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Research Article

SURVEY AND PARTIAL CHARACTERIZATION OF AGGLUTININS IN THE HEPATOPANCREAS EXTRACT OF THREE MARINE GASTROPODS, *TROCHUS RADIATUS* (GMELIN, 1791), *TURBINELLA PYRUM* (LINNAEUS, 1767) AND *BABYLONIA ZEYLANICA* (BRUGUIERE, 1789)

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ABSTRACT

The hepatopancreas exctract of three marine gastropods, *Trochus radiatus* (Gmelin, 1791), *Turbinella pyrum* (Linnaeus, 1767) and *Babylonia zeylanica* (Bruguiere, 1789), were studied for the presence of agglutinins using 10 different mammalian erythrocytes. *Babylonia zeylanica* agglutinated all the 10 erythrocytes but in varying degrees. Agglutinins with strong affinity for rabbit and rat erythrocytes, as revealed by high haemagglutination (HA) titre, were observed in *Trochus radiatus* and *Babylonia zeylanica*. Hepatopancreas extract of *Babylonia zeylanica* also showed high affinity for pig erythrocytes (HA = 2048). *Turbinella pyrum* showed a weak activity against rabbit (HA = 8) and rat erythrocytes (HA = 4) but no haemagglutinting activity was observed against all the other erythrocytes. Results revealed the pH and temperature sensitivity of the hepatopancreas agglutinins of all three species, as the HA titre varied with pH and temperature. Calcium ions at 10 mM concentration greatly enhanced the activity of the agglutinins of *Trochus radiatus* and *Babylonia zeylanica* but a slight decrease in activity was noticed for the agglutinin of *Turbinella pyrum*. The present work provides the basic information needed for further research on the isolation and purification of the agglutinins of *Trochus radiatus* and *Babylonia zeylanica*, The purified agglutinins can be studied for their pharmacological properties and tested for their biomedical potential in the diagnosis and treatment of several fatal and difficult-to-treat diseases.

Keywords: Hepatopancreas exctract, Haemagglutination, Tris-buffered saline, Calcium ions.

INTRODUCTION

The rapid increase in population, changing human life style and climate change impacts, have propelled the origin and spread of many incurable and fatal diseases like influenza, diabetes, coronary disorder, AIDS and cancer globally (Pati *et al*., 2015). Increasing emergence of multi-resistant strains of pathogenic bacteria, as well as new epidemics, i.e., dengue, chikungunya, bird flu, swine flu, etc. drive the need to develop new therapeutics. As disease resistance to antibiotics and other drugs continues to build, even new methods of discovery such as combinatorial chemistry may not be able to meet the ever-increasing need for more efficient and more effective compounds.

Oceans are considered as treasure house of valuable bioactive compounds. Exploration and exploitation of sea-based resources have witnessed a paradigm shift in recent years (Rinehart, 2000). Scientists believe that an untapped reservoir of powerful new medicines is in the oceans. Although many of these products are not likely to become therapeutics, the information gained from studying them is likely to lead the development and understanding of novel molecular targets, which in turn may lead to the development of new classes of therapeutic agents.

Marine organisms are a rich source of biologically active compounds of interest for development of pharmaceuticals and alternative medicines (Benkendorff, 2009). Natural antibody like humoral substances agglutinins, lysins and antimicrobial factors, capable of agglutination, hemolysis and antibacterial properties have been reported in the body fluids of many invertebrates (Moffett, 1995). Bioactive substances from marine

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organisms such as protozoans, poriferans, cnidarians, annelids, arthropods, molluscs and echinoderms have attracted attention, due to their antiviral, antimicrobial, antiprotozoal, antifungal, antihelminthic and anticancer activities (Zapata & Amemiya, 2000).

Molluscs, a wide group of invertebrates, constitute to about 23% of the animals inhabiting the marine hydrosphere among which 80% are gastropods. In fact a number of secondary metabolites from molluscs have been tested for their strong bioactive properties and reported to have valuable pharmaceutical applications. Many of these compounds have undergone preclinical assessment and some of them have entered clinical trials. Dolastatin 10, Ziconotide, Neosurugatoxin, Diemenensin A, Chromodorolide A, Ulapualide A, Onchidal, KLH (Keyhole Limpet Hemocyanin), Kahalalide F, Keenamide A, and Bursatellanin P are some of the products derived from marine gastropods. Considering the available richness in diversity of marine life in the ocean, number of pharmaceutically valuable products derived from marine organisms is meager and the marine resources remain rather under explored. It is hoped that the present work on agglutinins, would provide the basic information for new pharmaceutical discoveries from marine gastropods, in order to fight against many fatal and difficult-to-treat diseases.

MATERIALS AND METHODS

Animals studied

The present study was carried out on three species of marine gastropods (Table 1), collected from Arockiapuram, located in the southeast coast of Tamil Nadu. The species were identified (ZSI, Chennai) as *Trochus radiatus* (Gmelin, 1791), Turbinella pyrum (Linnaeus, 1767) and Babylonia zeylanica (Bruguiere, 1789).

Table 1. Systematic position of the marine gastropods studied.

Phylum	Mollusca		
Class	Gastropoda	Gastropoda	Gastropoda
Subclass	Vetigastropoda	Caenogastropoda	Caenogastropoda
Order	Trochida	Neogastropoda	Neogastropoda
Superfamily	Trochoidea	Turbinelloidea	Muricoidea
Family	Trochidae	Turbinellidae	Babyloniidae
Species	*Trochus radiatus* (Gmelin,1791)	*Turbinella pyrum* (Linnaeus, 1767)	*Babylonia zeylanica* (Bruguiere,1789)
			

Preparation of Hepatopancreas extract

Animals collected were brought to the laboratory, rinsed with sterile water to remove the debris and other adhering matter from outer surface of the shell and blotted to remove water prior to the experiment. After the careful removal of shell, the whole body of each snail was removed and washed thoroughly with normal saline solution and blotted. The hepatopancreas was isolated from other tissues, washed with sterile saline and an extract of hepatopancreas was then prepared by homogenizing 100 mg of the tissue 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 for further assays.

Haemagglutination (HA) assay

Agglutinins are capable of agglutinating a variety of foreign particles such as bacteria, yeasts, protozoans, vertebrate erythrocytes. Erythrocytes are particularly, the useful targets as they are readily available and agglutination is observable even with the naked eye. Hence in the present study, 10 different mammalian erythrocytes (human A, B and O, rabbit, rat, dog, pig, cow, goat and buffalo) were used to study the activity of the agglutinins in the hepatopancreas extract of the marine gastropods. Blood for haemagglutination assay was collected directly in cold Alsevier's medium. The erythrocytes were washed three times, twice with ten volumes of 0.9% saline and once with TRIS buffered saline (pH 7.5) and resuspended in the same as 1.5% suspension.

Haemagglutination assays were performed in microtitre plates with 'U' bottomed wells, by twofold serial dilutions of 25 µl of the sample with an equal volume of TRIS buffer (pH 7.5). After the dilution of the sample, 25µl of 1.5% erythrocyte suspension was added, mixed well and incubated for 1h at room temperature. The HA titre was determined as the reciprocal of the highest dilution of the sample that gave complete agglutination. Since the agglutinins in the hepatopancreas extract of all the animals showed high affinity for rabbit erythrocytes, for further studies on characterization of the agglutinins, rabbit erythrocytes were used in the hemagglutination assays.

Haemagglutination assay for pH and thermal stability

To study the effect of pH on the haemagglutinating activity, 25 µl of the sample was suspended in 25 µl of Tris-buffered saline (TBS) of varying pH (6.5, 7.5 and 8.5) and serially diluted in a microtitre plate. It was incubated for 1 h at room temperature and mixed with 25 µl of 1.5% rabbit erythrocyte suspension. The HA titre was determined as before. The thermal stability of agglutinin in the sample was studied by preincubating the sample at different temperatures 25ºC, 35ºC and 45ºC. The sample was then tested for haemagglutinating activity against rabbit erythrocytes.

Hemagglutination assay for divalent cation (Ca^{2+}) requirement

To study the influence of divalent cations such as calcium (Ca^{2+}) on the HA activity, haemagglutination assays were performed in TBS (pH 7.5) with (10 mM) or without calcium.

RESULTS AND DISCUSSION

The marine environment is a rich source of biologically active natural products, many of which have not been found in terrestrial sources. Many invertebrates (Boman, 1995; Moffett, 1995) have been reported to possess substances capable of agglutination, hemolysis and a number of marine-derived natural products have an extensive array of therapeutic properties, including anticoagulant, antimicrobial, wound healing and immune modulating, antioxidant, anticancer, anti-inflammatory, antihypertensive, and other medicinal properties (Senthilkumar & Kim, 2013). Agglutinins are proteins/glycoproteins that have the ability to recognize and bind reversibly to specific structural determinants (usually a carbohydrate) present on cell surfaces, extra cellular matrices, and secreted glycoproteins (Goldstein, 1980; Sharon & Lis, 1995; Weis, 1997). Invertebrate agglutinins form a major component of the innate immune system performing physiological functions like wound healing and immunological functions such as opsonization (Vasta, 1991; Wang & Wang, 2013). Agglutinins with sugar specificity are referred to as lectins, a group of molecules that have drawn the attention of scientists, as they have proved to be useful diagnostic tool for a number of dieases, like cancer (Mody *et al*., 1995) and some also have therapeutic activities.

The Indian coastline is rich in molluscan diversity (Apte, 2004). Natural products isolated from marine molluscs have been tested for their strong bioactive properties i.e., neuromuscular blocking action, anti-predator, antimicrobial, anti-neoplastic and cytotoxic activity. The most promising metabolite isolated from a marine mollusc is Dolastatin 10, an anti-neoplastic peptide isolated from the sea hare *Dolabella auricularia* (Pettit *et al*., 1987). Dolastatin 10 has recently reached clinical trials in the United States and is reported to be one of the most potent anticancer agents known (Carte, 1996). Ziconotide isolated from the the venom of the predatory Indo-Pacific marine mollusc, *Conus magus* shows remarkable analgesic activity, 1,000 times more active that morphine in animal models of nociceptic pain, due to the blockage of calcium channels (Olivera, 2000). Neosurugatoxin isolated from the Japanese ivory mollusc *Babylonia japonica*, is a reversible antagonist of acetylcholine receptors (Ireland *et al*., 1993). Diemenensin A is an antibiotic derived from the intertidal airbreathing gastropod, *Siphonaria diemenensis* (Hochlowski & Faulkner, 1983). Chromodorolide A, isolated from *Chromodoris cavae*, (colourful sea slug) exhibits *in vitro* antimicrobial and cytotoxic activity as well as moderate *in vivo* antitumor activity against P388 murine leukemia cells (Morris *et al*., 1991). Ulapualide A isolated from the egg masses of the brilliant red Spanish dancer nudibranch *Hexabranchus sanguineus*, exhibits potent cytotoxic activity against L1210 murine leukemia cells and antifungal activity that exceeds that of the clinically useful amphotericin (Chattopadhyay & Pattenden, 1998; Roesener & Scheuer, 1986). Onchidal from *Onchidella bieyi* is a useful probe for identifying the active site residues that contribute to binding and hydrolysis of acetyl cholinesterase (Ireland *et al*., 1993). KLH (Keyhole Limpet Hemocyanin), a copper containing extracellular respiratory protein present in *Megathura crenulata*, possess remarkable immunostimulatory properties and is under clinical trials for the treatment of bladder carcinoma. KLH, may also have significant potential for the treatment of other types of cancers, particularly the epithelially derived adenocarciomas, by using it as a carrier for carcinoma gangliosides and mucin-like epitopes (Wirguin *et al*., 1995). Kahalalide F, isolated from the Hawaiian marine gastropod slug, *Elysia rufescens* (López Macià *et al*., 2001) is a good anticancer agent showing excellent antitumour activity against various solid tumour models, including colon, breast, lung cancers and certain prostate cancers. Kahalalide F could cause oncosis in cancer cells by lysosomal induction and cell membrane permeabilization. It also inhibits the expression of certain genes involved in cell proliferation. Thus Kahalalide F could inhibit tumour spreading and growth. Keenamide A, a hexapeptide isolated from the marine mollusc, *Pleurobranchus forskalii*, exhibits significant activity against the P-388, A-549, MEL-20 and HT-29 tumour cell lines (Weiss *et al*., 2000) and so, could be a potential anticancer biomolecule of

molluscan origin. Bursatellanin-P, a protein purified from the purple ink of the sea hare *Bursatella leachii* (Rajaganapathi *et al*., 2002) exhibits anti-HIV activity.

Presence of agglutinins has been reported in a number of marine molluscs *Haliotis laevigata* (Weiss *et al*., 2000), *Pteria penguin* (Naganuma *et al*., 2006), *Turbo brunneus* (Thanalakshmi, 2006), *Mytilus edulis* (Espinosa *et al*., 2010) and *Crassostrea virginica* (Jing *et al*., 2011).

Table 2. Haemagglutination by the hepatopancreas extract of marine gastropods.

Erythrocyte	HA Titre		
	Trochus radiatus	*Turbinella pyrum*	*Babylonia zeylanica*
Human A	8	0	8
Human B	0	0	16
Human O	2	0	8
Rabbit	2048	8	1024
Rat	512	4	1024
Dog	0	0	16
Pig	8	0	2048
Cow	0	0	4
Goat	0	0	8
Buffalo	0	0	16

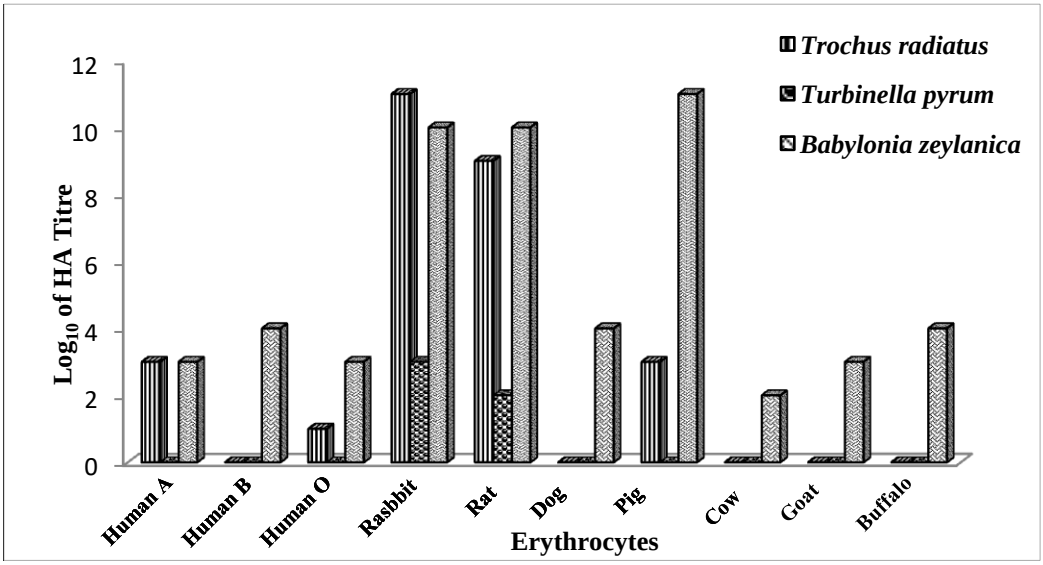

Figure 1. Haemagglutination by the hepatopancreas extract of marine gastropods.

Our study reveals the presence of agglutinins capable of recognizing and binding with the surface receptors of the mammalian erythrocytes, in the hepatopancreas all the three species of marine gastropods (Table 2 & Figure 1). Out of the three, the hepatopanctreas extract of two species, *Trochus radiatus* and *Babylonia zeylanica* showed the presence of agglutinins with strong affinity for rabbit and/or rat and/or pig erythrocytes. The HA titre of the hepatopancreas extract of *Trochus radiatus* against the different erythrocytes are as follows: 8 (human A), 2 (human O), 2048 (rabbit), 512 (rat) and 8 (pig). Maximum activity, a HA titre value of 2048 was observed with rabbit erythrocytes. *Turbinella pyrum* showed agglutinins that agglutinated only rabbit (HA = 8) and rat (HA = 4) erythrocytes but with weak potency. The hepatopancreas extract of *Babylonia zeylanica* agglutinated all the 10 erythrocytes used in the study, with a HA titre value of 8 against human A, 16 against human B, 8 against human O, 1024 against rabbit, 1024 against rat, 16 against dog, 2048 against pig, 4 against cow, 8 against goat and 16 against buffalo erythrocytes and the maximum activity was against pig erythrocytes.

The HA activity has been found to vary with the source of the sample (*Trochus radiatus*/*Turbinella pyrum*/*Babylonia zeylanica*) as well as with the type of erythrocyte used in the haemagglutination assay. The variation observed in HA activity against different erythrocytes reveals that the hepatopancreas agglutinins may probably share a common receptor that recognizes and binds to the surface residues of these erythrocytes but with a quantitative difference. The high HA titre values obtained for rabbit, rat and pig erythrocytes by the hepatopancreas agglutinin of *Trochus radiatus* and *Babylonia zeylanica* suggests that the receptor determinants preferentially recognized by agglutinins were either abundant or more accessible on these erythrocytes compared to other erythrocytes. The surface residues on some of the erythrocytes recognized by agglutinins have been reported for few mammalian erythrocytes. Rabbit erythrocytes contain NeuAc, 9-O-Ac NeuAc, NeuGc and 9-O-Ac NeuGc (Pfeil *et al*., 1980), rat erythrocytes contain NeuGc/ NeuAc/4(7)-0-acetylated sialic acids (Bhavanandan & Katlic, 1979), human A erythrocytes express NeuAc (Mercy & Ravindranath, 1993;Boman, 1995), dog erythrocytes express NeuGc/ NeuAc (Yasue *et al*., 1978). and buffalo erythrocytes express NeuGc (Chien *et al*., 1978). Differential affinity of agglutinins for diverse mammalian erythrocytes, has also been reported in various molluscs and other invertebrates (Bulgakov *et al*., 2000; Jayaraj *et al* ., 2008; Naganuma *et al*., 2006; Imamichi & Yokoyama, 2013; Yang *et al*., 2010; Zhang *et al*., 2009).

The activity of the agglutinins in the hepatopancreas extract of the three gastropods studied was found to be sensitive to pH as revealed by the variation in HA titre (Table 3 & Figure 2). The hepatopancreas agglutinin of *Trochus radiatus* showed maximum affinity (HA = 2048) for rabbit erythrocytes at pH 7.5. Maximum affinity was observed at pH 6.5 for *Turbinella pyrum* agglutinin and the activity decreased at pH 7.5 and pH 8.5. The activity of *Babylonia zeylanica* agglutinin remained the same at pH 6.5 and pH 7.5 (HA = 1024) but a decrease in affinity was observed at pH 8.5. This variation in the affinity of the agglutinin for rabbit erythrocytes in relation to pH reveals the pH sensitivity of the agglutinins.

Table 3. HA activity against rabbit erythrocytes in relation to pH.

Species	pH		
	6.5	7.5	8.5
Trochus radiatus	1024	2048	1024
Turbinella pyrum	16	8	8
Babylonia zeylanica	1024	1024	256

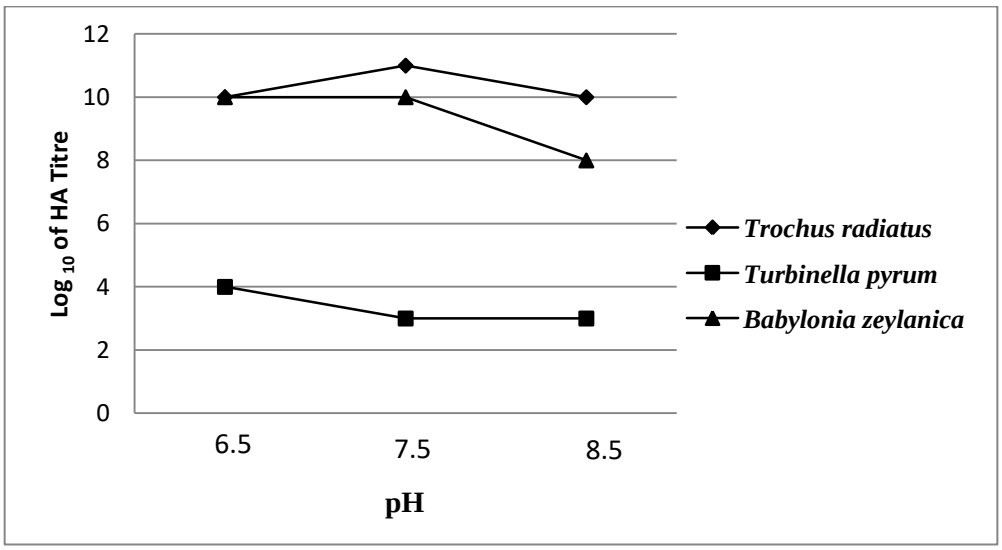

Figure 2. HA activity of *Trochus radiatus*, *Turbinella pyrum* and *Babylonia zeylanica* in relation to pH.

Temperature has been found to influence the affinity of the hepatopancreas agglutinins for rabbit erythrocytes. The activity of the agglutinins of all the three species decreased after exposure to 45ºC. The activity of

Trochus radiatus agglutinin remained high (HA = 2048) at 25ºC and 35 ºC but further increase in temperature weakened the binding affinity. HA titre for *Turbinella pyrum* agglutinin was 16 at 25ºC but activity was reduced at higher temperature (HA = 8). The haemagglutinating activity of *Babylonia zeylanica* showed an increase from 512 at 25ºC to 1024 at 35ºC but decreased to 256 at 45ºC (Figure 3).

Table 4. Hemagglutinating activity in relation to temperature.

Species	Temperature		
	25ºC	35ºC	45ºC
Trochus radiatus	2048	2048	1024
Turbinella pyrum	16	8	8
Babylonia zeylanica	512	1024	256

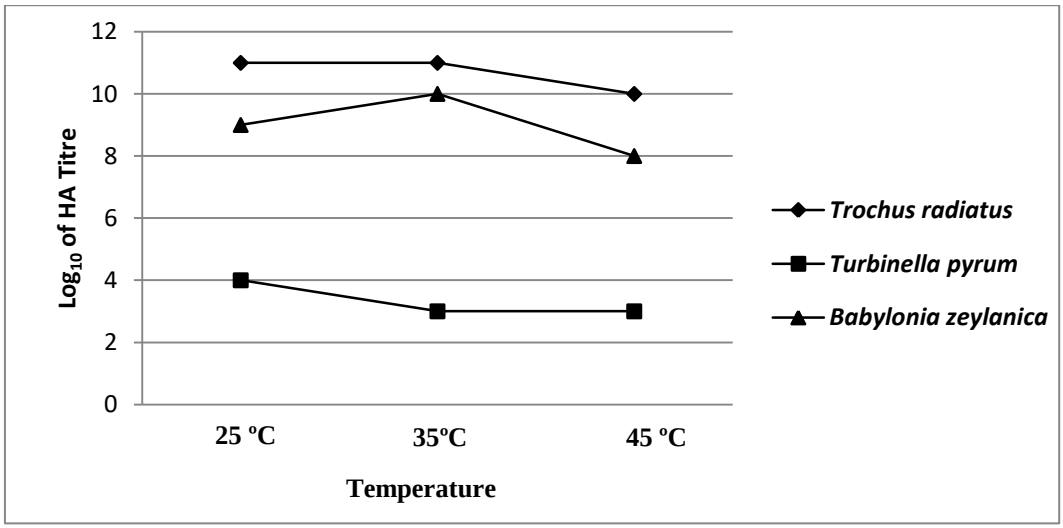

Figure 3. Hemagglutinating activity in relation to temperature.

Thus, the results reveal that the susceptibility of erythrocyte binding affinity of the hepatopancreas agglutinins to pH and temperature variation. The variation in HA activity may be due to alterations in conformation of the agglutinins as a result of change or dissociation of the binding sites caused by increase or decrease in pH and temperature, thereby the haemagglutinating activity may be suppressed or activated (Table 4). Singh & Saxena (2013) have also reported that variation in temperature and nature of medium affect the tertiary structure and henceforth haemagglutination activity of lectins.

Divalent cations are known to be important in stabilizing the primary structure of haemagglutinins (Acton & Weinheimer, 1974) and thereby enhance the binding affinity of the agglutinins with the specific receptors on the target molecules. In C-type lectins, calcium ion acts as a bridge between the protein (agglutinin/lectin) and the carbohydrate (receptor) through direct interactions with sugar hydroxyl groups (Berg *et al*., 2002). As reported, haemagglutinating activity may or may not require exogenous calcium (Anitha *et al*., 2018). The haemagglutinating activity of *Trochus radiatus* and *Babylonia zeylanica* showed a significant increase, in the presence of 10 mM calcium ion concentration, i.e., from 128 to 2048 by *Trochus radiatus* and from 256 to 1024 by *Babylonia zeylanica* agglutinin (Table 5 & Figure 4) indicating the calcium ion dependency of the agglutinins of these two species. In contrast, the HA activity of *Turbinella pyrum* agglutinin decreased from 16 to 8 in the presence of 10 mM Ca^{++} which reveals that the calcium requirement for the agglutinin may be provided by the available internal calcium and requires no exogenous calcium for its activity. An extra supply of calcium may even have a negative impact on the agglutinin's activity as revealed in the activity of *Turbinella pyrum* agglutinin.

Further work on the isolation and purification of the factors responsible for agglutination from *Trochus radiatus* and *Babylonia zeylanica* would help to investigate the nature, properties and biological activities of these active principles. Subjecting them to preclinical and clinical

investigation, their scope in the development of new drugs can be elucidated. These agglutinins, if identified to have sugar specificity, can be categorized under lectins, which are molecules having immense biomedical potential. Today, lectin research is gaining greater interest and importance, as they can serve as drug carriers and thereby play a major role in "Targeted Drug Therapy".

Table 5. HA activity of in relation to divalent cations (Ca^{++}) concentration.

Species	Concentration of Ca^{++}	
	0 mM	10 mM
Trochus radiatus	128	2048
Turbinella pyrum	16	8
Babylonia zeylanica	256	1024

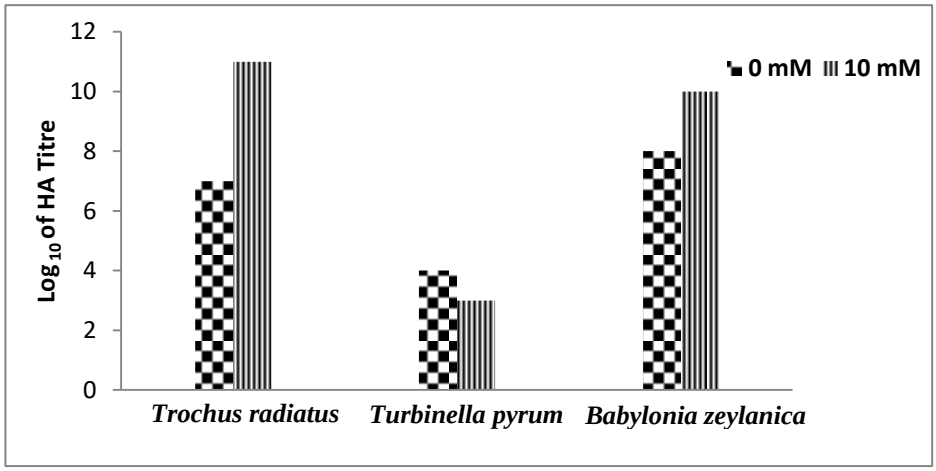

Figure 4. Effect of calcium ions on haemagglutinating activity of *Trochus radiatus*, *Turbinella pyrum* and *Babylonia zeylanica***.**

CONCLUSION

The present work on the screening of the extract of the hepatopancreas of 3 locally available of species marine gastropods, *Trochus radiatus*, *Turbinella pyrum* and *Babylonia zeylanica*, for agglutinins, has revealed the presence of powerful agglutinins with strong affinity for rabbit and rat erythrocytes in 2 species, *Trochus radiatus*, and *Babylonia zeylanica*. *Babylonia zeylanica* also possess agglutinins with high affinity for pig erythrocytes. The appropriate conditions (temperature, pH and calcium dependency) required for improving the activity of the agglutinin have also been elucidated. Thus the study provides information in identifying potential sources of agglutinins, the biomedically valuable molecules having immense diagnostic and therapeutic applications.

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