AGGLUTININS WITH BINDING SPECIFICITY FOR MAMMALIAN ERYTHROCYTES IN THE WHOLE BODY EXTRACT OF MARINE GASTROPODS

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Abstract

Presence of agglutinins in the whole body extract of some locally available species of marine gastropods was studied by adopting haemagglutination assay using 10 different mammalian erythrocytes. Of the animals surveyed, 14 species showed the presence of agglutinins for one or more type of erythrocytes. The agglutinating activity varied with the species as well as with the type of erythrocytes. Rabbit and rat erythrocytes were agglutinated by all the species studied. Highest activity of the agglutinins was recorded in the extract of *Fasciolaria tulipa* and *Fusinus nicobaricus* for rabbit erythrocytes, as revealed by a HA (Haemagglutination) titre of 1024, the maximum value obtained in the study. *Trochus radiatus*, *Tonna cepa*, *Bufornia echineta*, *Volegalea cochlidium*, *Chicoreus ramosus*, *Chicoreus brunneus*, *Babylonia spirata*, *Babylonia zeylanica* and *Turbinella pyrum* are among the other species, possessing strong (HA titre ranging from 128 to 512) anti-rabbit agglutinins. Agglutinins with binding specificity for rat erythrocytes have been observed in the extract of *Trochus radiatus*, *Fasciolaria tulipa* and *Fusinus nicobaricus*. None of the species agglutinated dog, cow, goat and buffalo erythrocytes. Agglutinins with weak activity against human erythrocytes were observed in *Chicoreus brunneus* (HA = 4 – 8). The present work has helped to identify potential sources of agglutinins among marine gastropods available in and around Kanyakumari District and thereby provides the baseline information, in the search for new pharmacologically valuable compounds derived from marine organisms. The agglutinins with high binding specificity, like those in the extract of *Fasciolaria tulipa* and *Fusinus nicobaricus* can be purified and studied further for evaluating their potential in the field of medicine and molecular biology.

Key words

Agglutinins, Haemagglutination, Erythrocytes, Gastropods, Lectins, Tris-buffered saline,

INTRODUCTION

Oceans serve as the natural habitat for a broad variety of living organisms, harbouring the largest diversity of invertebrates. To withstand a wide range of abiotic and biotic pressures, marine invertebrates possess specific adaptations. Many of these marine invertebrates are sessile or slow moving, and lack physical defense structures to protect themselves from potential predators and competitors. Despite being

constantly bathed in an environment of microorganisms, they lack adaptive immunity against pathogens and parasites and rely entirely on effective innate immune systems to defend themselves. Marine invertebrates produce an arsenal of bioactive secondary metabolites to protect themselves from infection. Some use water soluble secondary metabolites for chemical communication (pheromones, settlement cues) and neurotoxins (in venoms) to paralyze or kill their prey.

Thus, the marine environment is an exceptional reservoir of novel bioactive natural products, many of which exhibit structural/chemical features not found in terrestrial natural products. The rapid increase in drug resistance among disease causing pathogens coupled with the emergence of new epidemics have forced the scientists to focus their research on marine natural products for the discovery of new drugs. Another reason for the intensified search of pharmacologically active compounds from marine organisms is that, molecules of marine origin are accepted by humans with minimal manipulation (Vignesh *et al*., 2011).

India has over 8000 km of coastline with clusters of marine habitats like inter-tidal, rocky, muddy and sandy shores, coral reefs, and mangrove forests (Malve, 2016). Many classes of bioactive compounds exhibiting anti-tumor, anti-leukemic, antibacterial, anti-parasitic and antiviral activities have been reported worldwide in marine organisms (Grabley and Thiericke, 1999; Rajaganapathy *et al*., 2002; Fuesetani, 2000; Simmons *et al*., 2005). Bioactive compounds from marine organisms take the form of agglutinins, lysins, acute phase proteins or antimicrobial substances which play a crucial role in innate immunity and host defense. Such compounds occur in the haemolymph, mucus, tissues, shell or in egg masses.

Over 20,000 natural products have been isolated and identified from various marine organisms (Hu *et al*., 2011). Of these products isolated, 25% are from algae, 33% from sponges, 18% from coelenterates (sea whips, sea fans and soft corals) and 24% from invertebrates belonging to other phyla such as bryozoans (moss animals), opisthobranch molluscs (nudibranch, sea hares etc.) and echinoderms (Kijjoa and Sawangwong, 2004). Some of the marine natural products which are promising candidates for new drugs that have been discovered are Halichondrin B, Mycaperoxide B, Dithiocyanates, Manoalide, Discodermolide and Contignasterol (from sponges), pseudopterosins (from cnidarian); Bryostatin1 (from bryozoan); Ziconitide, Kahalaide F and Dolastatin 10 (from mollusc); Imbricatine and Lysastroside-A (from echinoderm); Aplidine, Ecteinascidin-743, Lamellarin a 20 sulfate, Didemnin B and Cyclodidemniserinol trisulfate (from tunicate). Commercialized products from marine organisms, available in the market include Cephalosporin from marine fungi, cytostatic Cytarabine from sponge, antihelminthic insecticide Kanic acid from red alga, Zincototide from mollusk, etc. (Thakur *et al*., 2005). Others are under clinical or preclinical trials. As the search for 'drugs from sea' progresses, the number of new compounds from marine organisms increases at the rate of 10 percent per year (Kijjoa and Sawangwong, 2004).

Among the compounds isolated from marine invertebrates, agglutinins have received great interest today because of their wide application in medicine and other applied branches of biology. Agglutinins are primarily an important class of recognition molecules (Vijayan and Chandra, 1999) and are known to occur ubiquitously, among microorganisms (Sasmal *et al*., 1992), plants and animals (Yeaton,

1981). They are capable of agglutinating a variety of foreign particles such as bacteria, yeasts, protozoans, vertebrate erythrocytes, normal and malignant cells and metazoan parasites or precipitate glycoconjugates, in a manner similar to antigen-antibody interactions.

A group of agglutinins, which have recently gained much attention and importance are lectins. As described by Sharon and Lis (2004), lectins are cell agglutinating sugar-specific proteins. These magic molecules are defined by their sugar binding specificity and have wide application in various fields of biological sciences. They serve as invaluable tools for blood typing, diagnosis of microorganisms in food and biological samples, discrimination of normal and malignant cells, as cytotoxic agents, as inflammalogens, for mitogenic stimulation of lymphocytes, to examine cell surface carbohydrates, for purifications of saccharide-containing substances including polysaccharides, glycopeptides and glycoproteins, viruses, subcellular fractions, bacteria and mammalian cells. Recently, they have been recognized as molecules that can serve as 'drug carriers', to carry biologically active molecules to target cells or organelles (Bies *et al*., 2004). Thus, lectins today find their application almost in every aspect of biology.

Considering the myriad exciting functions and of applications of lectins and the available rich marine biodiversity in and around Kanyakumari District, the present work was carried out to identify new sources of lectins, among the locally available marine gastropods. The first step in the identification of lectins is to identify them by their cell agglutinating property. Hence in the present work, the marine gastropods were screened for the presence of agglutinins capable of agglutinating the indicator cells, the erythrocytes.

MATERIALS AND METHODS

Animals studied and their systematic position

The marine gastropods for the present study were collected from coasts in and around Kanyakumari District. Collection was mainly by handpicking from the intertidal zone during low tide. Some were collected from crab net, trawl net etc. from regular fish landings. Species were identified with the help of Zoological Survey of India, Chennai (F.No. 12-18/2016-2017/SRC/Moll/ID/F.V./).

Preparation of whole body extract

Animals were brought to the laboratory and they were rinsed with sterile water to remove adhering mud and other debris from outer surface of the shell and blotted to remove water prior to the experiment. The shells were broken very carefully using a hammer. The whole body of each snail was removed and washed thoroughly with normal saline solution and blotted. The whole body (shell-free) of each snail macerated and ground with a mortar and pestle to make a fine paste. The body extract was then prepared by homogenizing 100 mg of the finely ground body in 1 ml of sterile saline. Homogenized extract was centrifuged at 4000 x g for 10 minutes at 4°C and the supernatant was pooled in small aliquots and stored at -20°C.

Gastropods studied and their systematic position

1. *Trochus radiatus* (Gmelin,1791)

Phylum : Mollusca
Class : Gastropoda
Sub class : Vetigastropoda
Order : : Trochida
Super family : Trochoidea
Family : Trochidae


2. *Tonna dolium* (Linnaeus, 1758)

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Littorinimorpha
Super family : Tonnoidea
Family : Tonnidae


3. *Tonna cepa* (Linnaeus, 1758)

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Littorinimorpha
Super family : Tonnoidea
Family : Tonnidae


4. *Bufornia echineta* (Link, 1807)

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Littorinimorpha
Super family : Tonnoidea
Family : Bursidae


5. *Volegalea cochlidium* (Linnaeus, 1758)

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Neogastropoda
Super family : Buccinoidea
Family : Melonginidae


6. *Fasciolaria tulipa* *(Linnaeus, 1758)*

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Neogastropoda
Super family : Buccinoidea
Family : Fasciolariidae


7. *Fusinus nicobaricus* *(Roeding, 1798)*

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Neogastropoda
Super family : Buccinoidea
Family : Fasciolariidae


8. *Mancinella echineta* *(Blainville,1832*

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Neogastropoda
Super family : Muricoidea
Family : Muricidae


9. *Purpura panama* *(Roeding , 1798)*

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Neogastropoda
Super family : Muricoidea
Family : Muricidae


10. *Chicoreus ramosus* *(Linnaeus, 1758)*

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Neogastropoda
Super family : Muricoidea
Family : Muricidae


11. *Chicoreus brunneus* *(Link, 1807)*

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Neogastropoda
Super family : Muricoidea
Family : Muricidae


12. *Babylonia spirata* *(Linnaeus1758)*

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Neogastropoda
Super family : Muricoidea
Family : Babyloniidae


13. *Babylonia zeylanica* *(Bruguiere,1789)*

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Neogastropoda
Super family : Muricoidea
Family : Babyloniidae


14. *Turbinella pyrum* *(Linnaeus, 1767)*

Phylum : Mollusca
Class : Gastropoda
Sub class : Caenogastropoda
Order : : Neogastropoda
Super family : Turbinelloidea
Family : Turbinellidae


Erythrocyte preparation

Blood for haemagglutination assay was obtained by venipuncture of the ear (rabbit) or forearm (man, dog and buffalo) or heart puncture (rat) or from slaughter house (pig, cow and goat) and collected directly in cold modified Alsevier's medium (Sodium citrate: 30 mM; Sodium chloride: 77 mM; Glucose: 114 mM; Neomycin sulphate: 100 µg/ml; Chloramphenicol: 300 µg/ml; pH 6.1). The erythrocytes were washed with ten volumes of 0.9% saline twice and with TRIS buffered saline (pH 7.5) once and resuspended in the same as 1.5% suspension.

Haemagglutination assay

Haemagglutination assays were performed in microtitre plates with 'U' bottomed wells by two fold serial dilutions of 25 µl sample with an equal volume of TRIS buffer (pH 7.5). After the dilution of the sample, 25 µl of 1.5% erythrocyte suspension was added, mixed well and incubated for 1h at room temperature (30° ± 1°C). The HA titre was determined as the reciprocal of the highest dilution of the sample giving complete agglutination.

RESULTS AND DISCUSSION

The ocean provides enormous opportunities to discover new compounds Though a large number of biologically active compounds of therapeutic interest have been isolated so far from marine organisms, the number is meager when compared to the rich biodiversity available in the Indian ocean and thereby the Indian marine habitat remains largely unexplored. Among the marine invertebrates, marine molluscs are the good source of bioactive metabolites (Kiran *et al*., 2014). These bioactive factors of invertebrates include agglutinins, lysins, antibacterial and antifungal proteins, phenoloxidase system, LPS binding protein and β-1, 3 glucan binding protein (Ofek and Sharon, 1988).

Extracts from marine molluscs are potential sources of bioactive compounds. Antibacterial agents have been reported in the marine mollusc, *Kelletia kelletii* (Tymiak and Rinehart, 1983). HIV virus-inhibiting compound from the green mussel *Perna viridis* has been studied and patented (Mitra and Chatterji, 2004). Highly active anti-tumor compounds Dolastatins have been isolated from the sea hare Dolabella (Pettit *et al*., 1987). A cardiotonic glycoprotein 'Striatoxin', was obtained from *Conus striatus* (Kobayashi *et al*., 1982). A steroidal glycoside 'Lysastroside-A' was isolated from the starfish *Lysastrosoma anthosticta* (Levina *et a*l., 2002). Antibacterial activity has been reported in *Trochus radiatus* (Mary Elezabeth *et al*., 2003), *Trochus tentorium* (Anbuselvi *et al*., 2009), *Lambis lambis* (Rohini *et al*., 2012), *Melo melo* (Sivasubramanian *et al*. 2011). *Nerita albicilla* and *Purpura bufo* (Sharmin Vini *et al*., 2018). Wide spectrum antibacterial activity has been reported in the shell powder extracts of marine mollusc *Donax faba* (Giftson and Patterson, 2014).

Naturally occurring agglutinins of red blood cells occur in the tissue and body fluids of several species of gastropod molluscs (Tyler, 1946). The ability of the agglutinins to bind to specific erythrocytes depends upon the presence of specific receptors on the surface of the erythrocyte membrane. The results obtained in our study reveal the presence of agglutinins for one or more types of erythrocytes in the whole body extract of the marine gastropods (Table 2). The agglutinins showed binding specificity for human A, human B, human O, rabbit, rat and pig erythrocytes, while dog, cow, goat and buffalo erythrocytes were not agglutinated by any of the species studied. This suggests that the agglutinins may share common binding receptor/receptors present on the surface of human ABO erythrocytes, rabbit, rat and pig erythrocytes. The variation in the HA titre may be due to the difference in the quantitative distribution of specific receptor

determinants on the erythrocyte membrane which are preferentially recognized by the agglutinins. High haemagglutinating activity was obtained with the extract of *Trochus radiatus*, *Tonna cepa*, *Bufornia echineta*, *Volegalea cochlidium*, *Fasciolaria tulipa*, *Fusinus nicobaricus*, *Chicoreus ramosus*, *Chicoreus brunneus*, *Babylonia spirata*, *Babylonia zeylanica* and *Turbinella pyrum*. All the species showed agglutinins for rabbit and rat erythrocytes but the degree of agglutinating activity varied. This may be due to more number of the specific receptors these erythrocytes or the receptors are more accessible on these erythrocytes. The predominant surface receptor determinants of some mammalian erythrocytes have been reported. Human erythrocytes display both NeuAcα2,6Gal and NeuAcα2,3Gal (Medeiros *et al*., 2004). Rabbit erythrocytes contain NeuAc, 9-O-Ac NeuAc, NeuGc and 9-O-Ac NeuGc (Pfeil *et al*., 1980). NeuGc/ NeuAc/4(7)-0-acetylated sialic acids occur on rat erythrocytes (Bhavanandan *et al*., 1964). NeuGcα2,6Gal are present on the surface of pig erythrocytes (Trombetta *et al*., 2018).

Table 2. Haemagglutination activity (expressed as HA Titre) of whole body extract of the marine gastropods

Species	Erythrocytes									
	Human A	Human B	Human O	Rabbit	Rat	Dog	Pig	Cow	Goat	Buffalo
Trochus radiatus	0	0	0	256	128	0	16	0	0	0
Tonna dolium	0	0	0	16	16	0	8	0	0	0
Tonna cepa	0	0	0	256	32	0	4	0	0	0
Bufornia echineta	0	0	0	128	64	0	16	0	0	0
Volegalea cochlidium	2	0	0	128	32	0	0	0	0	0
Fasciolaria tulipa	0	0	0	1024	128	0	0	0	0	0
Fusinus nicobaricus	0	0	0	1024	256	0	0	0	0	0
Mancinella echineta	0	0	0	32	8	0	0	0	0	0
Purpura panama	0	0	0	32	16	0	0	0	0	0
Chicoreus ramosus	0	0	0	512	32	0	0	0	0	0
Chicoreus brunneus	8	4	4	128	64	0	0	0	0	0
Babylonia spirata	0	0	0	256	64	0	64	0	0	0
Babylonia zeylanica	0	0	0	128	32	0	0	0	0	0
Turbinella pyrum	0	0	0	128	32	0	8	0	0	0

The extract of *Trochus radiatus* agglutinated rabbit, rat and pig erythrocytes. Maximum activity (HA titre 256) was shown against rabbit erythrocytes (Figure 1). *Tonna dolium* showed a comparatively weaker affinity for rabbit, rat and dog erythrocytes with the HA titres of 16, 16 and 8 respectively (Figure 2).

Figure 1

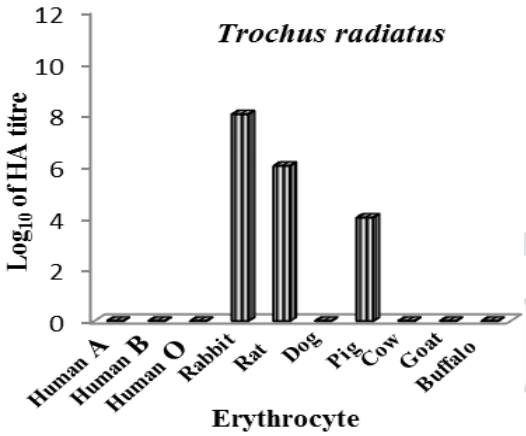
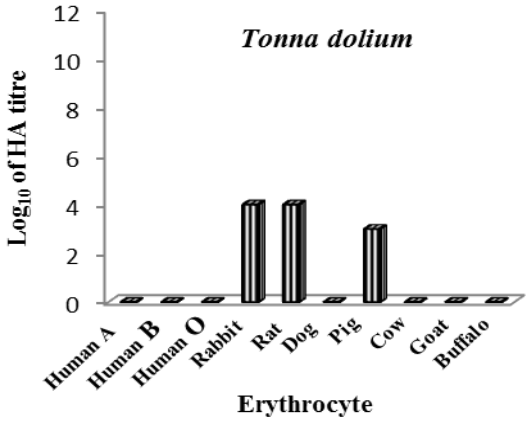


Figure 2



Tonna cepa agglutinated rabbit erythrocytes more efficiently (HA titre 256) than rat (HA titre 32) and pig (HA titre 4) erythrocytes (Figure 3). *Bufornia echineta* gave a HA titre of 128 with rabbit erythrocytes, 64 against rat erythrocytes and 16 against pig erythrocytes (Figure 4).

Figure 3

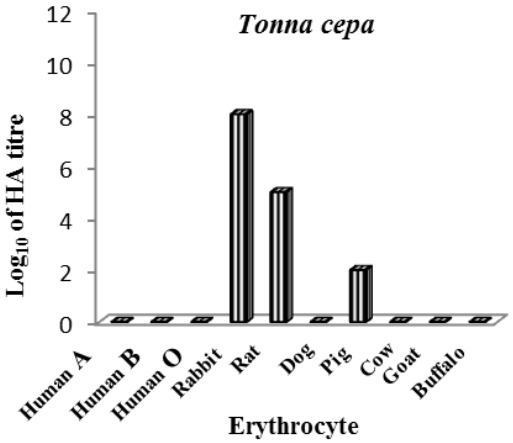
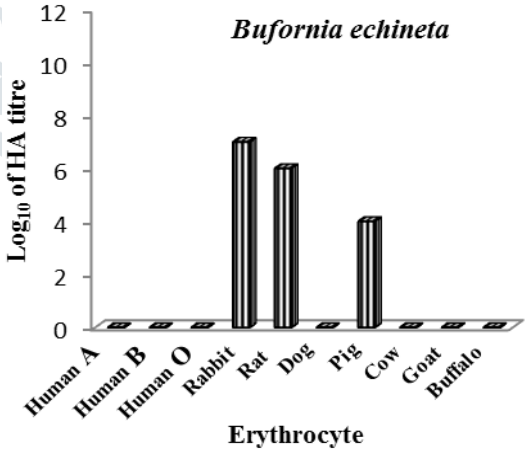


Figure 4



Volegalea cochlidium agglutinated human A (HA titre 2), rabbit (HA titre 128) and rat (HA titre 32) erythrocytes (Figure 5). *Fasciolaria tulipa* was one of species that revealed the presence of strong agglutinins for rabbit erythrocytes (HA titre 1024). Rat erythrocytes were also agglutinated (HA titre 128) but with a lesser potency (Figure 6).

Figure 5

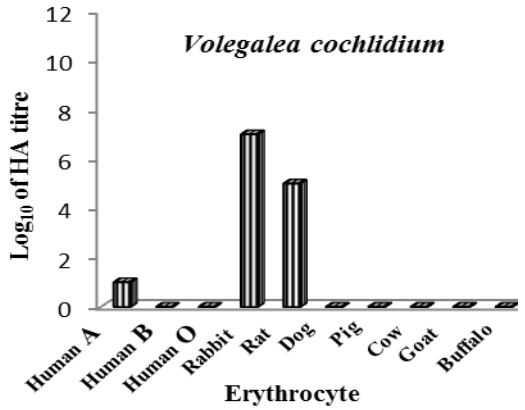
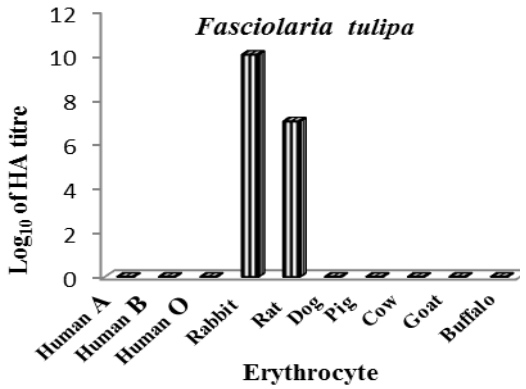


Figure 6



Fusinus nicobaricus also showed high binding specificity for rabbit erythrocytes (HA titre 1024) and a lesser affinity for rat (HA titre 256) erythrocytes (Figure 7). *Mancinella echineta* agglutinated rabbit (HA titre 32) and rat (HA titre 8) erythrocytes (Figure 8*).*

Figure 7

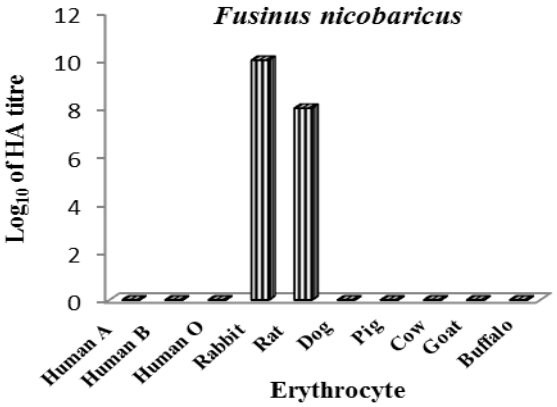
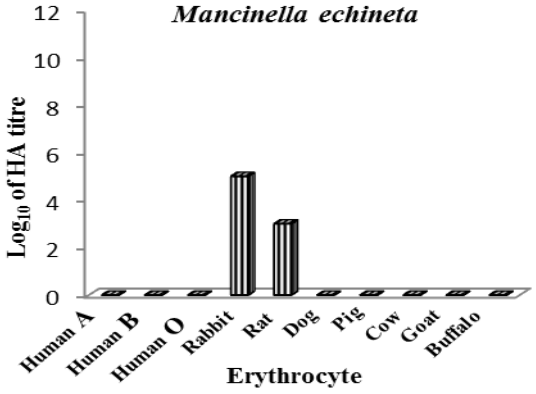


Figure 8



The whole body extract of *Purpura panama* showed affinity for rabbit (HA titre 32) and rat (HA titre 16) erythrocytes (Figure 9*). Chicoreus ramosus* showed higher affinity for rabbit (HA titre 512) but a weaker affinity for rat (HA titre 32) erythrocytes (Figure 10*).*

Figure 9

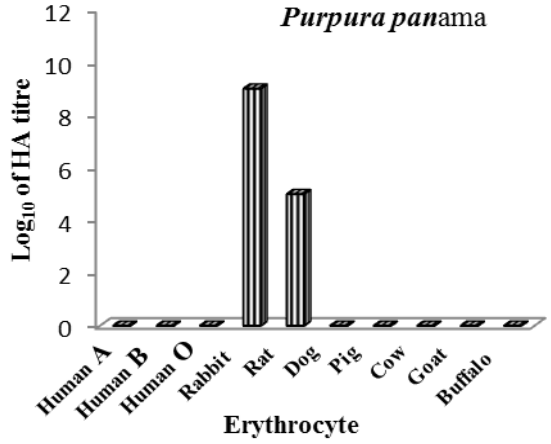
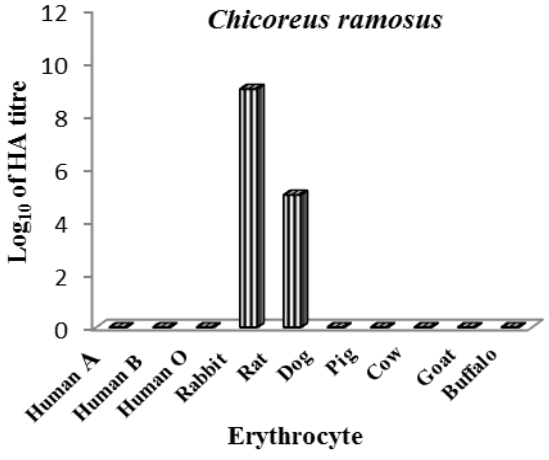


Figure 10



Specificity for human ABO erythrocytes, rabbit rat erythrocytes was observed with *Chicoreus brunneus* extract that gave a HA titre of 8 with human A, 4 with human B, 4 with human O, 128 with rabbit and 64 with rat erythrocytes (Figure 11*)*. *Babylonia spirata* agglutinated rabbit (HA titre 256), rat (HA titre 64) and pig erythrocytes (HA titre 64) (Figure 12*)*.

Figure 11

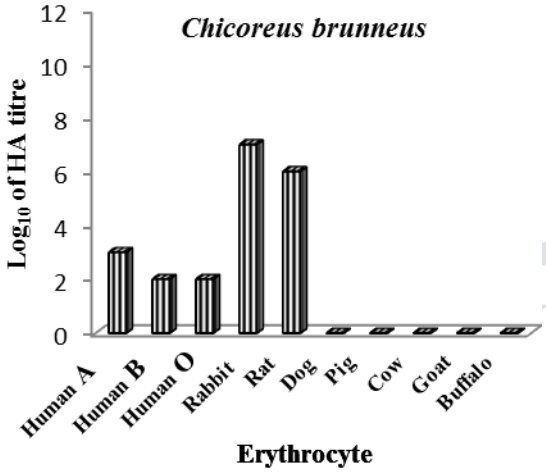
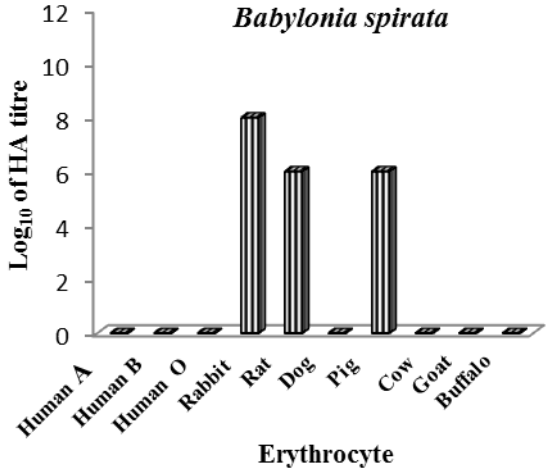


Figure 12



Babylonia zeylanica extract agglutinated rabbit (HA titre 128) and rat (HA titre 32) erythrocytes (Figure 13*)*. *Turbinella pyrum* agglutinated rabbit (HA titre 128), rat (HA titre 32) and pig (HA titre 8) erythrocytes (Figure 14*)*.

Figure 13

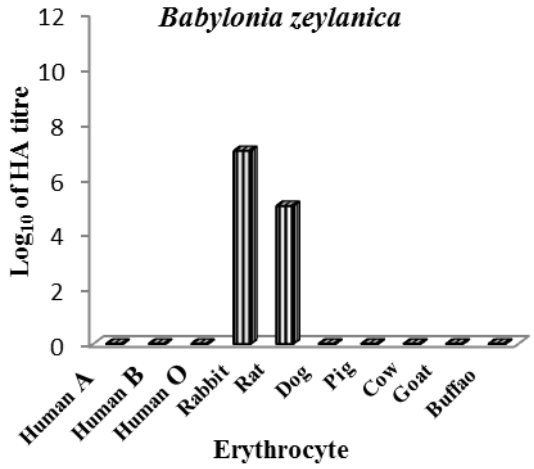
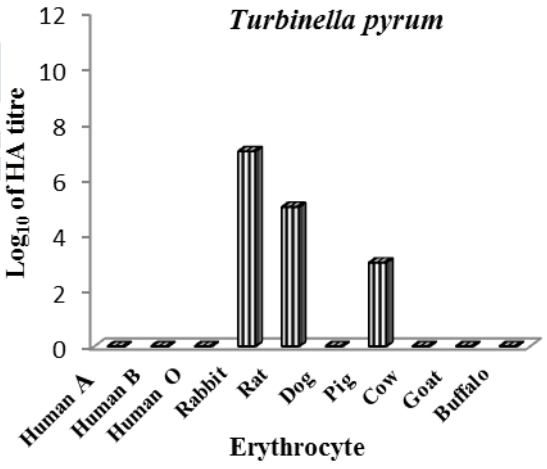


Figure 14



A perusal of literature reveals the occurrence of haemagglutinins in various molluscan species. Haemagglutinins have been reported to be present in the whole body homogenate of *Achatina fulica* and *Achatina granulata* (Gold *et al*., 1967; Brain and Grace, 1968; Khalap *et al*., 1970), common limpet *Patella vulgata* (Pemberton (1970) and *Pomacea flagellata* (Roberto Arreguin and Barbarín Arreguín-Lozano, 1997), haemolymph of *Viviparus malleatus* (Cheng and Sanders, 1962), *Australorbis glabratus* (Gilbertson and

Etges, 1967), *Biomphalaria sudanica* (Gilbertson and Etges, 1967), murray mussel *Velesunio ambiguous* (Jenkin and Rowley, 1970), sea snail *Dolabella* (Ishiyama *et al*., 1972), *Aplysia californica* (Pauley *et al*., 1971), *Biomphalaria glabrata* (Boswell and Bayne, 1984), cephalopod *Octopus vulgaris* (Rogener *et al*.,1985) and *Turbo brunneus* (Thana Lakshmi, 2006), eggs of the sea hare *Aplysia kurodai* (Kamiya and Shimizu 1961) and *Pomacea palucdosa* (Baldo and Uhlenbruck, 1974)., albumin gland of *Helix pomatia* (Uhlenbruck and Prokop, 1966), *Otala lactea* (Bhatia *et al*. (1967), *Helix pomatia* (Hammarstrom and Kabat, 1971), *Cepaea nemoralis* (Schnitzler *et al*., 1971), *Caucasotachea atrolabiata* (Schnitzler *et al*., 1971), *Helix aspersa* (Ishiyama *et al*., 1973), *Euhacira callizona amaliae* (Ishiyama *et al*., 1974), *Pomacea urceus* (Baldo and Uhlenbruck, 1974) and *Trachia vittata* (Thana Lakshmi, 2006), mucus of oyster *Crassostrea virginica* (Fisher, 1992), digestive diverticula of the scallop *Patinopecten yessoensis* (Mori *et al*., 1980), plasma of marine mussel *Perna viridis* (Jayaraj *et al*., 2008) and spermatheca of *Telescopium telescopium* (Maji *et al*., 2010).

Agglutinins with sugar specificity, referred to as lectins, are extremely useful tools for the investigation of carbohydrates on cell surfaces, in particular of the changes that the latter undergo in malignancy, as well as for the isolation and characterization of glycoproteins (Sharon and Lis, 2004). Lectins are versatile reagents that can be applied in certain instances to provide definitive identification and strain characterization of a particular infectious agent (Slifkin and Doyle, 1990). Lectins have been shown to be of value in the detection of intrastrain variations in their respective cell wall carbohydrate residues. Schalla *et al*. (1985) have shown that lectins are useful in the epidemiological characterization of *Neisseria gonorrhoeae*. Lectins are potential drugs for treatment of AIDS (Hamid *et al*., 2013). Recently it has been found that lectins could be used as drug carriers, for carrying biologically active molecules and directing them to specific cells or organelles (Lavelle, 2001; Lehr and Gabov, 2004; Bies *et al*., 2004) which signifies their role in 'targeted drug therapy'

Thus the current study reveals the presence of agglutinins among the local species of marine gastropods. Those agglutinins with strong erythrocyte-binding specificity if, characterized as lectins may be of great value in various fields of biology such as glycobiology, clinical pathology and other related branches of medical science.

CONCLUSION

Agglutinins with strong binding specificity for receptor determinants on rabbit and rat erythrocytes have been identified in the extract of *Fasciolaria tulipa* and *Fusinus nicobaricus*. Further studies are required to characterize and purify these agglutinins, so that their immunological role can be elucidated and their biological applications and pharmacological activity can be explored. If these molecules are found to be pharmacologically active and clinically safe, there is vast scope for these agglutinins to become potential 'drug candidates' or 'drug leads'.

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