted to five individual fractions. The resolved fractions were tested for both absorbance and activity. These fractions were pooled and stored at -20 C for further characterization after determining protein concentration and checking for hemagglutination activity.

Assay for agglutination of the column fraction gave results very similar to the positive control as seen in fig 4.7. First columns are positive control, second columns and fourth columns are purified factions and third row negative control.

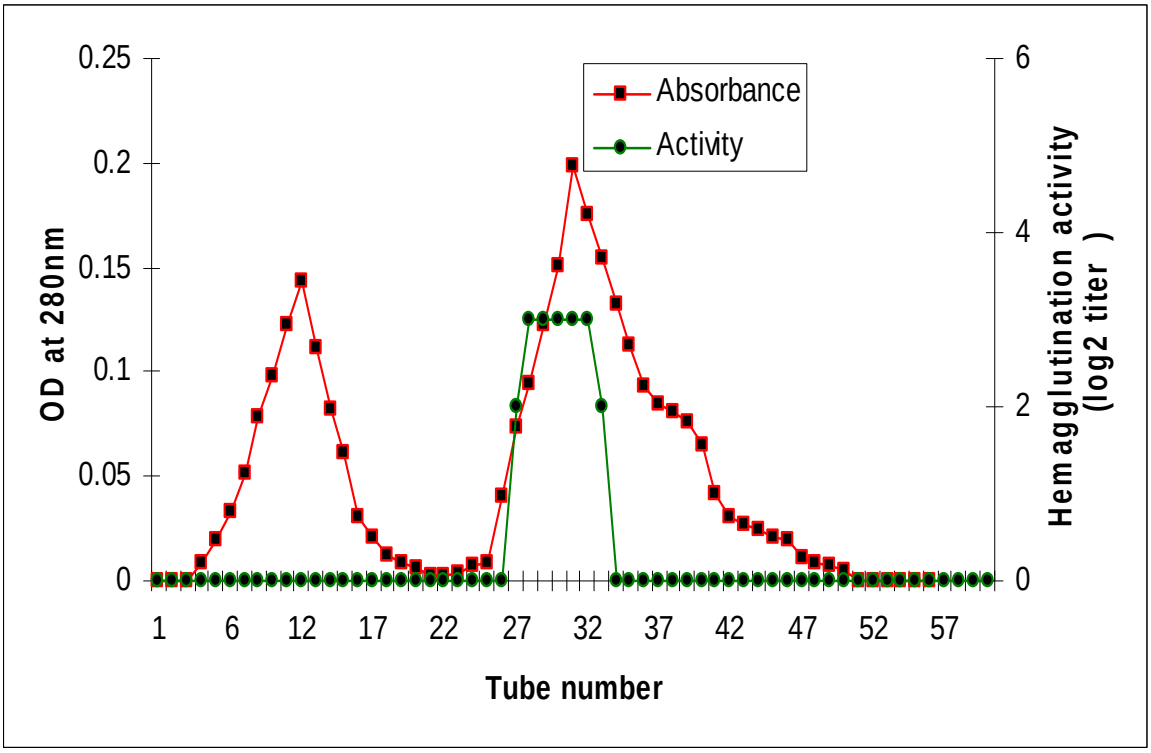


Fig 4.6:- DEAE -cellulose column chromatography of *C. sertularioides* 60-90 % saturated ammonium sulfate precipitate. ●——● absorbance at 280 nm; hemagglutination activity against human blood cells ◄——►

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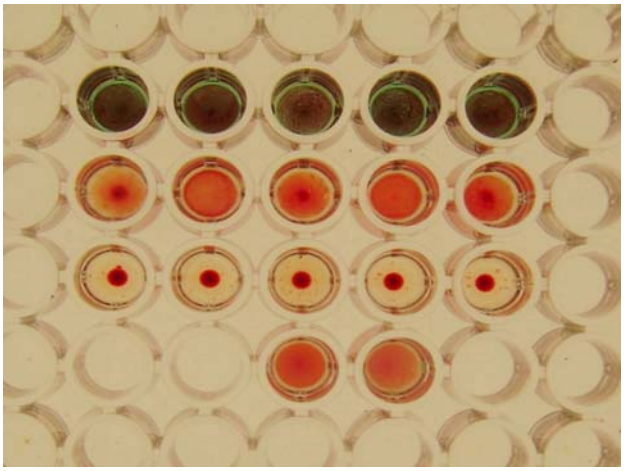

Fig 4.7:- Hemagglutination assay of fractions obtained 27 – 33

Determination of protein and yield

The concentration of protein recovered and the corresponding agglutinating activity during the sequential steps of purification is summarized in fig 4.8.

Protein extracts from *C. sertularioides* were precipitated with increasing quantities of ammonium sulfate and purified using column chromatography. During ammonium sulfate precipitation, highest hemagglutinating activity was observed in fractions precipitated with 60-90% ammonium sulfate whereas the 0-30% fractions showed moderate activity and minimal activity was observed in the 30-60% fractions.

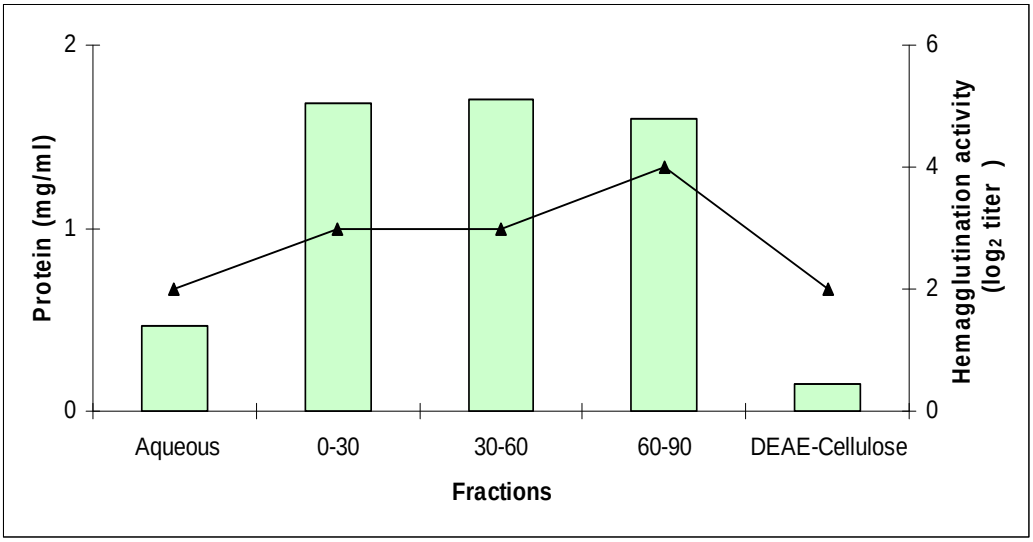


Fig 4.8 Bar graph of *C. sertularioides* hemagglutinins with respect to protein/titer activity, different fractionations of CSH with the associated yield of protein (bar) and the respective hemagglutination titer (triangle).

Estimation of protein concentrations was done using standard graph of BSA. The concentration of protein and yield is summarized in Table 4. 3.

Since fractions yielded in a single peak in observation and activity this column purified fraction was termed as UFH and CSH.

Table 4.3 Protein yields of CSH from *C. sertularioides*

| Fraction | Total Protein (mg) | Activity (HU) | Specific activity ($HUmg^{-1}$) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Aqueous extract | 20.1 | 60 | 2.985 | 1 | 100 |
| 60-90% of $(NH_4)_2SO_4$ | 2.16 | 24 | 11.11 | 3.7 | 40 |
| DEAE-Cellulose | 1.68 | 24 | 14.28 | 4.8 | 40 |

This chapter deals with various methodologies employed and results obtained in the attempt to characterize the agglutinins extracted from *Ulva fasciata* and *Caulerpa sertularioides* and purified by column chromatography.

This chapter is divided into three main sections as indicated below:

**5.1 Introduction**

**5.2 Materials and methods employed for both were similar**

5.2a. Determination of molecular weight

5.2b. Determination of optimal pH for lectin activity

5.2c. Study of thermal stability

5.1d. Effect of metal ions and chemical reagents

5.2e. Determination of Sugar specificity

5.2f. Amino acid analysis of UFH

5.2g. Qualitative detection of glycoproteins

5.2h. Estimation of carbohydrates

**5.3 Results**

## 5.1 Introduction

Lectins are present in a wide range of organisms from bacteria to animals, being present in all classes and families, although not in all the kinds and species (Sharon and Lis, 2003). Lectins from terrestrial plants and animals have been isolated, characterized and exploited extensively in many aspects of biochemistry and biomedicine. Compared with land plant lectins described as early as 19$^{th}$ century, the occurrence of lectins from marine algae, however, was first reported by Boyd et al (1966). The first agglutinin from marine algae was isolated and characterized by Rogers et al, (1977).

Smilar to land plants, lectins from marine alga reveal a proteinaceous nature but differ in some of their properties. They generally have lower molecular masses than most land plant lectins and are more specific for complex oligosaccharides or glycoproteins. Further, most of marine algal lectins do not require divalent cations for their biological activity (Rogers & Hori, 1993). They occur mainly in monomeric form and have a high content of acidic amino acid, with isoelectric points from 4 to 6 (Rogers & Hori 1993). Although several studies on lectins from marine algae have been reported till date, few lectins from algae have been characterized in detail.

Gel electrophoresis is often used after each step of a purification protocol to assess the purity of the extracted product. A pure protein gives a single band on a PAGE, unless the molecule is made up of two unequal subunits. Electrophoresis describes the migration of a charged particle under the influence of an electric field. Many important biological molecules posess

ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations or anions. Under the influence of an electric field these charged particles migrate either to the cathode or to the anode, depending on the nature of their net charge (Janson and Ryden, 1989).

SDS-PAGE is the most frequently used gel system for studying proteins, the method is of no use if one is aiming to detect a particular protein on the basis of its biological activity. In this case it is necessary to use native (non-denaturing) condition. In non-denaturing PAGE proteins separate according to their different electrophoretic mobilities and the sieving effect of the gel. It is not possible to predict the behavior of a given protein in a gel but, because of the range of different charges and sizes of proteins in a given protein mixture, good resolution is achieved. In non-denaturing gel SDS is absent and the proteins are not denatured prior to loading.

SDS-PAGE is the most widely used method for analyzing protein mixtures qualitatively. It is particularly useful for monitoring protein purification and, because the method is based on the separation of proteins according to size, the method can also be used to determine the relative molecular mass of proteins (Wilson and Walker, 2005).

The hallmark of hemagglutinins (also called anti carbohydrate antibodies) is the ability to bind carbohydrates specifically and reversibly (Sharon and Lis, 2003). Carbohydrate specificity of hemagglutinin is customarily performed by the hapten-inhibition techniques, in which different monosaccharides,

oligosaccharides or glycoproteins are tested for their ability to inhibit either hemagglutination or precipitation by the lectin.

Protein purification and characterizations are generally a multi-step process exploiting a wide range of biochemical and biophysical characteristics of the target protein. Each protein offers its own unique set of physicochemical characteristics which are important to be considered in it's application. This chapter describes the biochemical characterization in terms of physico-chemical properties as well as determination of the molecular weight of the agglutinins extracted from *Ulva fasciata* & *Caulerpa sertularioides* respectively.

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**5.2 Materials and Methods**

The chemicals used for the characterization of purified lectin Sodium citrate, Sodiium acetate, EDTA, Calcium chloride, Magnesium chloride, Manganese chloride, Mercuric chloride, Nickel, Zinc Chloride, Di-sodium Hydrogen Phosphate, Sodium Hydrogen Phosphate , Sodium Chloride, Cobaltous chloride, Tris-Base, β-mercaptoethanol electrophoresis grade, Ammonium persulfate (APS) (electrophoresis grade), N, N, N', N'-tetramethylethylenediamine (TEMED), Glycine (electrophoresis grade), Acetic acid, Ethanol, Methanol, Sodium thiosulphate, Silver nitrate, Formaldehyde, Sugars monosaccharide all were obtained from Himedia Labs, Mumbai, India. Folin-Ciacalteau's phenol reagent was obtained from Sisco Research laboratories PVT. Ltd. Potassium sodium tartarate, Sodium hydroxide, Sodium carbonate anhydrous, periodic acid, Thymol all were

obtained from Merk Pvt. Ltd. Glycoproteins (Avidin from egg white, Mannan from S. cerevisae, Trypsin inhibitor, Mucin from bovine submaxillary gland and Fetuin), Sodium Dodecyl sulfate, Acrylamide and Bisacrylamide were obtained from Sigma- Aldrich Chemicals, USA. Spacer, Comb, glass plate, notch glass plate, elecophoretic chamber and standard protein markers Carbonic Anhydrase (29000 Da), Soyabean Trypsin inhibitor (20100 Da), Lysozyme (14300 Da), Aprotinin (6500Da) and Insulin (3000 ka) were obtained from Genei, Banglore, India

**5.2a.** Determination of molecular weight

**5.2a1.** Non-denaturing PAGE (Native PAGE)

The confirmation of purification was done using 12 % native PAGE (appendix 8.1 I and II gel preparation) as described by Laemmli (1970). Samples were prepared in Tris-HCl buffer, pH 6.8 without SDS and mercaptoethanol. Purified proteins were loaded prior without heat treatment. The gel was electrophoresed at 50 Volt till the dye front reached the resolving. Then the voltage was increased to 100 volt and electrophorised until the dye reached the end of the gel. Protein bands were visualized by the standard silver staining (appendix 10) method described by Blum *et al.* (1987).

**5.2a2.** Denaturing PAGE (SDS-PAGE)

Molecular weight was determined using SDS- PAGE in a 12 % gel (appendix 9.1, 9.2) as described by Laemmli (1970). Samples and standards were prepared in Tris- HCl buffer, pH 6.8, containing 10% SDS and 5% 2-mercaptoethanol (appendix 9.3) and heated at 100 ºC for 5 minutes. Standard protein markers used was a mixture consisting of ovalbumin (MW 43,000),

carbonic anhydrase (MW 29,000), soyabean trypsin inhibitor (MW 20,100), lysozyme (MW 14,300), aprotinin (MW 6,500) and insulin (MW 3,000). The gel was run on 50 Volt till the dye reached stacking gel, followed by 100 Volt until dye reached the bottom. Protein bands were visualized by the standard silver staining (appendix 10) method described by Blum *et al.* (1987).

**5.2b** Determination of optimal pH for lectin activity

Activity of both agglutinins was tested at different pH using different buffers. 1ml each of the purified lectin was dialyzed, at 4$^{o}$C for 24 hrs, against 500 ml (100mM) of Sodium citrate, pH 9.0 and 10.5 (Appendix 11, 12 and 13 ), Phosphate buffer at pH 6.0, 7.0 and 8.0 and Sodium acetate at pH 5.0. Hemagglutinating activity was tested by addition of 2% erythrocyte suspension and incubating the mixture at room temperature for 1 hour as detailed in section 3.2.

**5.2c** Thermal stability

1ml of lectin was incubated at 40, 60, 80 and 100$^{o}$C for 10, 20 and 30 minutes respectively. It was cooled at room temperature for five minutes and then assayed for agglutinating activity and the titer was determined.

**5.2d** Effect of metal ions and chemical reagents

Effect of EDTA and divalent cations for lectin activity

To evaluate the effect of metal ions and EDTA on the activity (Kawakubo et al., 1999), each of the purified lectin was dialyzed against 5mM EDTA in 0.15 M PBS (pH 7.4) for 16 h at 4 $^{o}$C, the dialyzed fractions were used for

hemagglutination assay in the presence and absence of 5mM each of $CaCl_2$, $MnCl_2$, $Zn^+$, $Mg^+$, $Co_2^+$ and Ni.

**5.1e.** Determination of Sugar specificity

In an attempt to identify the specificity of these agglutinins, hemagglutination-inhibition test was done. The monosaccharides tested were D-glucose, D-galactose, D-mannose, L-fucose, D-fucose, L-rhamnose, N-acetyl-D-glucosamine, N-acetyl neuramic acid, D (+) galacturonic acid, D (+) glucosamine, D(+) gulactosamine, D (+) glucoronic acid. Few glycoproteins were also tested and these were avidin from egg white, mannan from *S. cerevisae,* trypsin inhibitor, mucin from bovine submaxillary gland and fetuin (Sigma Chemicals). The test was performed in a manner analogous to the hemagglutination test. Serial two-fold dilutions of test monosaccharides and glycoproteins were prepared in PBS, pH 7.4. Each dilution was mixed with an equal volume (50 μl) of a solution of the hemagglutinin. The mixture was allowed to stand for 30 min at room temperature before mixing with 50 μl of human erythrocyte suspension. The plates were mixed and allowed to sit at room temperature for 1 hr. Control designed to record positive was erythrocytes with PBS and negative was erythrocytes with Con-A and erythrocytes with anti-sera of respective blood groups. The minimum concentration of the test sugars and glycoproteins in the final reaction mixture, which completely inhibited the lectins were recorded.

**5.2f.** Amino acid analysis

Single band obtained on SDS-PAGE of UFH was carefully picked up and submitted for amino acid analysis and protein sequencing using Mass spectroscopy LCMS to TCGA, Delhi. Amino acid similarity search was carried out using MASCOT search. Proteins were identified through searching against the protein databases using MASCOT software (http://www.matrixscience.com).

**5.2g.** Qualitative determination of glycoprotein

Two different methods were used for the detection of glycoprotein using non-denaturing PAGE; Thymol-Sulphuric acid staining (TSA) method (Rauchsen, 1979) and Alcian blue staining.

TSA method

Non-denaturing PAGE was performed (Appendix 8.1). The gel was fixed by submerging in fixative (25% 2-proponal + 10% acetic acid + 65% distilled water), and rocking gently for 2 hr at room temperature. The gel was observed to have shrunk significantly. This procedure was repeated twice to leach maximum Tris-glycine from the gel. It was immersed in the solution (25% 2-proponal + 10% acetic acid + 65% distilled water) containing 0.2% (v/v) thymol for 90 minutes with gentle rocking. Then, the gel was soaked in 80% sulphuric acid + 20% ethanol until the gel clarified and glycoprotein appeared as pink/red band.

Alcian blue staining

12 % Non-denaturing PAGE was performed (Appendix 8.1) and the gel was stained with 0.25mg Alcain blue in fixative (Methanol: Acetic acid: Distilled water, 50:10:40) and further destained using Methanol: Acetic acid: Distilled water (50:10:40).

**5.2h.** Estimation of carbohydrates

Neutral sugar content of the different extracts obtained through sequential purification was estimated by the phenol-sulphuric acid method (Dubois et al. 1956) using D- glucose as a standard.

## 5.3 Results

### 5.3.A Characterization of *U. fasciata* hemagglutinin (UFH)

Determination of molecular weight

Non-denaturing (Native PAGE)

The extract obtained from sequential purification was resolved by native PAGE and stained by silver staining. The results obtained may be seen in fig 5.1. It was observed that the number of bands stained and detected decreased from aqueous extract in lane 4 to purified UFH in lanes 1-3. Lane 5 contains the ammonium sulfate fraction (60-90%). The single band that was observed with the purified extracts was seen to be present in all three extracts through the purification process.

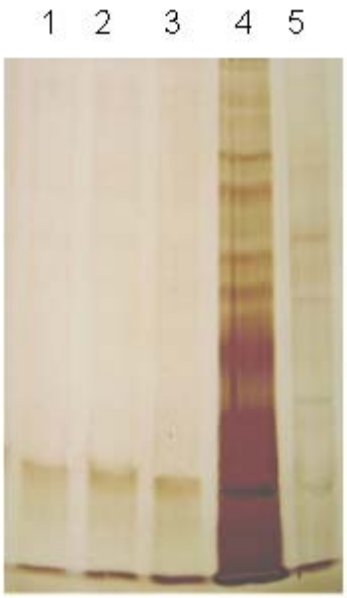


**Fig. 5.1** Native PAGE profile of *U. fasciata*.

Denaturing SDS-PAGE

Further the proteins were denatured using mercaptoethanol and run along with standard molecular weight marker on SDS-PAGE. As observed in the native PAGE profile the single band of purified protein lane 4 Fig 4.2 was coinciding with the bands in lane 1 and 3 (aqueous extract and ammonium sulfate fraction 60-90% ). The band of UFH in lane 4 coincided with the band equivalent to 14.3 kDa of the standard molecular weight marker that was run. Molecular weight of the purified lectin was thus estimated as ≈14.5 kDa. The single band in SDS-PAGE suggests that the lectin is purified and is a monomeric protein. This purified lectin from *Ulva fasciata* was designated as UFH (*Ulva fasciata hemagglutinin)*

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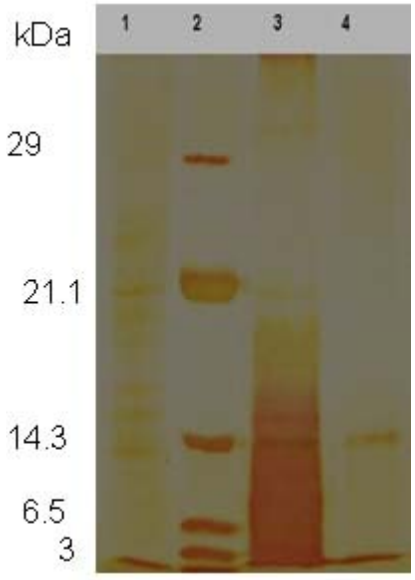



**Fig 5.2:-** SDS-PAGE profile of UFH.

Determination of optimal pH for lectin activity

The effect of pH on agglutination activity and stability of the UFH activity was assayed at pH values ranging from 5.0 to 10.5 as described earlier. The results obtained are presented in Figs 5.3 and 5.4. UFH was active over a fairly broad pH range of 6.0 to 10.5. This activity was lost at acidic pH below 6.0.

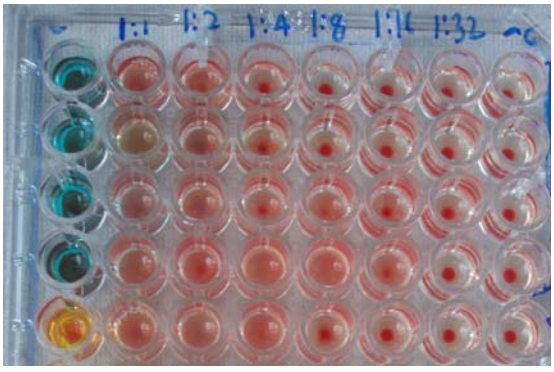


Fig 5.3:- Effect of pH on hemagglutination activity

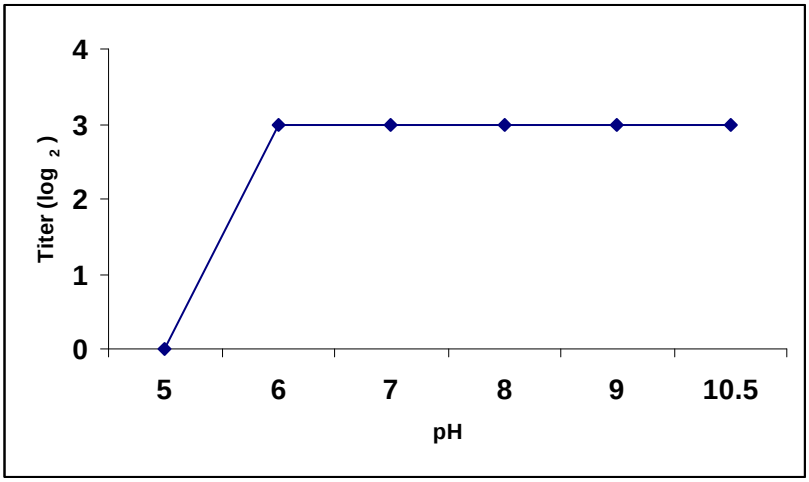



Fig 5.4 Effect of pH on hemagglutinating titer of UFH

Thermal stability

In assessing the heat stability of UFH the results obtained are tabulated in Table 5.1 and represented in Fig 5.6. As can be seen in Fig 5.5, activity as determined by hemagglutination titer remained stable and constant at temperatures 40-80 $^{o}$C even after warming for 30 mins. However, on heating at 100 $^{o}$C , for 30 mins, the activity was retained but seemed less effective in agglutinating giving a hemagglutination titer of 1:4 as seen in row 14 of the plate in Fig 5.5.

Table 5.1- Effect of temperature on hemagglutination titer activity

| **Temperature ($^{o}$C)** | **HA titer after 10 minutes** | **HA titer after 20 minutes** | **HA titer after 30 minutes** |
|---|---|---|---|
| 40 $^{o}$C | 1:8 | 1:8 | 1:8 |
| 60 $^{o}$C | 1:8 | 1:8 | 1:8 |
| 80 $^{o}$C | 1:8 | 1:8 | 1:8 |
| 100 $^{o}$C | 1:8 | 1:8 | 1:4 |

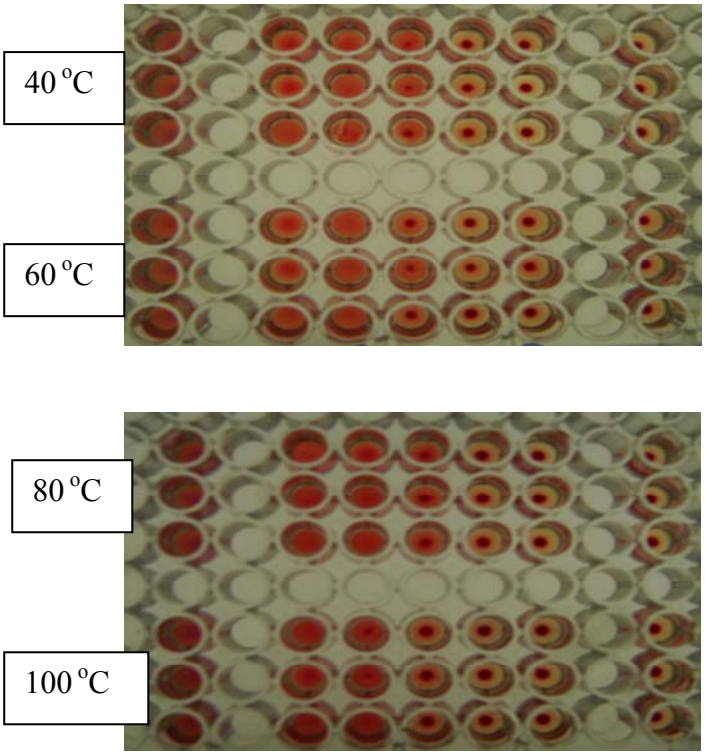



Fig 5.5:- Hemagglutination assay for thermal stability of UFH

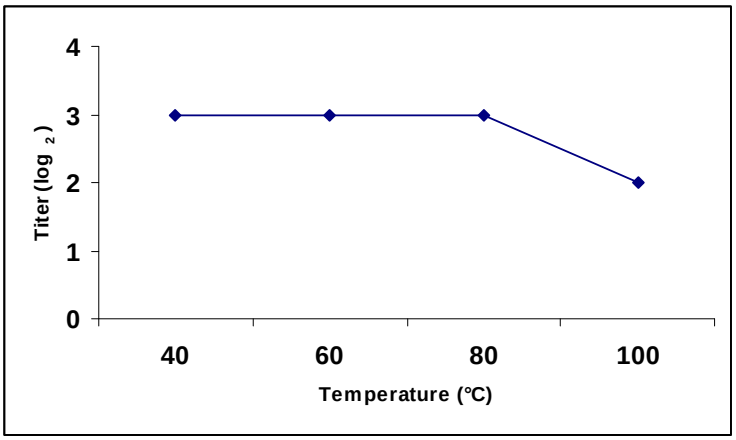



Fig 5.6 Effect of temperature (for 30 min) on hemagglutinating titer activity of UFH

Effect of metal ions and chemical reagents

Hemagglutinating activity appeared unaffected by treatment with EDTA or addition of divalent cations such as 5mM each of $CaCl_2$, $MnCl_2$, $Zn^+$, $Mg^+$, $Co_2^+$ and Ni (fig 5.7). The results obtained indicate that *U. fasciata* lectin does not require metal ions for it's agglutinating activity.

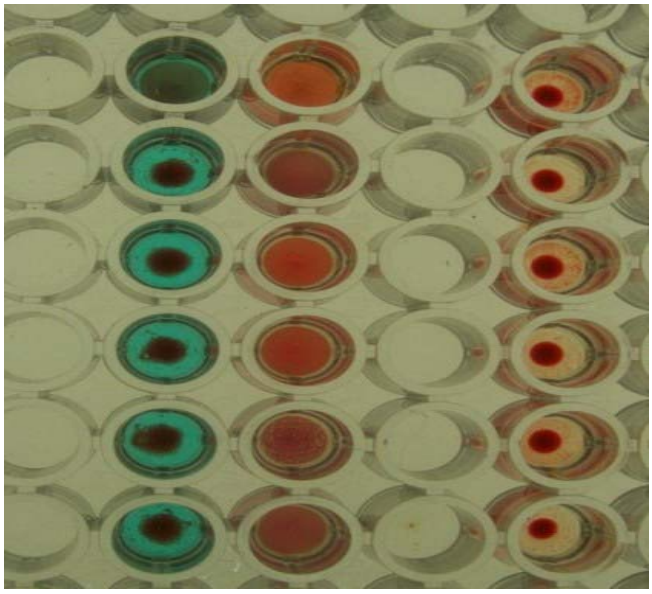


Fig 5.7:- Effect of metal ions on hemagglutination activity

Assay for sugar Inhibition

Binding specificity of UFH for carbohydrates was tested using a hemagglutination inhibition test, which revealed that the hemagglutination activity of UFH was not inhibited by any of the tested monosaccharides and glycoproteins. To confirm this experiment, Con A was used as a control and its agglutinating activity was inhibited (Fig 5.8). Binding of Con A to the RBC was inhibited by D-mannose and L-rhamnose as seen in lanes 3, row numbers 3 and 6. This experiment was done in duplicate.

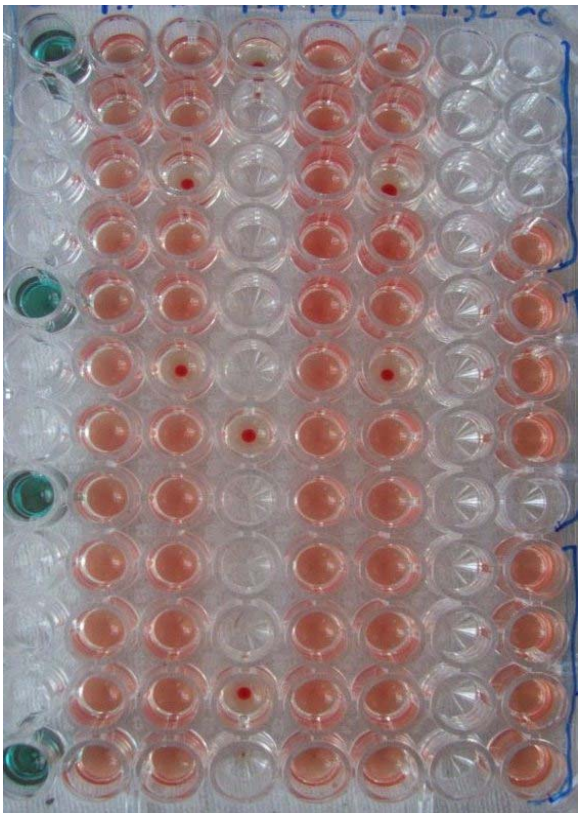


Fig 5.8:- Assay for Sugar inhibition

Amino acid analysis of UFH

Purified UFH migrated in SDS-PAGE as a single band of molecular weight 14.5 kDa and this band was used for analysis of N-terminal amino acid sequence.

It showed significant peptide hits:-

**gi|452341** type II light-harvesting chlorophyll a /b-binding protein [Zea mays]

**gi|1006704** light-harvesting chlorophyll a/b binding protein of photosystem II [Pseudotsuga**}**

**gi|3928891** chlorophyll a/b binding protein [Acetabularia acetabulum]

**gi|11467117** ribosomal protein L2 [Marchantia polymorpha]

**Peptide Summary Report**

Significance threshold p< 0.05 maximum number of hits 10

Standard scoring MudPIT scoring Ions score cut-off 0 Show sub-sets

It showed pop-ups suppress pop-ups sort unassigned decreasing Score

required bold red

**1.** gi|452341 **Mass:** 24824 **Score:** 106 **Queries matched:** 3

| Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|---|---|---|---|---|---|---|---|---|---|
| 553 | **939.07** | **1876.12** | **1874.98** | **1.14** | **0** | **70** | **0.00017** | **1** | **R.WAMLGALGCVFPEILAK.N** |
| 561 | **947.46** | **1892.90** | **1890.97** | **1.93** | **0** | **(61)** | **0.0015** | **1** | **R.WAMLGALGCVFPEILAK.N + Ox** |
| 683 | **947.46** | **2839.35** | **2838.49** | **0.86** | **1** | **36** | **0.28** | **1** | **R.ELEVIHSRWAMLGALGCVFPEILAK** |

type II light-harvesting chlorophyll a /b-binding protein [Zea mays]

**Proteins matching the same set of peptides:**

gi|3386569 **Mass:** 20513 **Score:** 106 **Queries matched:** 3

photosystem II type II chlorophyll a/b binding protein [Sorghum bicolor]

**2.** gi|1006704 **Mass:** 25506 **Score:** 106 **Queries matched:** 3

light-harvesting chlorophyll a/b binding protein of photosystem II

[Pseudotsuga menziesii]

| Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|---|---|---|---|---|---|---|---|---|---|
| 553 | **939.07** | **1876.12** | **1874.98** | **1.14** | **0** | **70** | **0.00017** | **1** | **R.WAMLGALGCVFPEILAK.N** |
| 561 | **947.46** | **1892.90** | **1890.97** | **1.93** | **0** | **(61)** | **0.0015** | **1** | **R.WAMLGALGCVFPEILAK.N + Ox** |
| 683 | **947.46** | **2839.35** | **2838.49** | **0.86** | **1** | **36** | **0.28** | **1** | **R.ELEVIHSRWAMLGALGCVFPEILAK** |

**Proteins matching the same set of peptides:**

gi|1262847 **Mass:** 26815 **Score:** 106 **Queries matched:** 3

type 2 light-harvesting chlorophyll a/b-binding polypeptide [Pinus palustris]

gi|9719392 **Mass:** 16723 **Score:** 106 **Queries matched:** 3

chlorophyll a/b-binding protein [Picea glauca]

gi|53627210 **Mass:** 27222 **Score:** 106 **Queries matched:** 3

chloroplast light harvesting chlorophyll a/b binding protein [Pinus chiapensis]

gi|53627212 **Mass:** 27248 **Score:** 106 **Queries matched:** 3

chloroplast light harvesting chlorophyll a/b binding protein [Pinus flexilis]

**3.** gi|3928891 **Mass:** 26917 **Score:** 58 **Queries matched:** 1

Chlorophyll a/b binding protein [Acetabularia acetabulum]

| Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|---|---|---|---|---|---|---|---|---|---|
| 210 | 485.50 | 968.98 | 967.51 | 1.47 | 0 | 58 | 0.0034 | 1 | R.ETELIHAR.W |

**Proteins matching the same set of peptides:**

gi|145079101 **Mass:** 21324 **Score:** 58 **Queries matched:** 1

TPA: TPA_inf: chloroplast light-harvesting complex II protein precursor

Lhcbm1 [Acetabularia acetab

gi|145079310 **Mass:** 22369 **Score:** 58 **Queries matched:** 1

TPA: TPA_inf: chloroplast light-harvesting complex II protein precursor

Lhcbm3 [Acetabularia acetab

gi|145079419 **Mass:** 26613 **Score:** 58 **Queries matched:** 1

TPA: TPA_inf: chloroplast light-harvesting complex II protein precursor

Lhcbm2 [Acetabularia ac

**4.** gi|11467117 **Mass:** 55989 **Score:** 50 **Queries matched:** 2

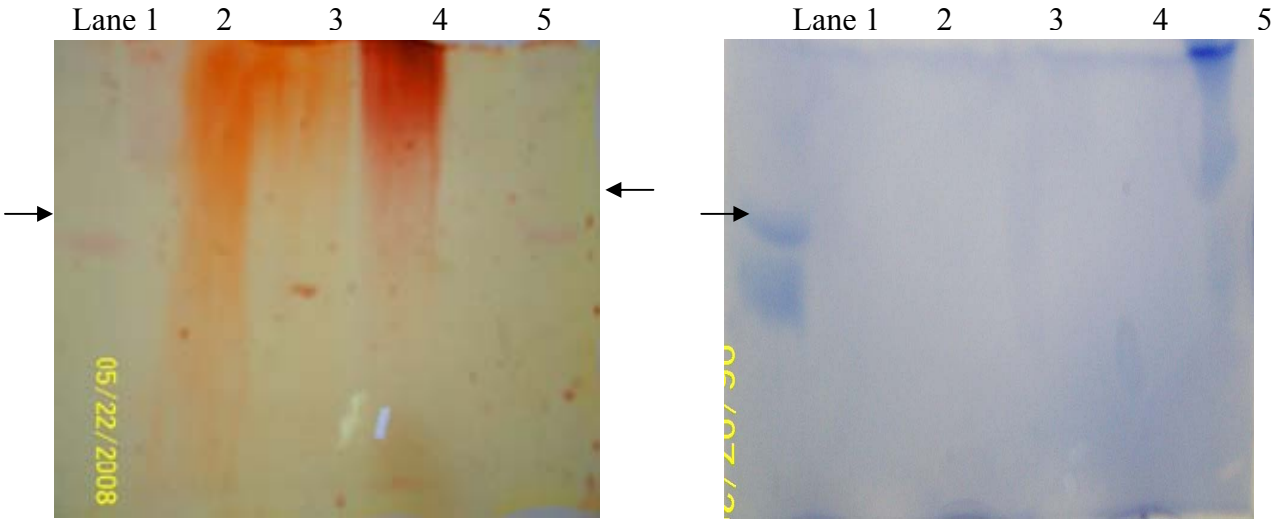
Ribosomal protein L2 [Marchantia polymorpha]

| Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|---|---|---|---|---|---|---|---|---|---|
| 507 | **844.53** | **1687.05** | **1685.77** | **1.28** | **0** | **46** | **0.044** | **1** | **R.GVAMNPVDH PHGGGEGR.T** |

The N- terminal peptide sequence of the UFH lectin does not show amino acid sequence similarity to any known plant and animal lectins. Hence, this protein might be the paradigm of a novel lectin family.

Quantitative detection of glycoprotein

Lectins being glycoproteins a qualitative assay for the detection of glycoproteins was done on non denaturing PAGE along with standard glycoprotein obtained from Sigma chemicals. Staining of the gel with both thymol sulphuric acid as well as alcian blue yielded a single band respectively, for the standard glycoprotein in lane 1 (fig 5.9a) and lanes 1 and 5 (fig 5.9b). UFH could not be resolved as a single band by either of the staining procedures. However, a smear was observed in lanes 2 to 4 in the gel stained by thymol sulphuric acid. No smear nor band was stained by alcian blue.




5.9a 5.9b

Fig 5.9 Glycoprotein profile of UFH

5.9a. Thymol sulphuric acid stain:- Lane 1 and 5 standard glycoprotein, lane 2 Aqueous, lane 3 ammonium sulfate fraction (60-90%), lane 4 column purified.

5.9b. Alcian blue stain:- Lane 1 and 5 standard glycoprotein, lane 2 Aqueous extract, lane 3 ammonium sulfate fraction (60-90%), lane 4 column purified.

Estimation of carbohydrates

Since the results of the qualitative test for confirmation of glycoproteins was not conclusive, content of neutral sugars in UFH was estimated by phenol sulphuric acid and the amount estimated is summarized in Table 5.2.

Table 5.2:- Estimation of neutral sugar in UFH

| Fraction | Neutral sugar mg/ml | Total sugar (mg) |
|---|---|---|
| Aqueous extract | 0.82 | 24.6 |
| 60-90% of $(NH_4)_2SO_4$ | 0.84 | 3.36 |
| DEAE-Cellulose | 0.76 | 3.04 |

### 5.3.B Characterization of agglutinin from Caulerpa sertularioides (CSH)

Determination of molecular weight

Non-reducing (Native PAGE) and denaturing SDS-PAGE

The extracts obtained through the sequential purification were loaded on native PAGE and stained by silver staining after completing the run. The results obtained may be seen in fig 5.10a. It was observed that the number of bands detected decreased from aqueous extract in lane 2 to purified CSH in lane 3. In lane 1was loaded the ammonium sulfate fraction.

Further the proteins were denatured and run along with standard molecular weight marker. On denaturation, the single band that was detected in native PAGE resolved into two bands (Fig 5.10b) coinciding with molecular weight markers of 16 kDa and 31.3 kDa, respectively. These results indicate that the agglutinin may be a dimmer with intra chain disulfide bonds.


Fig 5.10a


Fig 5.10b

**Fig. 5.5** PAGE profile of CSH. 5.10a Native PAGE, 5.10b SDS-PAGE

Determination of optimal pH for lectin activity

The effect of pH on agglutination activity and stability of the CSH, activity was assayed at pH values ranging from 3.0 to 10.5 as described earlier. The results obtained are presented in Fig 5.11 and 5.12. CSH was active over a fairly broad pH range of 5.0 to 10.5. This activity was drastically lost at pH below 5.0.

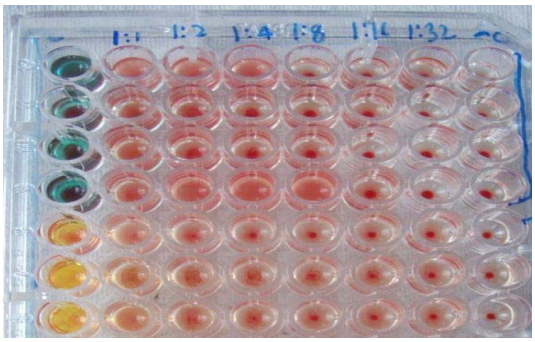



Fig 5.11:- Effect of pH on CSH titer activity

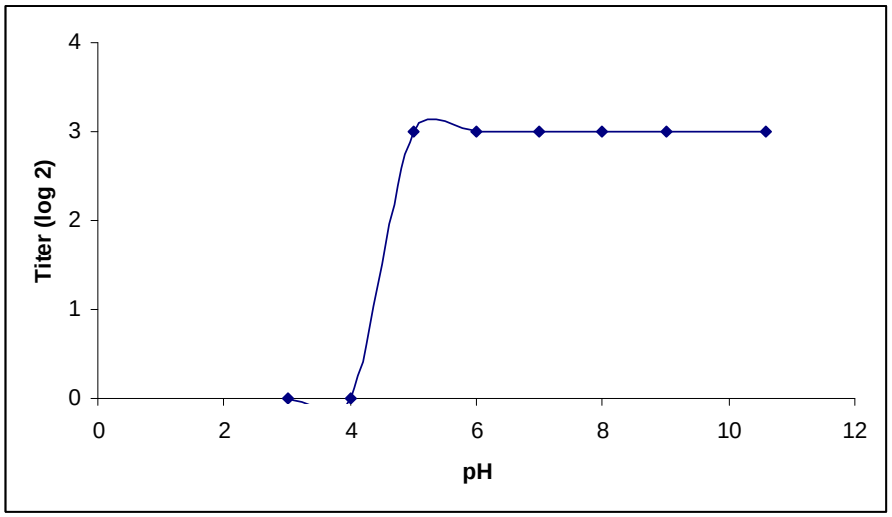



Fig 5.12 Effect of pH on hemagglutinating activity of CSH

Thermal stability of CSH

In assessing the heat stability the results obtained are represented in Fig 5.13 and 5.14. As can be seen in Fig 5.13, the activity as determined by hemagglutination titer remained stable and constant at temperature 40°C to 100°C even after worming for 30 min.

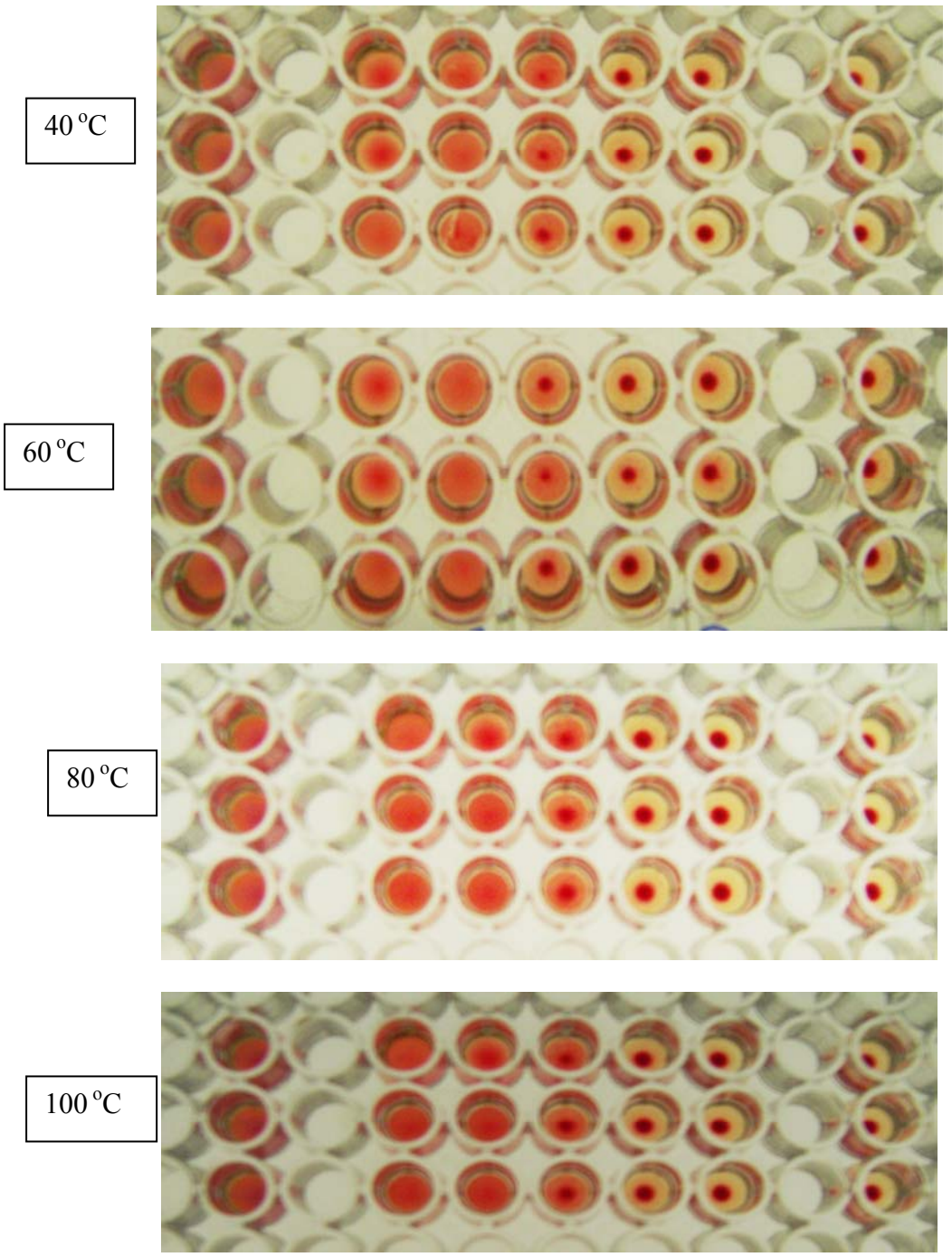



Fig 5.13:- Effect of thermal stability on CSH titer assay

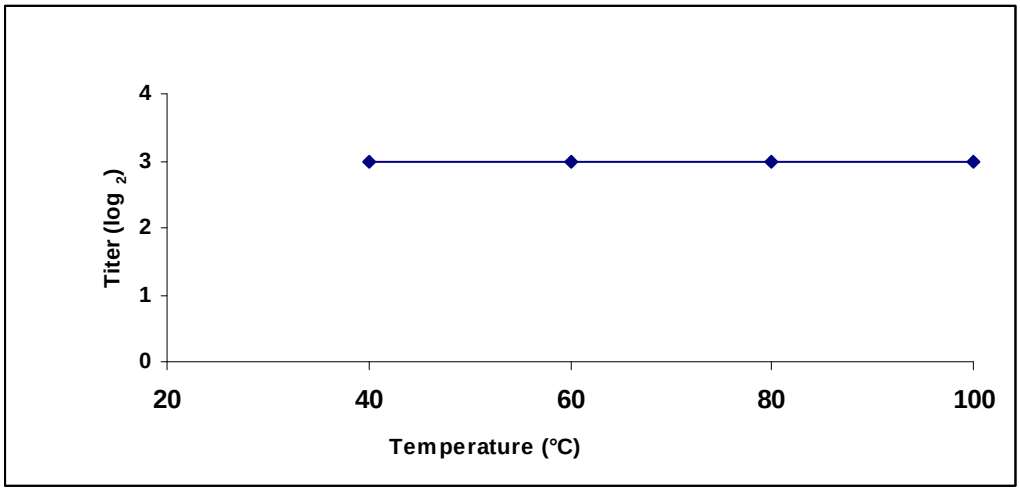



Fig 5.14 Effect of temperature (for 30 min) on activity of CSH

Effect of metal ions and chemical reagents

Hemagglutinating activity appeared unaffected by treatment with EDTA or addition of divalent cations such as 5mM each of $CaCl_2$, $MnCl_2$, $Zn^+$, $Mg^+$, $Co_2^+$ and Ni. The results obtained indicate that *C. sertularioides* lectin may not require metal ions for its agglutinating activity.

Sugar-inhibition assay

Twelve monosaccharides and six glycoproteins were included to be screened for specificity of CSH. The results obtained of the assay are shown in Fig 5.15 and Table 5.3. Screening of monosaccharides may be seen in plate 1 and glycoprotein in plate 2; lanes 1 and 2 in both plates contain negative and positive control respectively. Four different concentrations were tested and placed in lanes 3 to 6. In plate A inhibition is observed in row 7 to 10 though not all the concentrations and therefore not in all the lanes. These are

corresponding to N-acetyl–D-glucosamine, N-acetyl neuramic acid, D-galacturonic acid and D-glucosamine.

In plate B lane 1 and 2 are negative and positive controls respectively. Inhibition is observed in 1, 2 and 5 row of the experiment corresponding to glycoproteins avidin, fetuin and mucin from bovine submaxillary gland. Since agglutination by CSH was inhibited by specific monosaccharides and glycoproteins, the results indicate that CSH may be recognizing this carbohydrate when present on cell and by binding causes the agglutination.

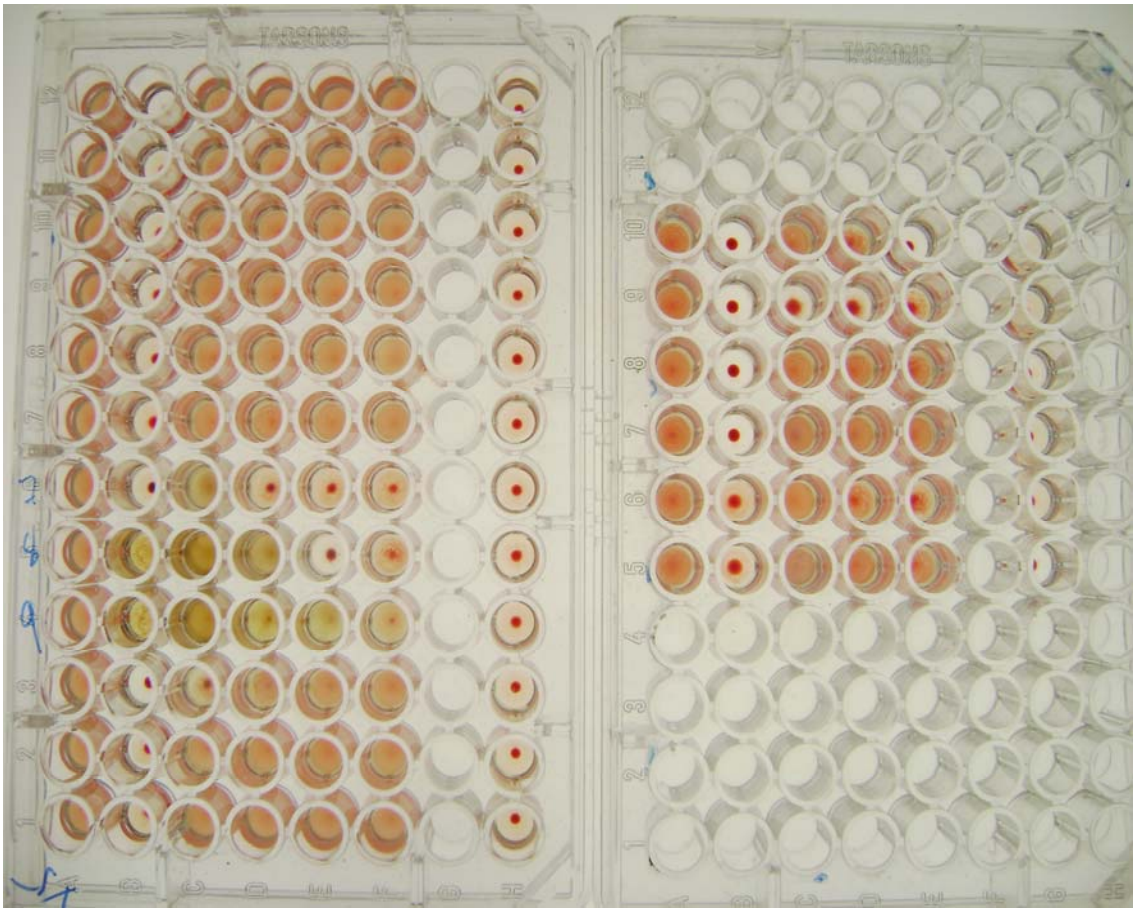


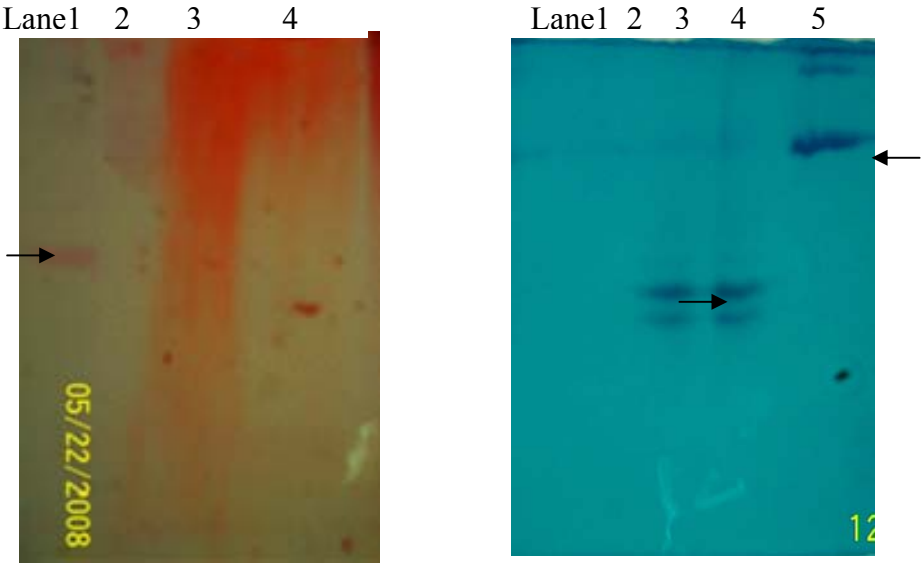
Fig 5.15 Hemagglutination-inhibition assay of CSH

Table 5.3:- Hemagglutination-inhibition test was carried out as described in materials and methods. Each value indicates the lowest concentration of sugar (mM) and glycoprotein (mg $ml^{-1}$) at which complete inhibition of agglutination was achieved.

| Monosaccharide and glycoprotein | Minimum inhibitory concentration |
|---|---|
| D-glucose | - |
| D-galactose | - |
| D-mannose | - |
| L-fucose | - |
| D-fucose | - |
| L-rhamnose | - |
| N-acetyl-D-glucosamine | 100mM |
| N-acetyl neuramic acid | 50mM |
| D-galacturonic acid | 50mM |
| D-glucosamine | 200mM |
| D-gulactosamine | - |
| D-glucoronic acid | - |
| Avidin | 0.5mg $ml^{-1}$ |
| Fetuin | 0.125 mg $ml^{-1}$ |
| Mannan from *S. cerevisae* | - |
| Trypsin inhibitor | - |
| Mucin from bovine submaxillary gland | 0.25 mg $ml^{-1}$ |

Qualitative detection of glycoprotein

Lectins being glycoproteins a qualitative assay for the detection of glycoprotein was done on non denaturing PAGE along with standard glycoprotein obtained from Sigma chemicals. Staining of the gel with both thymol sulphuric acid as well as alcian blue yielded a single band respectively, for the standard glycoprotein in lane 1 (fig 5.16a) and lanes 3-5 (fig 5.16b). CSH could not be resolved as a single band by either of the staining procedures. However, a smear was observed smear in lanes 2 to 4 in the gel stained by thymol sulphuric acid. No smear nor band was stained by alcian blue.




5.16a 5.16b

Fig 5.16: Glycoprotein profile of CSH.

5.16a. Lane 1 standard glycoprotein, lane 2 aqueous extract, lane 3 ammonium sulfate fraction (60-90%) and lane 4 columns purified.

5.16b. Lane 1 ammonium sulfate fraction (60-90%), lane 2 purified lectin, lane 3 and 4 standard glycoprotein and lane 5 antiserum.

Estimation of carbohydrate content

Since the results of the qualitative test for confirmation of glycoproteins were not conclusive content of neutral sugars in CSH was estimated by phenol sulphuric acid and the amount estimated is summarized in Table 5.4. CSH contain fairly smaller components of neutral sugar.

Table 5.4:- Estimation of neutral sugar in CSH

| Fraction | Neutral sugar mg/ml | Total sugar (mg) |
|---|---|---|
| Aqueous extract | 0.024 | 13.8 |
| 60-90% of $(NH_4)_2SO_4$ | 0.049 | 1.92 |
| DEAE-Cellulose | 0.045 | 1.8 |

The latest tendencies in biotechnology research point to marine ecosystems as a source of immeasurable value for obtaining molecules with extraordinary properties that are used in the development of drugs and other products of high added value for multidisciplinary purpose. The extensive biodiversity of submarine environment and the secondary metabolites produced by the marine organisms as a result of the environmental conditions in which they are found make them completely different from terrestrial organisms. With this aim, *Ulva fasciata* hemagglutinin (UFH) and *Caulerpa sertularioides* hemagglutinins (CSH) were evaluated for their potential In-Vitro anticancer, antimicrobial and antioxidant role in the development of new natural products.

This chapter deals with the application studies of the two isolated lectins in cancer cells, their antioxidant potential and antimicrobial studies.

This chapter is divided into the following sections:

**6.1 Introduction**

**6.2 Materials and methods**

**6.3 Results of testing with cancer cell lines**

**6.4 Results of antimicrobial studies**

**6.5 Results of antioxidant tests**

## 6.1 Introduction

Drug discovery has been developed greatly in the process of screening large numbers of pure organic compounds or crude extracts to provide new leads. Large-scale screening still continues to play an important role in the procedure for developing new drugs. Nature has been the source of medicinal treatment for thousands of years and plant- based systems continue to play a pivotal and essential role in the primary health care of 80% of world's population (Mukherjee et al., 2001)

The National Cancer Institute has taken up a major screening programme to isolate potential anticancer drugs from natural products since 1960. A large number of compounds have been shown to have the ability to block cell division and inhibit cell growth of rapidly dividing cells. Both natural and synthetic drugs in cancer therapy raise a question of life threatening risk due to their inherent toxicity to the host. So the interest is centered in developing or isolating natural compounds, which selectively kill tumor cells (www.nci.org)

Apoptosis is a form of cell death that leads to elimination of excess or damaged cells. Apoptosis contributes to tissue homeostasis and embryonic development. A range of stimuli including DNA damage, growth factor withdrawal, anticancer drugs and members of the tumor necrosis factor can induce apoptotic signals. The apoptotic cascade in mammalian cells is a multi step process. In most cases, the apoptotic cascade is initiated by loss of integrity of the outer mitochondrial membrane, accompanied by release of cytochrome C from the inter membrane space of mitochondria to cytosol (Lewin, 2000).

An increasing number of studies have demonstrated that certain compounds produced by marine organisms have potential therapeutic uses. Certain seaweeds contain significant amounts of essential proteins, vitamins, and minerals, but also several species of algae have been found to produce or contain secondary metabolites, lectins (hemaggutinins), polysaccharides (PSs), and glycoproteins with anti-tumoral, antiviral, or immunostimulatory activities (Gustafson, 2003). Thus, marine algae have been historically an exceptionally rich source of pharmacologically active metabolites with anti-neoplastic, antimicrobial and antiviral effects (Faulkner, 2000; Tziveleka et al., 2003). Random screenings were effective in finding marine algae with various biological activities (Gerwick et al., 1994; Harada et al., 1997), and many of these reports have been reviewed (Cannell et al., 1993; Nekhoroshev, 1996). In addition, some natural products previously ascribed to marine invertebrates were proved to be algal secondary metabolites (Scheuer, 1990).

Antitumor activity is one of the most important activities in marine drugs, and a number of algae and their metabolites have shown potent cytotoxic activities (Fuller et al., 1994; Harada et al., 2002; Mayer and Gustafson, 2003). These metabolites have played an important role in leading to new pharmaceutical compounds for antitumor drugs (Luesch et al., 1999; Patterson et al., 1994; Yoo et al., 2002).It is reported that several representative antitumor compounds from algae, such as Halomon, have been developed to clinical phase (Egorin et al., 1996).

Phytochemical screening for the discovery of novel anticancer drugs is a long-term, time-consuming process. Considerable efforts have been made by public organizations and private companies to speed up drug discovery, by expanding on promising results from preliminary *in vitro* screening tests. Hallmarking this progress, the United States National Cancer Institute (NCI) has set forward exemplary strategies for the discovery and development of novel natural anticancer agents. The NCI has been involved with the preclinical and/or clinical evaluation of the overwhelming majority of compounds under consideration for the treatment of cancer. During the past 45 years, more than 400,000 chemicals, both synthetic and natural, have been screened for antitumor activity (www.nci.org)

Plant materials under consideration for efficacy testing are usually composed of complex mixtures of different compounds with different solubility in aqueous culture media. The first step is the selection of starting materials, primarily based on ethobotanical information (i.e., reputation of therapeutical action in a traditional medicine sense). The second step is the identification of biological activity (in the case of cancer chemotherapy, this certainly includes selective cytotoxicity) of the extracts derived from the selected plant material. Frequently, extracts are pre- fractionated by means of chromatography and then fractions are screened for biological activity *in vitro* (Constant and Beecher, 1995). The combination of different *in vitro* assay systems may not only enhance the capacity to screen for active compounds, but may also lead to better conclusions about possible mechanisms and therapeutic effects. Thus, preclinical tests usually evaluate the cytotoxicity of a candidate antitumor agent *in vitro*. Certain

neoplastic animal cell lines have been repeatedly used for this purpose. Alternatively, animal systems bearing certain types of cancer have been used (Gebhardt, 2000). The availability of a wide variety of human tumor cell lines representing many different forms of human cancer, however, offers a suitable basis for development of a disease-oriented *in vitro* primary screening (Miyairi *et al.*, 1991; Mockel *et al.*, 1997; Gebhardt, 2000).

Although lectins have been known from several decades, interest in them has recently been revived because of the potential for lectin-based diagnosis and therapy in cancer. Within the past few years, lectins have become a well-established means for elucidating various aspects of tumorigenesis and metastasis (Gabious et al., 1998; Caselitz, 1987; Misra et al., 2000; Gorelik et al., 2001). Lectins are dynamic contributors to tumor cell recognition (surface markers), cell adhesion, membrane signal transduction, augmentation of host immune defense, cytotoxicity and apoptosis. The initial step for these activities seems to be initiated by binding lectins to the cell surface carbohydrate chains, a component part of lectin-specific receptors. So, together with antibodies, the plant lectins constitute the family of carbohydrate-binding proteins and are considered a model system for protein-carbohydrate interactions (Buts et al., 2001), which are involved in many and various physiological processes.

Current research into tumor lectinology includes the design of custom-made carrier-immobilized carbohydrate ligands (neoglycoconjugates), their application for detection of specific binding sites and evaluation of potential therapeutic approaches by blocking access or by directing drug conjugates to

cell surface lectin receptors, correlation of their expression with clinical parameters such as prognosis and their biochemical characterization (Gabious, 1997)

Exposed cell surface carbohydrate-containing macromolecules have been implicated in growth, morphogenesis, differentiation, recognition, intercellular interactions, and adhesion. Certain surface changes associated with transformation and neoplasia may lead to alterations of the above fundamental cellular processes (Hokamori, 1996). Therefore, the study of tumor cell surface characteristics is an important approach to the understanding of factors which influence the expression of the malignant phenotype. The surface properties of tumor cells play a major role in tumor growth at the primary site, invasion into surrounding host tissue, dissemination, embolization, and implantation at distant secondary sites to form metastases (Hokamori, 1996; Nicolson et al., 1977).

The mammalian cell surface is decorated with a dense layer of carbohydrates, which are attached to membrane glycoproteins and glycolipids. These cell surface carbohydrates are cell-type specific and participate in a wide variety of biological processes including cell adhesion, fertilization, differentiation, development, and tumor-cell metastasis (Bertozzi et al., 2001). Abnormal glycosylation has been associated with many diseases, especially cancers, such as breast cancer, prostate cancer, lung cancer, and colon cancer (Redondo et al., 2004; Kakari et al., 1991; Lin et al., 2002). Moreover, tumor-associated alterations of cell surface glycosylation plays a crucial role in metastasis of carcinoma cells by altering tumor cell adhesion or motility in a manner that

either promotes or inhibits invasion and metastasis (Hekomn et al., 1996; Lin et al., 2002). The ability to characterize cell surface carbohydrate expression patterns is thus critical both to understanding their role in disease development as well as to provide diagnostic tools to help guide treatment.

Specific carbohydrate-binding ligands such as monoclonal antibodies and lectins have proven to be invaluable tools for the analysis of complex glycoconjugates. Monoclonal antibodies are generally directed towards terminal carbohydrate structures, which limit their utility to the analysis of these terminal components (Dubelsteen et al., 1996). In contrast, lectins, which are proteins of non-immune origin recognize and bind to specific carbohydrate structural epitopes and thus can detect changes in the carbohydrate core structures (Nilson, 2003 and Wearne et al., 2006). Both antibodies and lectins can be tagged with a fluorophore or enzyme and used to stain tissue sections to provide information on the distribution of carbohydrate structures within the sample.

MTT-tetrazolium salts are reduced to formazan by cellular respiratory enzymes. The MTT assay is a colorimetric assay based on the ability of viable cells to reduce a soluble MTT to blue formazan crystals. This activity is performed only by viable cells, and thus the method may indicate the amount of viable cells present in culture.

Trypan blue assay is used to determine cell viability. Dead cells take up the dye, trypan blue, and appear blue under the microscope. Living cells exclude tyrpan blue, and appear normal. Thus, the percentage of viable cells can be calculated.

### 6.1b. Anti-microbials

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased in India.

Herbal medicine involves the use of plants for medicinal purposes. The term "Herb" includes leaves, stems, flowers, fruits, seeds, roots, rhizomes and bark. There can be little doubt that the use of plants for healing purposes is the most ancient form of medicine known. The quest for plants with medicinal properties continues to receive attention as scientists are in need of plants, particularly of ethno botanical significance for a complete range of biological activities, which ranges from antibiotic to anticancerous. Several plants and herb species used traditionally have potential antimicrobial and antiviral properties (Shelef, 1983; Zaika, 1988) and this has raised the optimism of scientists about the future of phyto-antimicrobial agents (Das et al., 1999).

Several phytochemical surveys have been published, including the random sampling approach which involved some plant accessions collected from all parts of the world. The major chemical substances of interest in these surveys were the alkaloids and steroidal sapogenins. However, other diverse groups of naturally occurring phytocomponents such as flavonoids, tannins, unsaturated sterols, triterpenoids, essential oils etc., have also been reported (Lozoya et al., 1990). There is currently a large and ever expanding global population base that

prefers the use of natural products in treating and preventing medical problems because herbal plants have proved to have a rich resource of medicinal properties.

According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance (Diallo et al., 1999).

Seaweeds have been used as food in the Asian diet for centuries as it contains carotenoids, dietary fibres, proteins, essential fatty acids, vitamins and minerals. Marine algae are exploited mainly for the industrial production of phycocolloids such as agar-agar, alginate and carrageenan but not for health aspects. Fresh and dry seaweeds are extensively consumed by people especially living in the coastal areas. From literature, it is observed that the edible seaweeds contain a significant amount of the protein, vitamins and minerals essential for the human nutrition (Fayaz et al., 2005). The nutrient composition of seaweed varies and is affected by the species, geographic areas and seasons of the year and temperature of the water.

Biostimulant properties of seaweeds are explored for use in agriculture and the antimicrobial activities for the development of novel antibiotics. Seaweeds have some components of great medicinal value such as antibiotics, laxatives, anticoagulants, anti-ulcer products and suspending agents in radiological preparations (Smit, 2004). Seaweeds have recently received significant attention for their potential as natural antioxidants. Most of the compounds of marine algae show anti-bacterial activities (Vairappan et al., 2001, Vlachos et al., 1996). Many metabolites isolated from marine algae have been shown to possess bioactive efforts (Oh et al., 2008, Venkateswarlu et al., 2007 and Yang et al., 2006).

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds (Edeoga et al., 2005). Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents (Cohen, 1992). Such a fact is cause for concern, because of the number of patients in hospitals who have suppressed immunity, and due to new bacterial strains, which are multi-resistant. Consequently, new infections can occur in hospitals resulting in high mortality.

The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be

taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient and seek novel, natural products.

**6.3c. Anti-oxidants**

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells**.** Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols**,** ascorbic acid or polyphenols. Antioxidants act as "free radical scavengers" and hence prevent and repair damage done by these free radicals. Antioxidants occur naturally as components or nutrients in our foods which can prevent or slow the oxidative damage to our body. When our body cells use oxygen, they naturally produce free radicals (by-products) which can cause damage. Health problems such as heart disease**,** macular degeneration, diabetes**,** cancer etc are all contributed by oxidative damage (Becker et al., 2004).

The term antioxidant is not restrained by any international accepted definition. An antioxidant is defined as a chemical substance that delays or prevents oxidation of a substrate. Chipault (1962) defined antioxidants in foods as

"substances that in small quantities are able to prevent or greatly retard the oxidation of easily oxidisable materials such as fats". Another widely used definition, which covers all oxidisable substrates, i.e. lipids, proteins, DNA and carbohydrates suggested by Halliwell and Guteridge (1989) is "any substance that when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance".

Various reports in the field of research and nutritional studies indicate that there is an inverse relationship between the dietary intake of antioxidant rich foods and the incidence of human diseases (Halliwell, 1991). Oxidative stress is a very primary indication in critically ill patients, also it is associated with age related diseases, rheumatoid arthritis, lung fibrosis, neurological disorders like Alzheimer's Disease, wherein the antioxidants are known to have beneficial health effects (Day, 2008; Opii *et al.* 2008; Abilés *et al.* 2006, Foronjy *et al.* 2008; van Vugt *et al.* 2008). Antioxidants prevent ethanol-associated apoptosis in Fetal Rhombencephalic Neurons (Antonio and Druse, 2008).It has been studied that dietary antioxidants protect hematopoietic cells and improve animal survival after total-body irradiation (Wambi *et al.* 2008). Antioxidants are nueroprotective (Kamat *et al.* 2008) and also beneficial in HIV treatment (Gaalen and Wahl, 2009).

Till now many different and complex methods for assessing free radical activity have been described depending on experimental conditions, the availability of analytical facilities, and the investigator's interest (Jackson, 1999). The antioxidant properties can be measured or apprised in terms of free radical

scavenging activity, reducing power, ion chelating activity, polyphenolic contents, inhibition of lipid peroxydation, xanthene oxidase inhibition and many of these techniques have already been used by different researchers for studying the antioxidant and bio-protective properties of the different classes of sea weeds (Meenakshi et al., 2009; Devi *et al.* 2008; Matanjun et al. 2008; Shanab, 2007; LIM *et al.* 2002 Ismail and Hong, 2002; Yan *et al.* 1998). The antioxidant also some times acts as pro-oxidants if taken in very high amounts from brown algae (Tutour *et al.* 1998). It has also been reported that Vitamin C exhibits pro-oxidant properties if its consumption is more than RDA value (500mg/day) so precaution should be taken in the use of antioxidants and moreover it is important that the antioxidant is thoroughly studied (Podmore *et al.* 1998).

The method for the evaluation of antioxidant potential is normally based on the identification of different anti-oxidative mechanisms under variable conditions, reflecting the multifunctional properties of antioxidants in both physiologically and food-related oxidative processes. Conflicting results have often been obtained as illustrated for single polyphenolic compounds (Moure et al., 2001), for herbs and spices (Madsen and Bertelsen, 1995) and for antioxidants. These conflicting results obtained for antioxidant capacity and activity in different assays may be attributed to several factors such as a) physical structure of the test system, b) nature of the substrate for oxidation, c) presence of interacting components, d) mode of initiating oxidation, and e) analytical method for measuring oxidation (Frankel and Meyer, 2000). Although the influence of some factors have been elucidated in simple model systems, the effect in a complex heterogeneous system such as a food matrix or the human body still

cannot be predicted and the need for a standardized testing protocol for evaluating antioxidant effects has been recognized (Frankel, 1993).

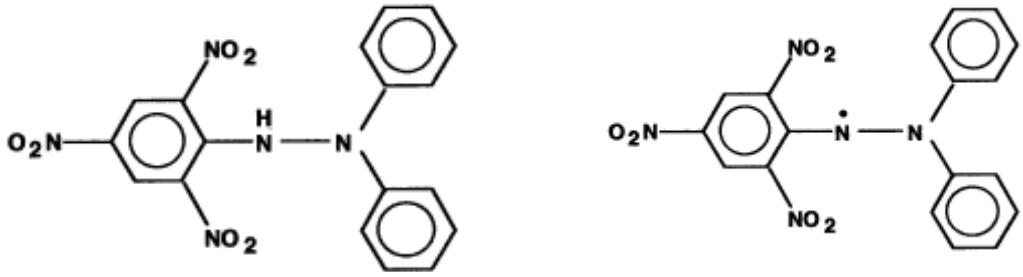
A factor that provides a distinct challenge in the assay of antioxidant capacity is that within biological systems, there are at least four general sources of antioxidants: (1) enzymes, for example, superoxide dismutase, glutathione peroxidase, and catalase; (2) large molecules (albumin, ceruloplasmin, ferritin, other proteins); (3) small molecules [ascorbic acid, glutathione, uric acid, tocopherol, carotenoids, (poly)phenols] (4) some hormones (estrogen, angiotensin, melatonin, etc.). On the other hand, there are multiple free radical and oxidant sources [e.g., O2-, 1O2, HO, NO, ONOO-, HOCl, RO (O), LO (O)], and both oxidants and antioxidants have different chemical and physical characteristics (Becker et al., 2004)

Seaweeds have been consumed in Asia since ancient times, but to a much lesser extent in the rest of the world (Chapman and Chapman, 1980; Indergaard and Minsaas, 1991). Edible seaweeds are a rich source of dietary fiber, minerals, and proteins (Lahaye, 1991; Ruperez and Saura-Calixto, 2001). Cell walls from marine algae characteristically contain sulfated polysaccharides, which are not found in land plants and which may have specific functions in ionic regulation (Kloareg and Quatrano, 1998). Nevertheless, there are very few reports in literature on the antioxidant capacity of algae. Alcoholic and aqueous extracts of seaweeds have been evaluated for antioxidant activity by lipoxygenase inhibition, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity and deoxyribose assays (Matsukawa et al.,1997; Yan et al.,1998).

DPPH assay measures by spectrophotometer the ability of antioxidants to reduce 2,2- diphenylpicrylhydrazyl (DPPH), another radical not commonly found in biological systems. The DPPH radical is one of the few stable organic nitrogen radicals, which bears a deep purple color. It is commercially available and does not have to be generated before assay like ABTS. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH. The ability can be evaluated by electron spin resonance (ESR) or by measuring the decrease of its absorbance. The widely used decoloration assay was first reported by Brand-Williams and co-workers (Brand-Williams et al., 1995). Antioxidant assays are based on measurement of the loss of DPPH color at 515 nm after reaction with test compounds (Bondet et al., 1997), and the reaction is monitored by a spectrometer. The percentage of the DPPH remaining is calculated.

2, 2-diphenyl-1-picryl-hydrazylhydrate (DPPH) is a stable, free radical. Though DPPH is not biologically important, its reactions help to understand the mechanism of antioxidant action (Naik *et al*., 2006). The measure of its content can also be used to estimate the total free radical scavenging activity of the extracts. Because of the odd electron, DPPH shows a strong absorption at 517nm and its alcoholic solutions are deep violet in color but when these electrons are paired off decolorization takes place and the absorption is reduced and can be measured spectrophotometrically. Thus DPPH offers a convenient way for detecting and measuring the oxidizable groups of natural and synthetic antioxidants (Blois, 1958). When a solution of DPPH is mixed with that of a substance that is a hydrogen donor, it gets reduced resulting in the loss of violet

colour (although there would be expected to be a residual pale yellow color from the picryl group still present) (Molyneux, 2003).



**Diphenylpicrylhydrazyl (free radical)** **Diphenylpicrylhydrazine (nonradical)**

(Molyneux, 2003)

$EC_{50}$ value is used to determine the reducing capacity of the test solution/extract of an antioxidant. $EC_{50}$ value is defined as the concentration of antioxidant required to 50% reduction of DPPH radical (Molyneux, 2003).

### 6.2. Materials and Methods

In vitro screening of cytotoxicity

The cytotoxic potential of both the lectins UFH &CSH was tested against three different human cancer cell lines breast cancer (MCF7), cervical cancer (Hela) and prostrate cancer (PC3). The cells tested originated from breast, prostate and cervical cancers. This work was done at National Center of Cell Science (NCCS). *In-Vitro* anticancer activities of purified lectins were carried out in NCCS Pune, under the guidance of Dr S. G. Kundu.

Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), Hanks F-12 and Trypan blue all were obtained from Sigma- Aldrich Chemicals, USA. (3-(4-5-Dimethylthiazol-2-yl)-2-5-Diphenyltetrazolium (MTT) was obtained from Bio-Rad Laboratories, CA.

Antimicrobial assay

Nutrient agar was obtained from Himedia Laboratories PVT Ltd, Mumbai and all microorganisms were obtained from Department of Biotechnology, Goa University, Goa.

Antioxidant assay

Ascorbic acid and DPPH were obtained from the Sigma- Aldrich Chemicals, USA. For the measurement of DPPH free radical scavenging activity, a photometric method was employed. UV-VIS Spectrophotometer (UV mini 1240, SHIMADZU). Ethanol was obtained from Changshu Yangyuan Chemicals, China. Methanol from (SDFCL) S. d. fine Chemicals Limited

**6.2 a**. Cell culture

Human cancer cell lines MCF7, Hela and PC3 were tested, cells were grown in DMEM, MEM and Hanks F12-K media respectively and supplemented with 10% (v/v) Foetal Bovine Serum and 100mg/ml tetracycline was used as the antibiotic. 200 µl of cell suspension ($8\times 10^3$) was seeded in the each well of a 96-welled plate, after 24 hours different concentrations of UFH and CSH were added to the grown cells. Concentration of lectin was in the range of 10-100 µg/ml. The plates were incubated at 37° C in a 5% Carbon dioxide incubator and

assayed/ terminated after 24, 48 and 72 hrs, respectively. All experiments were performed in triplicates.

**6.2b.** In vitro testing for cytotoxicity using MTT Assay (Cell Viability Assessment)

Tetrazolium derivative reduction (MTT) assay was used to determine the number of live cells (MCF7, Hela and PC3) surviving under experimental condition. Absorbance was measured at 570 nm using a spectrophotometer (Spectra max 250, Molecular Devices, Sunnyvale, CA) with background absorbance measured at 570-690 nm.

The cell survival ratio was calculated according to the following equation:

Percentage cell survival =Mean OD of wells receiving each lectin dilution / Mean OD of Control x 100

Absorbance of treated cells was expressed as percent of control survival. Controls used were 200µl of the respective culture and the other control was 200µl culture + PBS.

**6.2c**. Measurement of viability using Trypan blue exclusion assay

Efficacy of UFH & CSH as cytoxic agents was tested against 2 cell lines i.e Hela and PC3 using trypan blue exclusion. Hela cells were grown in MEM and PC3 in Hanks F12-K media. The media in each case was supplemented with 10% (v/v) Foetal Bovine Serum and 100mg/ml tetracycline was added as antibiotic.

Cell suspension ($8\times 10^3$) was seeded in the each well of 24-well plate. Subsequently, the grown cells were treated with purified extracts from UFH and

CSH at different concentrations (10µg/ml -100µg/ml). After incubation at 37° C for 24 and 48 hrs, respectively, the cell viability was assesed. Equal volume (1:1) of treated culture and trypan blue was mixed and allowed to stand for 10 min. 10µl was loaded on a hemocytometer and checked for cell survival using light microscope. All experiments were done in duplicates.

The percentage cell survival was calculated according to the following equation:
Percentage cell survival = Number of viable cells/Total number of cells (Viable and nonviable cells) ×100%

**6.2d.** Test for Antimicrobial activity

Test organisms screened for the antimicrobial assay were *Salmonella typhii, Pseudomonas aeruginosa, E. coli, Flavobacterium species, Micrococcus species* and *Streptococcus aureus*.

For preparation of inoculum, test culture strains were transferred from nutrient agar slants into test tubes containing nutrient broth, and grown overnight on a shaker at 37 ºC. An inoculum of the culture was then aseptically introduced on nutrient agar plate by three-dimension swab technique. The filter paper discs were each impregnated with 20 µl of purified lectins and placed on the inoculated agar plate. The plates were then incubated at 37 ºC for 24 h. Filter paper disc containing the reference PBS was used as a control.

**6.2e**. DPPH Radical Scavenging Activity

Antioxidant activity of the purified hemagglutinins was tested using the DPPH free radical scavenging assay. All assays were carried out in triplicate and average values were considered.

DPPH solution (0.004% w/v) was prepared in 95% methanol. Stock solutions (1mg/mL) of the both purified hemagglutinins were prepared in 95% methanol. From stock solution 10µg, 20 µg, 30 µg, 40 µg, 50 µg, 60 µg, 70 µg, 80 µg, 90 µg & 100 µg were taken. 1.2 ml of freshly prepared DPPH solution (as above) was added to each of these test tubes containing *extracts* (10 µg – 100 µg), vortexed and incubated in dark for 10 min. Absorbance was measured at 517 nm on adjusting zero by using the blank. Ascorbic acid was used as a reference standard and dissolved in distilled water to make the same concentration (10 µg - 100 µg). Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as a blank.

Percentage of scavenging activity was calculated by the following equation:

DPPH scavenging activity (%) = $[(A_{cont} - A_{test}) / A_{cont}] \times 100$

Where,

$A_{cont}$ = Absorption of control (DPPH + methanol).

$A_{test}$ = Absorption of sample reaction – Absorption of Sample blank.

Sample reaction = DPPH + Sample.

Sample blank = methanol + Sample.

### 6.3 Results of MTT assay

To investigate the in-vitro anti-cancer activity of UFH, viability tests were applied using MTT assay and the effect of UFH was examined on the cell survival. When MCF7, Hela and PC3 cell viability were assayed after exposure to 10-100µg/ml UFH for 24 h, 48 h and 72 h, a concentration-dependent inhibitory effect on cell growth was observed.

As it is shown in Fig 6.1, exposure of MCF7 cells to UFH resulted in cell death. Significant cell death was observed in cells that were exposed to UFH ($p<0.01$) 48 h, cell death was also observed in 72 h and this was more than cells exposed for 24 h. The correlation and significance of cell survival data is represent in Table 6.1.

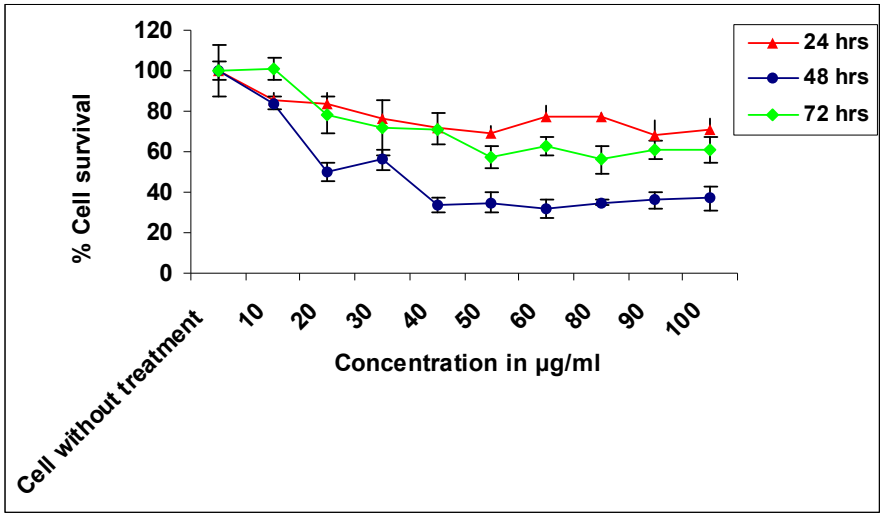



Fig 6.1 Effect of UFH on MCF7, results represent the mean ± SD of the three independent experiment; $p< 0.05$ compared with control (no treatment with UFH and PBS treatment).

| Time period exposure | Correlation | Significance |
|---|---|---|
| 24 hrs | -0.71655 | 0.013103 |
| 48 hrs | -0.81937 | 0.002026 |
| 72 hr | -0.81276 | 0.002358 |

Table 6.1 Correlation and significance of UFH against MCF7

As seen in the Fig 6.2 exposure of Hela cells to UFH resulted in a drop-in cell survival. Significant cell death were observed in cells that was exposed 24 h and 48 h to UFH ($p<0.01$). The correlation and significance of cell survival data is represent in Table 6.2.

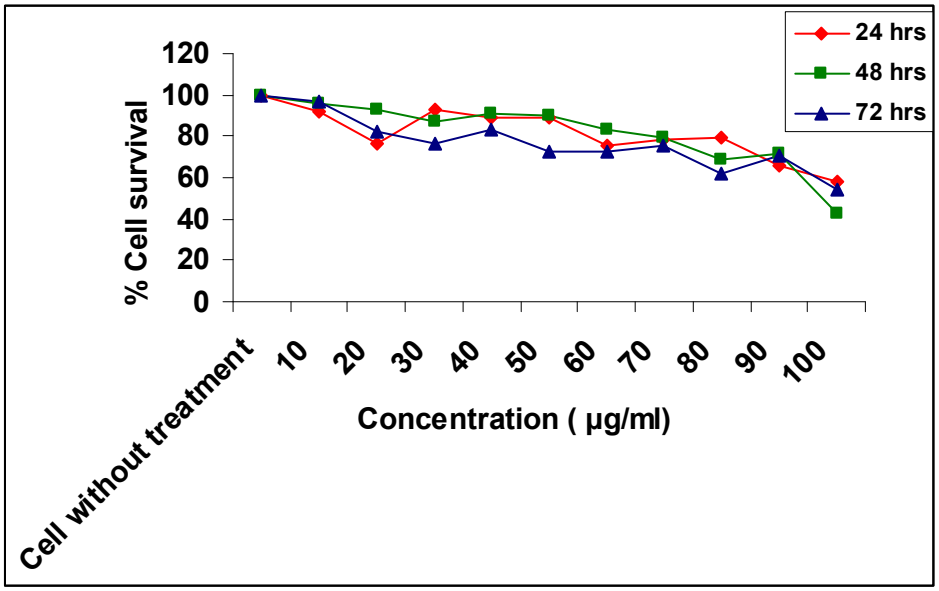



Fig 6.2 Effect of UFH on Hela, results represent the mean ± SD of the three independent experiment; $p< 0.05$ compared with control (no treatment with UFH and PBS treatment).

| Time period exposure | Correlation | Significance |
|---|---|---|
| 24 hrs | -0.83683 | 0.001317 |
| 48 hrs | -0.89007 | 0.000241 |
| 72 hr | -0.91115 | 0.004836685 |

Table 6.2 Correlation and significance of UFH against Hela

As seen in Fig 6.3 exposure of PC3 cells to UFH resulted in cell death. Significant cell death was observed in cell that was exposed 48 h ($p<00000$). Cell death was also observed in 72 hr ($p<0.01$).

The correlation and significance of cell survival data is represent in Table 6.3.

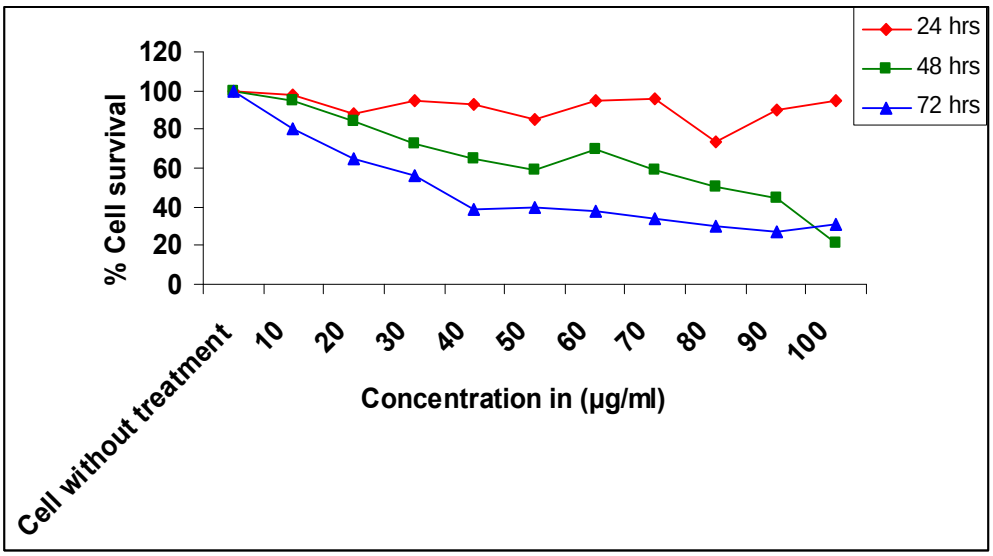



Fig 6.3 Effect of UFH on PC3, results represent the mean ± SD of the three independent experiment; $p< 0.05$ compared with control (no treatment with UFH and PBS treatment).

| Time period exposure | Correlation | Significance |
|---|---|---|
| 24 hrs | -0.28479 | 0.39599 |
| 48 hrs | -0.95367 | P< 00000 |
| 72 hr | -0.89988 | 0.000161 |

Table 6.3 Correlation and significance of UFH against PC3

To investigate the in-vitro anti-cancer activity of CSH, viability tests were applied using MTT assay and examined the effect of CSH was examined on the cell survival. When MCF7, Hela and PC3 cell viability were assayed after exposure to 10-100μg/ml CSH for 24 h, 48 h and 72 h, a concentration-dependent inhibitory effect on cell growth was observed.

As it is shown in Fig 6.4, exposure of MCF7 cells to CSH resulted in cell death. Significant cell death was observed in cell that was exposed 24 h and 48 h ($p<0.01$).

The correlation and significance of cell survival data is represent in Table 6.4.

| Time period exposure | Correlation | Significance |
|---|---|---|
| 24 hrs | -0.72269 | 0.01199 |
| 48 hrs | -0.67541 | 0.022563 |
| 72 hr | -0.9791 | 0.017839 |

Table 6.4 Correlation and significance of CSH against MCF7

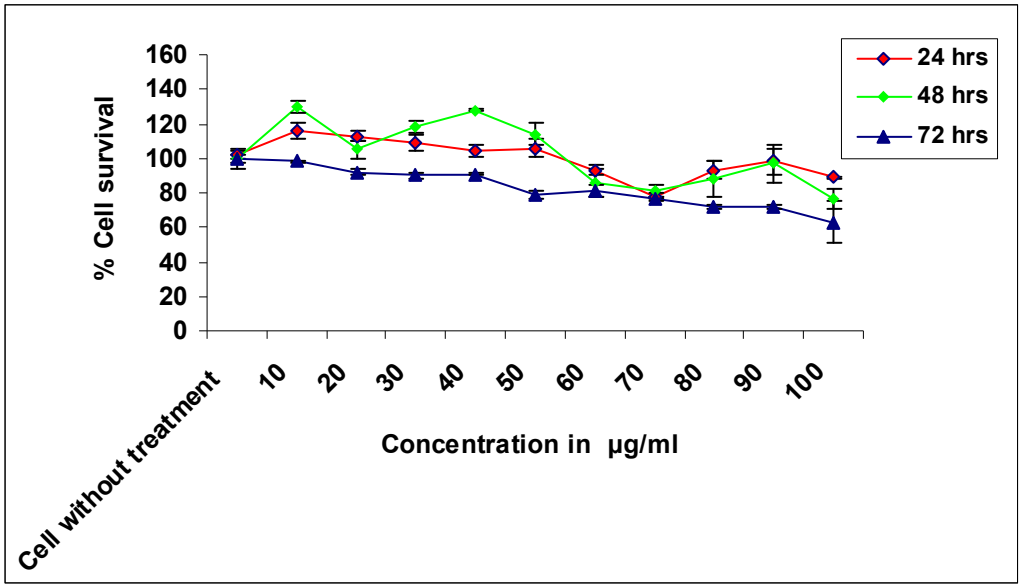



Fig 6.4 Effect of CSH on MCF, results represent the mean ± SD of the three independent experiment; $p< 0.05$ compared with control (no treatment with CSH and PBS treatment).

As seen in the Fig 6.5 exposure of Hela cells to CSH resulted in cell death. Significant cell death was observed in cells that was exposed 24 h and 72 h to CSH ($p<0.01$). The correlation and significance of cell survival data is represent in Table 6.5.

| Time period exposure | Correlation | Significance |
|---|---|---|
| 24 hrs | -0.94155 | P < 0000 |
| 48 hrs | -0.86932 | 0.000509 |
| 72 hr | -0.88354 | 0.00031 |

Table 6.5 Correlation and significance of CSH against Hela

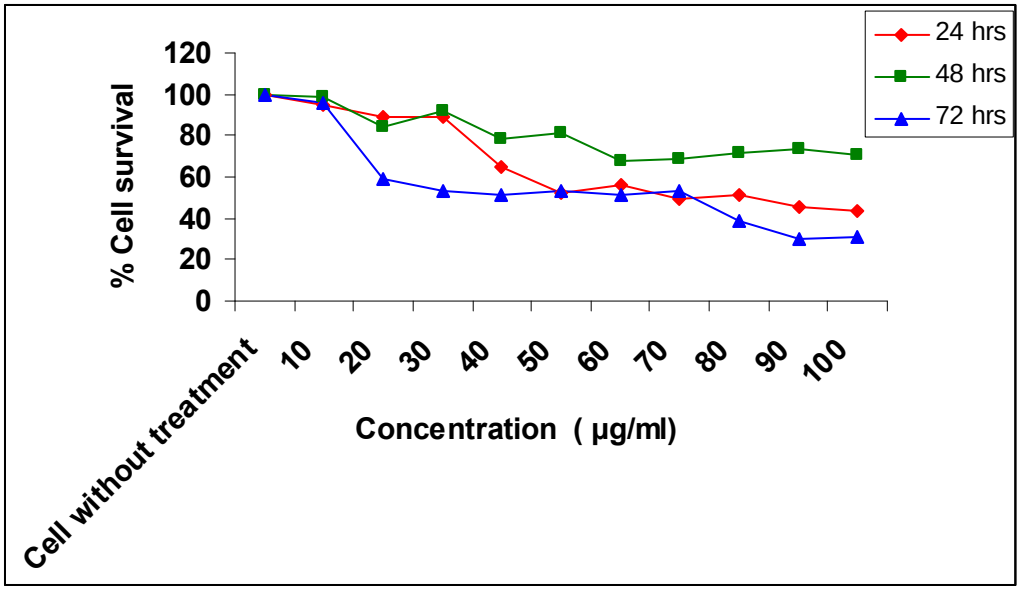



Fig 6.5 Effect of CSH on Hela, results represent the mean ± SD of the three independent experiment; $p < 0.05$ compared with control (no treatment with CSH and PBS treatment).

As seen in Fig 6.6 exposure of PC3 cells to CSH resulted in cell death. Significant cell death was observed in cell that was exposed 24h, 48h and 72 h ($p<0.01$).

The correlation and significance of cell survival data is represent in Table 6.6.

| Time period exposure | Correlation | Significance |
|---|---|---|
| 24 hrs | -0.73224 | 0.010398 |
| 48 hrs | -0.70496 | 0.015402 |
| 72 hr | -0.76959 | 0.005609 |

Table 6.6 Correlation and significance of CSH against PC3

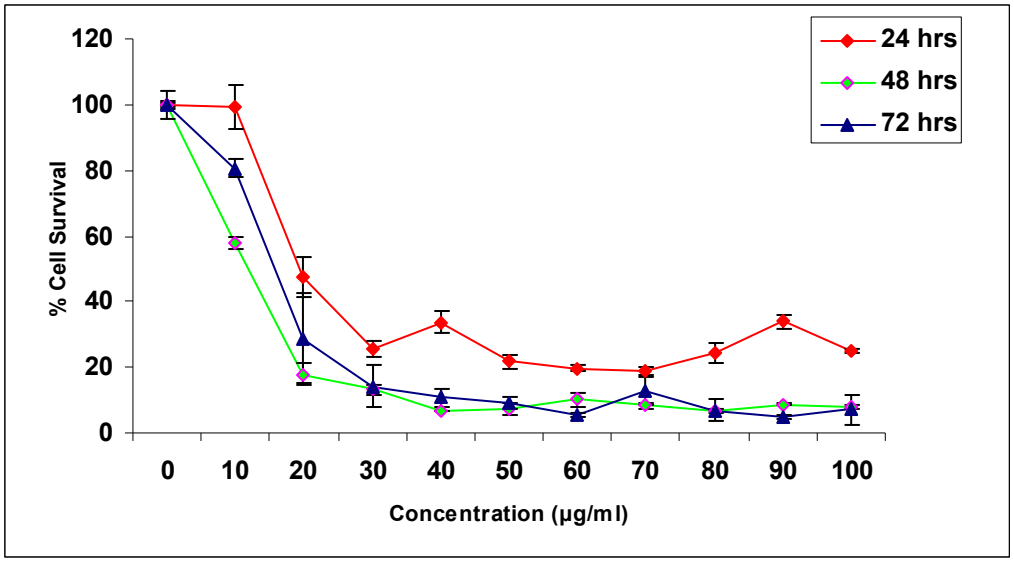



Fig 6.6 Effect of CSH on PC3, results represent the mean ± SD of the three independent experiment; $p < 0.05$ compared with control (no treatment with CSH and PBS treatment).

Trypan blue assay

Since MTT assay showed significant fall in cell survival on treatment with lectins, trypan blue assay was done to confirm the cell death. Actual numbers of dead cells were counted and these results are presented in Fig 6.7a, b and 6.8 a, b.

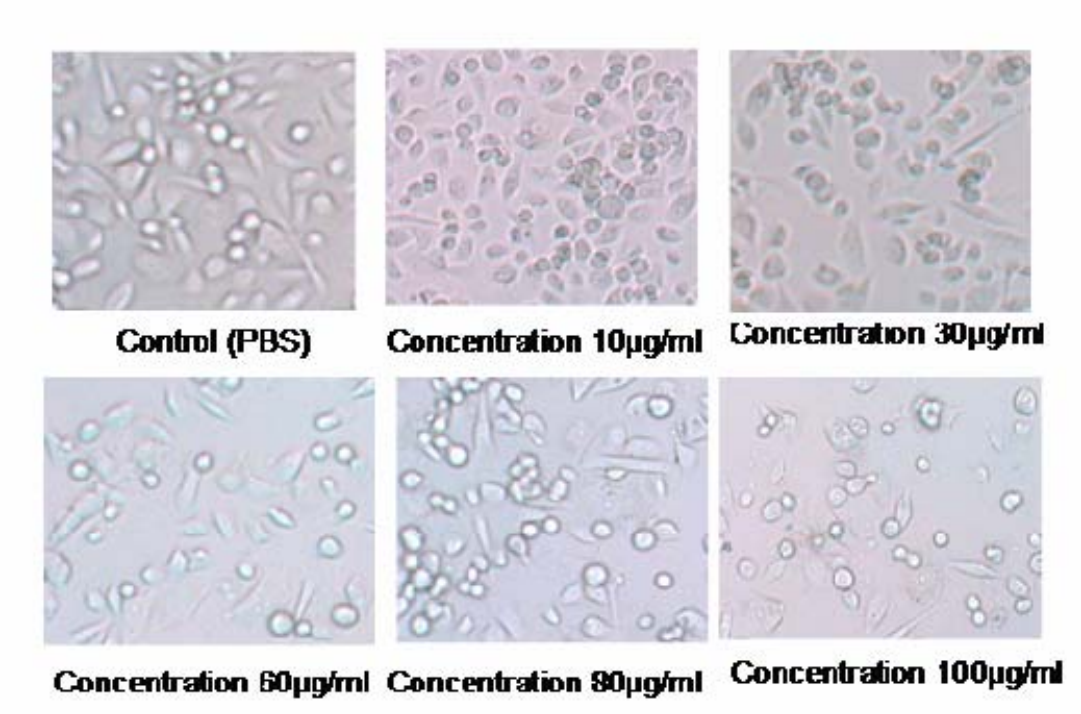



Fig 6.7a Microphotograph of Trypan blue assay of UFH vs PC3 cell line.

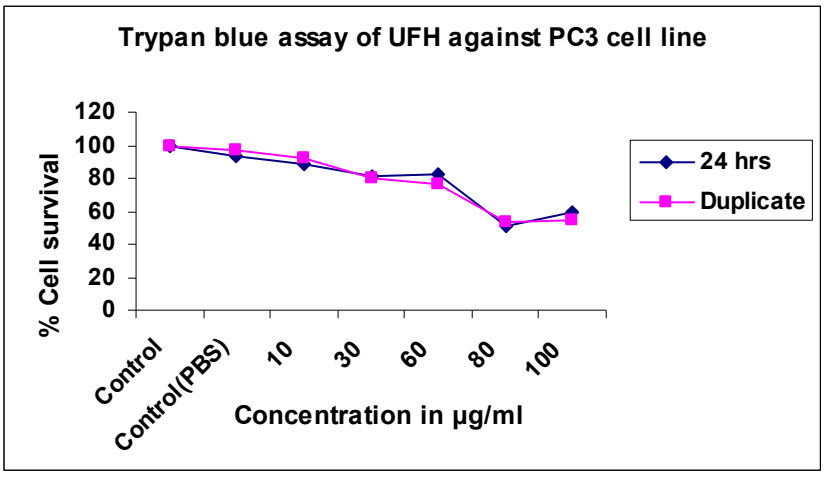



Fig6.7b Trypan blue assay of UFH vs PC3 cell survival

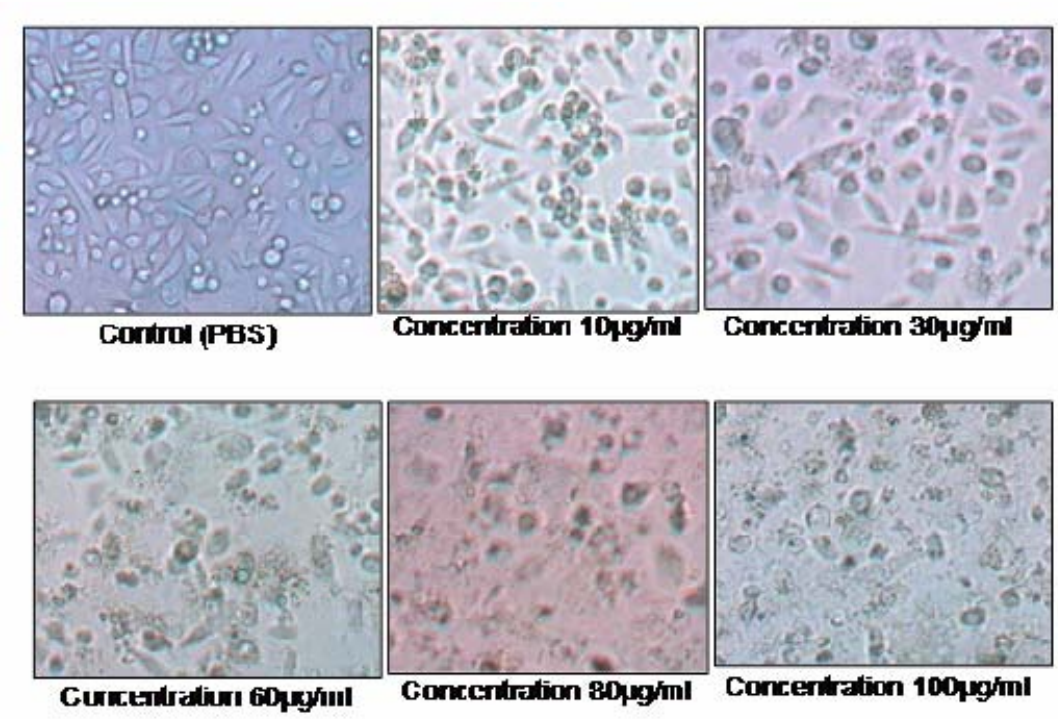



Fig 6.8a Microphotograph of Trypan blue assay of CSH vs PC3 cell line

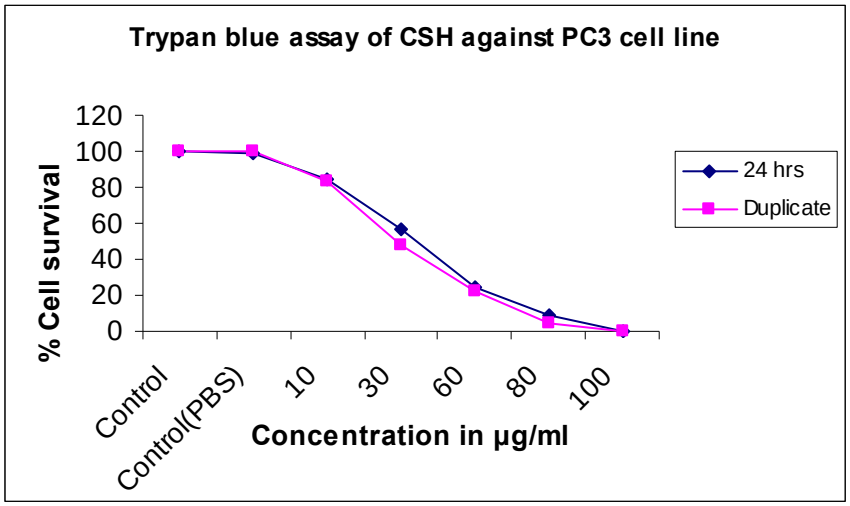



Fig 6.8b Trypan blue assay of CSH vs PC3 cell survival

### 6.4 Anti-microbial studies

No zone of clearance was seen in any of the culture tested against UFH and CSH. As shown in the Fig 6.9.

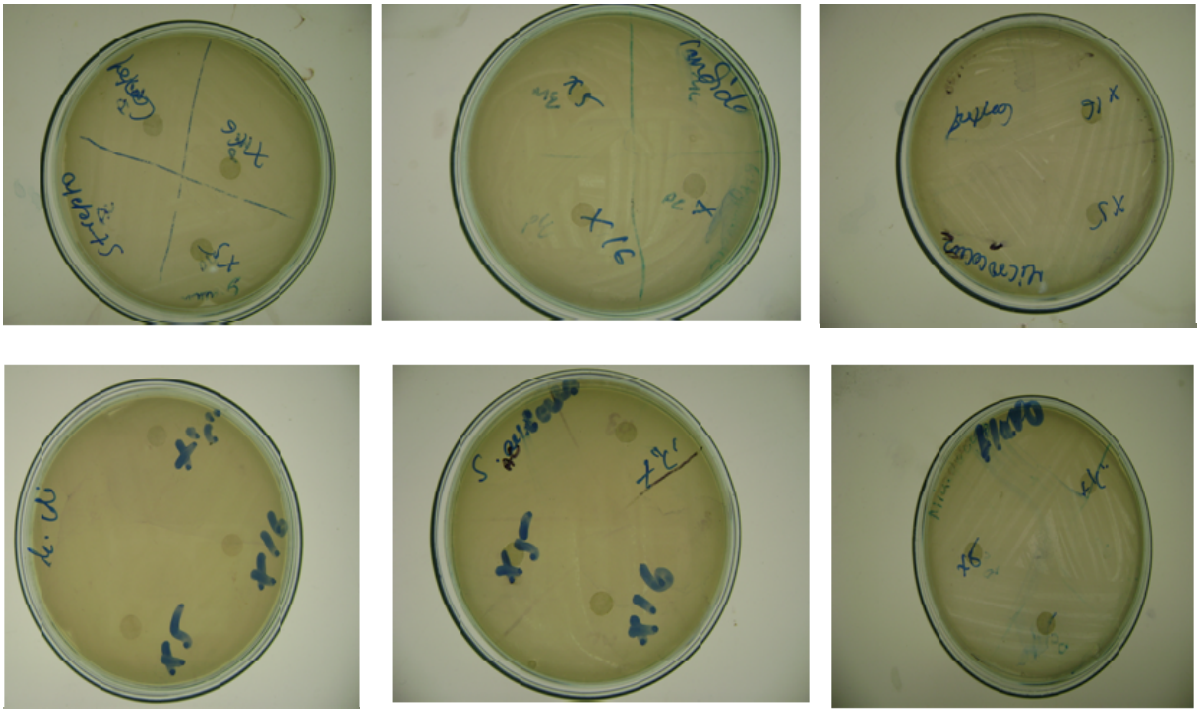


Fig 6.9:- Test for antibacterial activity

## 6.5 Antioxidant potential

The DPPH assay is the most widely used method for screening antioxidant activity, since it can accommodate many samples in a short period and detect active ingredients at low concentration (Piao et al., 2004). The absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It is visually noticeable as a color change from purple to yellow. The purified extract of UFH and CSH and ascorbic acid showed DPPH radical scavenging activity in a concentration–dependent manner (Fig.6.10).

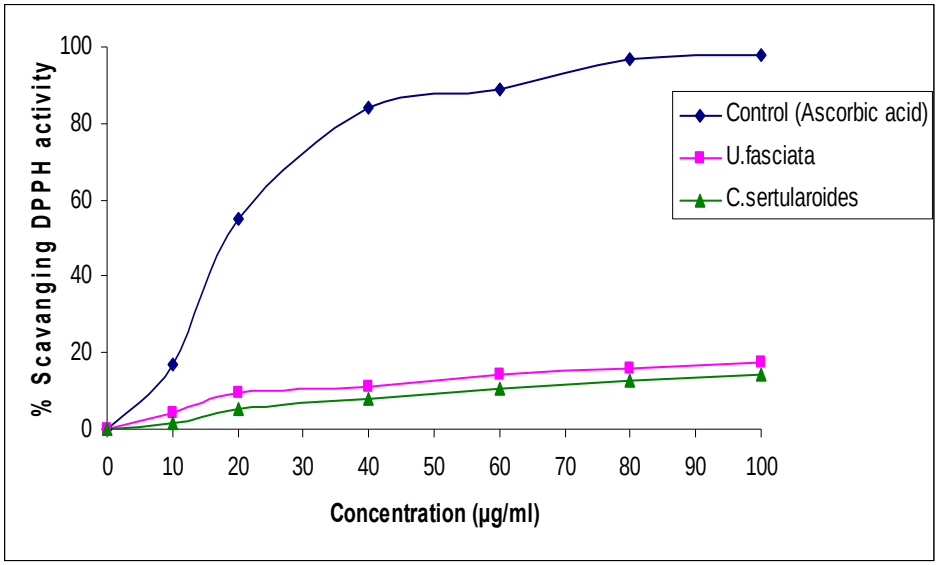



Fig 6.10 Free radical scavenging capacity of UFH and CSH

The long history of utilization of marine macroalgae for a variety of purposes has led to the gradual realization that some of their constituents are more superior and valuable in comparison to their counterparts on land. Seaweeds synthesize a wide range of chemicals, some of which stand the only natural resource, e.g. agar, carrageenan and alginates etc. The history of Indian seaweed research is not more than eighty years. The state of Indian marine macro algae resources was last reviewed in 2006 and subsequently a lot of new information relating to resources, utilization and commercial cultivation has been added.

There is a rich marine macroalgae resource in India. Marine macroalgae belonging to 217 genera and 844 species have been spotted along the coast of India (Rao and Mantri, 2006). Altogether 77 species of algae were reported along the coast of Goa and their grouping was Cyanophyceae 23, Phaeophyceae 22, Chlorophyceae 23 and Rhodophyceae 29 (Ambiye et al, 1992).

In this study, twenty one species of marine algae were collected from only four different beaches of Goa, in the vicinity of Goa University. Seven were green, eight were red and six were brown algae. All marine macroalgae were collected mainly during monsoon and post monsoon period based on observation of rich blooms growing during this period. These algae were identified based on morphological characters and by referring to books, monographs and consulting resource persons skilled in algal taxonomy.

Among these twenty one algae, *Caulerpa peltata* and *Hypnea valentiae* were found in scarce amounts during collection and thus could not be considered as a good choice of candidates for research in the long run as accessing resource as

and when required may have proved to be a difficult task. Similarly, *Amphiroa fragilissima* and *Grateloupia lithophila* were observed to grow at restricted time of the years being in bloom for only three months of the year. In contrast to these, *Ulva fasciata* and *Caulerpa sertularioides* were found to be blooming for longer time and at many sites.

Three different methods were employed for extraction and proteins soluble in Phosphate buffered saline, Tris-buffered saline and 0.85% Sodium chloride were extracted and checked for agglutination of human erythrocytes. All three buffers used for extraction were maintained at pH 7.4. However, aqueous extracts from each of these buffers for the same algae resulted in differential activity, albeit to a small degree, in agglutination. It would be interesting to determine the role played by buffer in enhancing the hemagglutination activity. There has been no report of extraction in different buffers nor a comparative report. However, each of these buffers have previously been used in isolation and have yielded different results. Chiles and Bird (1989) used TBS whereas Freitas et al., (1997) screened thirty species using 0.85% Sodium Chloride extract. Sodium Chloride was also used for extraction by Hori et al., (1981). Dinh et al., (2009) screened 44 species of macroalgae using PBS to extract agglutinins.

For the first time in India, macroalgae have been systematically screened for hemagglutinins along the coast of Goa. Macroalgae have earlier been screened in Japan (Hori et al.,1981) , Britain (Blunden et al., ) South Eastern Florida (Chiles and Bird.,1989), Brazil (Freitas et al., 1997) , Russia (Chernikov et al., 2007) and Vietnam (Dinh et al., 2009) to cite a few.

Hori et al., (1981) proposed the use of rabbit erythrocytes rather than human erythrocytes in screening for marine algal hemagglutinins. Chiles and Bird (1989) analysed 15 algal species to rabbit , sheep and human erythrocytes and highest agglutination was observed in rabbits and least in human. It was thus postulated that rabbit erythrocytes are most suitable cells for initial screening of agglutination (Ainouz and Sampaiao, 1991; Dalton et al., 1995). All assays in this study were tested against human RBCs but fairly high titers were obtained. Perhaps if other erythrocytes or treated erythrocytes were used, assays may have yielded higher activity. Ainouz and Sampaiao, (1991), Benevides et al., (2001) have reported higher activity against trypsin-treated erythrocytes. Thus employing treated RBCs may have also resulted in higher activity.

The screening study cannot be conclusive about the preferential reaction with a particular human blood group as not a single extract particularly agglutinated a specific blood group. Similar observations have been reported earlier by Hori et al., (1981); Sampaio et al., (1998); Wang et al.( 2004) and Dinh et al., (2009). Although, some algal lectins are indicated to be highly specific to either A, B and / or O erythrocytes (Hori et al., 1981; Chiles and Bird, 1989, Benevides et al., 2001). Higher titers were observed in a particular blood group but in order to confirm this observation, it is important to increase the sample size of this specific blood group and perhaps treat the RBCs before assay.

Pilot studies indicated the possibility of a lectin-like activity in some of the screened macroalgae but only six of the species that showed strong and good

agglutination reaction were further processed for purification by ammonium sulfate fractionation. It was further observed that the fraction precipitated with 60-90% ammonium sulfate had higher hemagglutination activity than the aqueous extract, suggesting that salt precipitation is an useful method for removal of inhibitory compounds from the aqueous extract. Highest hemagglutination titer was obtained in the PBS extract of *Ulva fasciata* and NaCl extract of *Caulerpa sertularioides*. Thus, these two extracts were selected for further study. In the initial part of this study, these two alga also seemed to be the best choice for study. The higher occurrence of these two species in time and space deem them to be the best choice as it would facilitate availability of study sample.

Species of the green marine alga genus *Ulva* are widely distributed throughout the world. The attention of several research groups have been attracted to *Ulva* species, a potential source of exploitable lectin. For example Gilboa-Garber et al., (1988) have identified fucose-binding lectin from *U. lactuca*. Other *Ulv*a species have yielded lectins that are not inhibited by simple sugars, but are inhibited by more complex glycoproteins (Chiles and Bird, 1989). Most of those lectins had been examined in the crude or semi-purified extracts of the marine alga. The first description of purification of a lectin from the genus *Ulva* was reported only in 1996 by Sampaio et al. Since then, three lectins have been purified from the genus Ulva (Sampaio et al., 1998; Li et al., 2000 and Wang et al., 2004).

For the first time, marine algae inhabiting the Indian shores have been examined for hemagglutinins. Hemagglutinating activity present in aqueous extracts of *U. fasciata* was first detected by Barros and Himanshu (2005). This study sought to purify and characterize the relevant hemagglutinin using ion-exchange chromatography on DEAE-cellulose column, of ammonium sulfate (60-90%) precipitated cell extract and confirmed by SDS-PAGE. Ion-exchange chromatography has proved to be an useful procedure to isolate lectins from marine algae (Shiomi et al., 1981, Calvete et al., 2000). It was observed that the fraction precipitated with 60-90% ammonium sulfate had higher hemagglutination activity as compared to the aqueous extract, suggesting that salt precipitation is a useful method for concentrating the lectin protein from the algal homogenate. However, it was found that the hemagglutination activity decreased following the column chromatography step, suggesting that the last step of the purification step could be improved for better yield of active UFH.

Most characteristics of the lectin UFH are consistent with previous descriptions about other *Ulva* lectins and other marine alga lectins. Like most lectins from *Ulva* species, UFH is a small, single- banded monomeric protein with a relative molecular weight of 14,500 Dalton, stable at pH 6-10.5 and is thermo-stable at temperatures 40-100ºC losing part of it's capacity after heating for over 10 mins at 100ºC. Unlike other *Ulva* lectins, activity, albeit reduced, was also observed at 100ºC. This observation of the activity at high temperatures may be attributed to the fact that *Ulva fasciata* has been isolated from tropical waters along the shores of the Arabian Sea. This unusual thermo-stability has also been reported in *H. Japonica* (Hori et al., 1990) and *H. musciformis* (Nagano et al., 2002). The

first report of Dinh et al., (2009) on tropical algae has also reported activity at 100°C of a few species.

The hemagglutination activity of UFH was not affected by the presence of EDTA and independent of divalent cations as also observed in other lectins (Hori et al., 1990). The requirement for metal ions is not a general characteristic of most algal lectins (Rogers and Hori, 1993). For UFH, all glycoproteins that were tested failed to inhibit the lectin. It has been reported that many algal lectins have affinity for glycoproteins but not for monosaccharides (Hori et al., 1990; Sampaio et al., 2002). The lack of detecting sugar-binding specificity from UFH may be the result of a more complex recognition process by the agglutinins, one which may involve oligosaccharides instead of simple monosaccharides (Rogers and Hori, 1993; Calvete et al., 2000 and Nagano et al., 2005). However, the activity of Concanavalin A, used as a control in the test, was inhibited by D-mannose and N-acetyl-D-glucosamine.

Thus, UFH is small monomeric glycoprotein, similar to other agglutinins from marine algae, although it remains to be determined how such a monomeric form can causes the agglutination of cells. The results imply that *U. fasciata* could be a valuable source of a lectin that works even at high temperature and over a wide pH range. In sum, we herein purified and characterized a novel hemagglutinin from *U. fasciata* with pH and temperature stability making it a strong candidate in lectin research and biomedical applications.

The lectin CSH was purified similarly as *UFH* using Ion-exchange chromatography of ammonium sulfate 60-90% precipitated cell extract. SDS-

PAGE indicated that CSH is a dimeric glycoprotein similar to the lectin isolated from *Caulerpa cupressoides* (Benevides et al., 2001). Most of the lectins isolated from marine algae are low molecular weight. Lectins with low molecular weights (dimeric and tetrameric) were isolated from *Gracilaria veruucosa* (Shiomi et al., 1981), Hypnins (Hori et al., 1990) and *Palmaria palmate* (Kamiya et al., 1982).

CSH appears to be a stable lectin showing good activity over a pH range 5.0-10.5.CSH was even more stable as compared to UFH, retaining it's agglutinating capacity after heating at 100 ºC for 30 mins. These results coincide with the blooms of *Caulerpa sertularioides* being more in the drier (thus hotter) period of the year than *Ulva fasciata* which is more prevalent in the rainy season that is cooler. The hemagglutination activity of CSH like UFH did not require metal ions for hemagglutination suggesting that this lectin is not a metalloprotein, similar to many red algal lectins (Hori et al., 1990; Oliveira et al., 2002; Lima et al., 2005).

The hemagglutination inhibition activity of CSH was inhibited by N-acetyl–D-glucosamine, N-acetylneuramic acid, D-galacturonic acid of the monosaccharides and avidin, fetuin and mucin among the tested glycoproteins. Similar observations of inhibition of *Caulerpa sertularioides* lectins by fetuin, bovine submaxillary mucin were reported (Dinh et al., 2009) and *Caulerpa cupressoides* inhibited by monosaccharides (Freitas et al., 1997). Sugar-inhibition of green algal lectins by porcine stomach mucin was reported in U.lactuca (Sampaio et al. 1998a) and in *Caulerpa cupressoides* (Vahl) C,Agardh

(Benevides et al., 2001). Molchanova et al (2010) reported inhibition of agglutination by fetuin in red alga. It has been reported that red algal lectin bind to the small molecule of N-acetylglucosamine (Rogers and Hori, 1993). Chiles and Bird (1989), however, have reported that the lectins from *Gracilaria tikvahiae* bind the small molecule N-acetylneuramic acid. These recent findings indicate that CSH is a valuable source of lectin that has unique carbohydrate-binding specificity that could be used as new probes for carbohydrate research. Carbohydrate analysis by the phenol-sulphuric acid method suggests that CSH is a glycoprotein as are other agglutinins from marine algae, such as *Cystoclonium purpureum* (Kamiya et al.,1980), *Gracilaria bursa-pastoris* (Okamoto et al., 1988) and *Gracilaria ornate* (Leite et al., 2005).

The amino acid sequence of the lectin from marine algae was first reported recently (Calvete *et a*l., 2000). Up to now, there are only few full amino acid sequences of marine algae lectins reported from the marine red alga *Bryothamnion triquetrum* and *Hypnea japonica* and green algae *Ulva pertusa* (Calvete *et a*l., 2000, Hori et al., 2000, Wang et al., 2004). Marine algal lectins show similarities to lectins from terrestrial plants. However the results obtained on amino acid sequences (Calvete et al., 2000; Hori et al., 2000, Wang et al., 2004) and their N-terminal sequence (Kawakubo et al., 1999; Nagano et al., 2002) show no similarity with other sequences deposited in public databases. Characterization of those lectin genes may help researchers to further understand the difference between land plant and marine alga lectins.

The N-terminal amino acid sequence of the UFH does not show amino acid sequence similarity to known plant and animal lectins. Hence, this protein might be the paradigm of a novel lectin family. Apparently there is still much work to do on the sequences of algae lectins since only few amino acid sequences have been determined so far. More sequence studies will contribute to understanding their biochemistry and evolution differences between lectins present in land plants and those in marine algae.

Lectins have captured the interest of a large number of researchers on account of the various exploitable activities that they exhibit, including their proliferative effects on various cell types. Compared to plant lectins, there are only few reports on the use of marine algal lectins. For example, some marine algal lectins have antibiotic activity against marine *vibrios* (Liao et al., 2003), a novel lectin possessing anti-HIV activity was detected in the red alga *Griffithsia sp*. (Mori et al., 2005) and the agglutinin from *Euchema serra* had anti-tumour effects on colon cancer cells in vivo and in vitro (Fukuda et al., 2006)

UFH and CSH were tested for their effects on the growth of human cancer cell lines MCF7, Hela and PC-3 in vitro using MTT assay and confirmed with trypan blue staining. Dose response curves constructed between 10-100µg/ml and control without treatment respectively, expressed a significant drop in number of viable cells with increasing concentrations and exposure time to UFH and CSH. The percentage of cell survival decreased and was observed to retain trypan blue indicating cell death. On comparison, CSH was more cytotoxic as compared to UFH for the same cell line and dose. The reasons for the observed

differences in cytotoxic potency cannot be explained until detection tests and structural studies have been involved. These interesting observations could be useful in drug development strategies. One drawback is that non- cancer cells were not used as a negative control in this study.

Many marine algae produce antibiotic substances capable of inhibiting bacteria. It appears that the antibiotic characteristic is dependent on many factors, including the particular alga, the microorganisms, the season, and the growth conditions (Centeno and Ballantine, 1999; Hornsey and Hide, 1974 &1976; Pesando and Caram, 1984).

The antibacterial activity of marine algae has been assayed using extracts in various organic solvents, e.g., acetone (Hornsey and Hide, 1976), methanol–toluene (Caccamese et al., 1980), ether (Chesters and Stott, 1956), ethanol (Pesando and Caram, 1984), and chloroform-methanol (Centeno and Ballantine, 1999). Several extractable compounds, such as cyclic polysulfides and halogenated compounds are toxic to microorganisms and therefore responsible for the antibiotic activity of some marine algae (Fenical, 1975; Ohta, 1979; Wrattens and Faulkner, 1976). However, an antibiotic assay of extracts in aqueous solutions probably does not reflect adequately the antibacterial activity of marine algae under natural conditions. UFH and CSH did not show any antimicrobial activity to the bacterial strains tested. Similar results have been reported in BPL-2, a lectin isolated from *Bryopsis plumose* that did not show any antimicrobial properties (Han et al., 2010). More comprehensive studies are necessary to determine the possible medical use of these lectins.

It has been reported that reactive oxygen species contribute to various patho-physiological conditions and endogenous defense mechanisms have evolved to offer protection in these conditions. An increase in the antioxidant reserves of the organism can reduce oxidative stress and some of the plant derived agents may help to reduce it. Determination of the natural antioxidant compounds of plant extracts will help to develop new drug candidates for antioxidant therapy (Prior, 2003; Hasan et al., 2006; Erdemoglu et al., 2006). Plants may be considered as good sources of natural antioxidants for medicinal uses such as against aging and other diseases related to radical mechanisms (Augustin et al., 2005). Further investigation on the UFH and CSH of antioxidant component(s) in the marine algae lectins may lead to chemical entities with potential for clinical use. Purified extracts of UFH and CSH showed low radical scavenging activity as compared to standard anti-oxidant ascorbic acid. Judging from the results of ascorbic acid inhibition and radical scavenging activity, it may be possible to find effective antioxidants among these algae when other solvents are used for extraction.

Based on the results obtained from this study and the earlier studies that have been reported, it is probable that lectins share some important role in the algae. It is therefore also important to establish the physiological properties of these lectins. In spite of the progress made in the biochemical characterization of marine algal lectins, additional information is required for further understanding of their properties, structures and biological functions.

The results obtained in this study indicate that Indian marine algae are a good source of novel and useful lectins.

Coast of Goa is abundant in algal distribution. It is worth isolating and study of lectins from these untapped abundant resources. The list of application of lectins is growing rapidly and the potential of this glycoprotein is far from completely exposed. Further scope of this study can be listed as follows:

- Tendency for O specificity to be checked with a larger sample as well as by treating the RBCs.
- Further characterization studies.
- It is therefore also important to establish the physiological properties of this lectin.
- It is important to elucidate the complete primary structure of this hemagglutinin and compare it to other *Ulva* lectins.
- The analysis of primary sequence of this purified lectin.
- Preparation of affinity column using CSH for purifications of glycoprotein
- Detailed investigation against cancer cell lines.
- To check the sugar specificity of isolated lectins and develop applications in the field of Biotechnology.
- Detection of mitogenic activity.
- Screen more algae for blood group specificity.
- Detection of the insecticidal activity of screened algae.
- Purification and characterization of more species especially from red algae.

The main objective of the present studies was to isolate lectin from untapped marine algae along the coast of Goa.

- Twenty one different species of marine algae prevalent among Goa coast were collected, screened for agglutinins.
- Three different buffers were used for isolating the aqueous extract and PBS extract gave better agglutination against human RBCs.
- Pilot studies indicate the possibility of a lectin-like activity from screened macroalgae. No blood group specificity was observed.
- Aqueous extract of six species that showed strong agglutination were processed for purification to extract protein by ammonium sulfate precipitation and activity was characterized by hemagglutination.
- The extract of *Ulva fasciata* and *Caulerpa sertularioides* precipitated out in 60-90% fraction yielded highest hemagglutination titers.
- Above fractions process for DEAE-Cellulose chromatography.
- Two lectins isolated namely UFH and CSH.
- UFH: monomeric, glycoproteins with molecular weight appears to be approximately 14.5 kDa, effective over pH range 6 to 10.5 and with good thermal stability and not require metal ions for its activity and was not inhibited by any of the sugars and glycoproteins tested.
- Amino acid analysis of UFH showed the similarity from *Zea mays, Oryza sativa* and *Pseudotsuga*
- CSH: dimeric, glycoprotein with molecular weight appears to be approximately1y kDa and 31.3kDa, effective over pH range 5 to 10.5 and

with good thermal stability and not require metal ions for activity and activity were inhibited by sugars N-acetyl –D-glucosamine, N-acetyl neuramic acid, D-galacturonic acid , Avidin from egg white, Fetuin and Mucin type 1

- UFH Strongly cytotoxic for MCF7 and Hela cell lines 48 h and 24 h to 48 h has exposure.
- CSH strongly cytotoxic for MCF7 and PC3 cell lines 24 h and 48 h has exposure.

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# Appendix

**1. Phosphate buffered saline**

Nacl 8.0g

$Na_2HPO_4$ 1.2g

$KH_2PO_4$ 0.2g

pH 7.4

**2. Tris-buffered saline**

10mM Tris base (Tris (hydroxymethyl) aminomethane)

150mM Nacl

pH 7.4

**3. 0.85% Sodium chloride**

**4. Dialysis bag cut-off 12 kDa**

**5. Ammonium sulfate precipitation**

0-30% - 1.64 gm in 10 ml

30-60% - 1.81 gm in 10ml

60-90% - 2.01 gm in 10 ml

**6. Folin-Lowary assay reagent**

**Reagent A**

2% $Na_2CO_3$ in 0.1 M NaOH

**Reagent B**

1% $CuSo_4$ in DW

**Reagent C**

2% ($NaKC_4H_4O_6.4H_2O$)

**Lowary stock solution**

4.9ml A
0.5ml B
0.5ml C

**Folin reagent- (Folin-ciocalteau reagent)**

1:1 in DW (Freshly prepared)

**BSA**

1mg/ml

**7. Neutral sugar (Phenol sulphuric acid)**

Glucose 1mg/ml

80% phenol solution

2.0 ml concentrated sulphuric acid

**8. Native PAGE**

30% Acrylamide mix (29g acrylamide and 1g Bis-acrylamide in 100ml DW)
1.5M Tris (pH 8.8)
10% ammonium persulfate
TEMED
1.0M Tris (pH 6.8)

**8.1. 12% Resolving gel (10ml)**

$H_2O$ 3.3ml
30% acrylamide mix 4.0ml
1.5 Tris (pH 8.8) 2.5 ml
10% ammonium persulfate100 µl
TEMED 0.5 µl

**8.II. Stacking gel (5ml)**

$H_2O$ 3.4ml
30% acrylamide mix 0.83ml
1.0 Tris (pH 6.8) 0.63 ml

10% ammonium persulfate 50µl

TEMED 0.5 µl

**8.III. 5X Native gel-loading buffer (5ml)**

0.3ml -1M Tris (pH 6.8)

2.5ml- Glycerol

0.5ml – 1%Bromophenol blue

**9. SDS- PAGE**

30% Acrylamide mix (29g acrylamide and 1g Bis-acrylamide in 100ml DW)

1.5M Tris (pH 8.8)

10% SDS

10% ammonium persulfate

TEMED

1.0M Tris (pH 6.8)

**9.1 Resolving Gels 12% (10ml)**

$H_2O$ 3.3ml

30% acrylamide mix 4.0ml

1.5 Tris (pH 8.8) 2.5 ml

10% SDS 100 µl

10% ammonium persulfate100 µl

TEMED 0.5 µl

**9.2 Stacking gel (5ml)**

$H_2O$ 3.4ml

30% acrylamide mix 0.83ml

1.0 Tris (pH 6.8) 0.63 ml

10% SDS 50 µl

10% ammonium persulfate 50µl

TEMED 0.5 µl

**9.3 5X SDS gel-loading buffer (5ml)**

0.3ml -1M Tris (pH 6.8)

2.5ml- Glycerol

1ml- 10% SDS

0.25 ml-mercaptoethanol

0.5ml – 1%Bromophenol blue

**9.4 1X Tris-glycine electrophoresis buffer**

25mM Tris

250mM glycine

0.1 (w/v) SDS

**10. Silver stain**

**A) Fixing solution**

Glacial acetic acid-10ml

DW-90ml

**B) Washing solution**

Absolute ethanol and DW (1:1)

**C) Sodium thiosulphate**- 0.002mg in DW

**D) Silver nitrate solution (100ml)**

Silver nitrate- 0.2g

Formaldehyde-0.075ml

**E) Developing solution (100ml)**

Sodium carbonate-6g

Sodium thiosulphate-0.004g

Formaldehyde-0.05ml

**F) Stop solution**

Glacial acetic acid-7ml

DW-93ml

**11. 100mM Sodium citrate at pH 9.0 and 10.5**

**12. 100mM Phosphate buffer at pH 6.0, 7.0 and 8.0**

**13. 100mM Sodium acetate at pH 5.0**

**14. Alcian blue stain (4:1:5)**

Alcian blue 0.2mg

Methanol 40ml

Glacial acetic acid 10ml

DW 50 ml

**15. Destain solution**

Methanol 40ml

Glacial acetic acid 10ml

DW 50 ml

**16. Thymol-Sulphuric acid staining for glycoprotein**

2-proponal, acetic acid and DW (25:10:65)

2-proponal, 10% acetic acid and DW (25:10:65)

Thymol (0.2% (v/v))

Sulphuric acid and ethanol (80:20)

**17. 5mM EDTA**

**18. 5mM $CaCl_2$**

**19. 5mM $MnCl_2$**

**20. DMEM**

**21. MEM**

**22. Hanks F12-K**

**23. Foetal Bovine Serum and 100mg/ml**

**24.Tetrazolium derivative reduction (MTT)**

**25. DPPH (0.004%)**

**26. Gallic acid 1mg/ml**

**27. Ascorbic acid 1mg/ml**

# List of abbreviation

**Nacl** - Sodium chloride

**PBS** - Phosphate buffered saline

**$Na_2HPO_4$** - di-Sodium hydrogen phosphate anhydrous

**$KH_2PO_4$** - Potassium dihydrogen orthophosphate

**TBS** - Tris-buffered saline

**ConA** - Concanavalin A

**D/W** - Distilled water

**BSA** - Bovive serum albumin

**g** - Gram

**mg** - Milli gram

**µg** - Micro gram

**µl** - Micro litre

**L** - Litre

**RBC** - Red blood cell

**rpm** - Revolution per minute

**M** - Molar

**mM** - Milli molar

**kDa** - Kilo Dalton

**w/v** - Weight/volume

**v/v** - Volume/volume

**v/w** - Volume/weight

**EDTA** - Ethylenediamine tetraacetic acid

**$CaCl_2$** - Calcium chloride

**$MnCl_2$** - Magnise chloride

**APS** - Ammonium persulphate

**TEMED** - N,N,N',N'-tetramethylethylenediamine

**PAGE** - Polyacrylamide gel electrophoresis

**SDS** - Sodium dodecyl sulphate

**$Na_2Co_3$** - Sodium carbonate anhydrous

**NaOH** - Sodium hydroxide

**$CuSo_4$** - Cupper sulphate

**$NaKC_4H_4O_6.4H_2O$- Sodium potassium tartrate**

**DMEM - Dulbecco's modified eagle medium**

**MEM -Minimum essential medium**

**Hanks F12-K**

**FBS - Foetal Bovine Serum**

**MTT - Tetrazolium derivative reduction**

**DPPH - 1, 1-diphenyl-2-picrylhydrazyl**