Fish and Shellfish Immunology 94 (2019) 896–906

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Anti-biofilm properties and immunological response of an immune molecule lectin isolated from shrimp *Metapenaeus monoceros*

Elumalai Preetham[a,b,*], Abdul Salam Rubeena[b], Baskaralingam Vaseeharan[c], Mukesh Kumar Chaurasia[a], Jesu Arockiaraj[d], Rolf Erik Olsen[e]

[a] Department of Processing Technology (Biochemistry), Kerala University of Fisheries and Ocean Studies, Panangad, 682 506, Kochi, Kerala, India
[b] School of Ocean Science and Technology, Kerala University of Fisheries and Ocean Studies, Panangad, 682 506, Kochi, Kerala, India
[c] Crustacean Molecular Biology and Genomics Division, Biomaterials and Biotechnology in Animal Health Lab, Department of Animal Health and Management, Alagappa University, Science Block 4th Floor, Burma Colony, Karaikudi, 630 004, Tamil Nadu, India
[d] SRM Research Institute, SRM Institute of Science and Technology, Kattankulathur, 603 203, Chennai, Tamil Nadu, India
[e] Norwegian University of Science and Technology, Department of Biology, 7491, Trondheim, Norway

ARTICLE INFO

Keywords:
Metapenaeus monoceros
Lectin
Antibiofilm
Agglutination
Immune molecule

ABSTRACT

The study is carried out to understand the antimicrobial and immunological response of a potential immune molecule lectin, *Mm*Lec isolated from haemolymph of Speckled shrimp, *Metapenaeus monoceros*. *Mm*Lec was purified using mannose coupled Sepharose CL-4B affinity chromatography, which was further subjected on SDS-PAGE to ascertain the distribution of their molecular weight. Sugar binding specificity assay was conducted at various pH and temperatures to investigate the binding affinity of *Mm*Lec towards the specific carbohydrate molecule. Functional analysis of immune molecule *Mm*Lec included haemagglutination assays performed using human erythrocytes and yeast agglutination activity against *Saccharomyces cerevisiae* which, were analyzed using light microscopy. In order to study the antimicrobial activity, two Gram-negative (*Vibrio parahaemolyticus* and *Aeromonas hydrophila*) and two Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) bacteria were treated with purified *Mm*Lec. Moreover, these bacterial species were also treated at different concentration of the *Mm*Lec to speculate the antibiofilm properties of *Mm*Lec which was analyzed under Light Microscopy and Confocal Laser Scanning Microscopy. In addition, other functional characterization of *Mm*Lec showed the uniqueness of *Mm*Lec in agglutination of human erythrocyte as well as the cells of yeast *Saccharomyces cerevisiae*. Also, the phenoloxidase activity and encapsulation assay was evaluated. MTT assay displayed that *Mm*Lec are potent in anticancer activity. The study will help to understand the immunological interference and antimicrobial nature of *Mm*Lec which would be supportive in establishing a potential therapeutic tool and to develop better and novel disease control strategies in shrimp and farmed aquaculture industries as well as in health management.

1. Introduction

Aquaculture has become one of the fastest emerging and most demanded sectors globally. It is especially increasing the supply of foods in developing countries like China, India, and many others [1,2]. The farming activities spans from shellfishes, finfishes, shrimps, molluscs and plants [3]. Among them, shrimps are now getting more attention world-wide and contribute to nearly 32% of total global production [4]. In recent time, a penaeid shrimp *Metapenaeus monoceros* commonly called as brown/pink shrimp or Speckled shrimp and locally known as Choodan chemmeen in Kerala state of India has become a potential candidate for commercial importance because of their high economical and nutritional value [5,6]. This shrimp family is native of the Indo-West Pacific region ranging from South Africa to the Red Sea along the central west coast of India. They preferably inhabits estuaries, estuaries flood plains and mangrove swamps [7,8].

Considering their importance in shrimp aquaculture industry, *M. monoceros* is a cheap and valuable natural food source for lower and middle income people. The protein level is high and the essential amino acids profiles are well balanced. They are being commercially cultivated and harvesting from estuarine and costal water largely in western and southern region of India [9,10]. Though, shrimp aquaculture is the

* Corresponding author. Department of Processing Technology (Biochemistry), Kerala University of Fisheries and Ocean Studies, Panangad, 682 506, Kochi, Kerala, India.
E-mail address: preetham@kufos.ac.in (E. Preetham).

https://doi.org/10.1016/j.fsi.2019.09.032
Received 1 April 2019; Received in revised form 7 September 2019; Accepted 14 September 2019
Available online 15 September 2019

fastest growing sector, reports from several Southeast-Asian countries have indicated several biotic and environmental factors that may hamper the development of a sustainable shrimp industry [11,12]. In 2012, Flegel et al. [13] reported several infections caused by bacteria (*Aeromonas* and *Vibrio sps*) and viruses (*Baculovirus sps*). These contribute to nearly 82% of disease outbreaks in wild and farmed shrimps. Other than this, the existence of *Monodon baculovirus* (MBV) in the culture of *Metapenaeus sps* has been also reported by Chen et al. [14] in Taiwan. These diseases are consistently affecting the shrimps leading to massive economic losses to shrimp farmers as well as industry [15–17]. Therefore, it becomes imperative to find a robust immuno-defensive tool for the protection and prevention of disease outbreaks in shrimps. This can be achieved by understanding the molecular mechanism of their immunological cascade.

Shrimps, as invertebrates, lack adaptive immunoglobulin and memory cells. They are consequently solely dependent on innate immune cells to protect and defend against pathogens. Innate immune responses are more generalized, vigorous and potentially distinguish the hazardous pathogenic invaders [18,19] than the other one. This process frequently progresses through host-pathogen based particular-recognition proteins, often through the recognition of specific sugar moieties on the surfaces of microbial intruders [20]. Most of the time occurs in the haemolymph which carries most of the immune response mechanisms in shrimp [21]. The principal innate immune elements which recognize those sugar molecules are universally known as lectins, these therefore has a central role in eradicating the invaders [22,23].

Lectins are a large group of specific sugar-binding proteins. They possess potential ability to discriminate peculiar glyco-conjugates or oligosaccharides from the vast array expressed on the invader surfaces [24]. Dutta et al. [25] stated that lectins are ubiquitous and abundantly found from lower invertebrates to higher vertebrates including plants also. Lectins are involved in cellular and biological processes including host-defence, cellular interactions, proliferation, opsonization, signal transduction, metastasis, apoptosis, agglutination and folding of glyco-conjugates [26–28]. Interestingly, lectins significantly interfere with the host immune response and have been considered as the first line of defence especially in those who lacks acquired immunity [29]. It has been also reported that lectins play important roles in nodule formation, phenoloxidase (PO) system activation and antimicrobial activity including antibacterial and antiviral. Therefore, it can quickly abolish invaders through enhanced agglutination, PO system, macrophages and phagocytosis [30–33]. Also, Sharon and Lis [24] have reported about their importance in diagnosis of cancer research consequently; can be treated as a powerful therapeutic tool in shrimp aquaculture.

Lectin families are categorized based on their specific organization of carbohydrate recognition domain i.e., CRD. Although, in vertebrates, lectins are well classified and their 17 distinct classes have been reported in wide range of animals so far. But, in invertebrates the information is not adequate yet, even after characterization of numerous invertebrate lectins [34–36]. However, lectins in invertebrates are more ample and widely diverse. So far, several lectin families have been speculated in variety of marine invertebrate animals such as sponges, annelids, echinoderms, mollusks, ascidians and arthropods and their role in agglutination, opsonic activity as well as PO system was assessed [37–43]. Consequently, lectins from many marine shrimps such as *Litopenaeus vannamei* [44], *Eriocheir sinensis* [45] and *Penaeus japonicas* [46] have been also investigated and reported. Other than this, a Ca^{2+} reliant lectin from haemolymph of *Fenneropenaeus merguiensis* have been described and their role in immune defence mechanisms has been speculated [47]. Additionally, few researchers have also stated that most of the lectins from marine animals functions as an important immune asset and exhibit antimicrobial properties [48,49]. But, through the best of our knowledge and available literature, the report about the lectin from shrimp *M. monoceros* are either nil or scare. Hence, seeing the significance of shrimp aquaculture and threats escalated by pathogens at present, it becomes imperious to report and characterize the lectin from shrimp *M. monoceros* to establish a comprehensive eco-friendly and more robust disease control strategies.

Therefore, in this study, we have characterized a purified lectin from the haemolymph of Speckled shrimp *M. monoceros* (denoted as *Mm*Lec) at molecular level. The lectin was then tested for its ability to agglutinate potential pathogens, stimulate encapsulation and PO activity and cytotoxicity towards cancer cell lines. We have also examined antibiofilm activity of *Mm*Lec against *Aeromonas*, *Vibrio*, *Staphylococcus* and *Enterococcus* bacterial species as well as characterization of specific sugar binding affinity towards purified *Mm*Lec.

2. Materials and methods

2.1. Sample collection and haemolymph preparation

Live speckled shrimps *M. monoceros* with an average body weight of 25 ± 10 g and a length of approximately 7 cm were obtained from a shrimp hatchery at Kumbalangi, Kochi, Kerala, India. The shrimps were transferred into a 30 L aerated plastic container and carried to the laboratory of Department of Processing Technology (Biochemistry), Kerala University of Fisheries and Ocean Studies. In laboratory, haemolymph was collected and extracted under aseptic conditions using gauge 23 hypodermic needles and diluted with 1:1 anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA at pH 7.5). A total of 30 ml haemolymph was pooled for purification of lectin. The haemolymph was immediately centrifuged at 1000 rpm for 10 min at 4 °C and the supernatant was transferred into fresh tubes and stored at −20 °C until further purification.

2.2. Purification of MmLec from shrimp haemolymph

Lectin was purified according to the method of Jayanthi et al. [50] with minor modifications. Briefly, a mannose coupled Sepharose CL-4B affinity column was first washed with a Tris Buffered Saline (TBS)/$CaCl_2$ buffer (10 mM Tris–HCl, 150 mM NaCl, 10 mM $CaCl_2$ at pH 7.4). The haemolymph was then equilibrated with equal volumes of TBS/$CaCl_2$ buffer. Then 20 ml of the equilibrated sample was gently loaded onto the column. The purified *Mm*Lec was eluted with elution buffer (10 mM Tris − HCl, 140 mM NaCl, 3 mM EDTA at pH 8.0) containing EDTA.

2.3. Characterization of purified MmLec

2.3.1. Molecular weight determination of MmLec

Polyacrylamide gel electrophoresis (10%) was performed on the eluted *Mm*Lec under reduced condition in the presence of β-mercaptoethanol as described by Laemmli [51]. The gel was stained with Coomassie brilliant blue (GE Healthcare Bio-Sciences, India) and the image captured through ChemiDoc XRS + system (Bio-Rad, USA). The molecular mass of the *Mm*Lec was determined by comparison to the molecular mass of marker proteins (Takara BIO INC, Japan). Total protein concentration was determined by Lowry's method [52] using bovine serum albumin (BSA) as standard. For the molecular mass confirmation, the band was excised and mass spectrophotometry was done.

2.3.2. High performance liquid chromatography (HPLC) and X-ray diffraction (XRD) of MmLec

HPLC separation was carried out using a reversed phase C_{18} column (7.8 mm × 30 cm). Previously, equilibrated with TBS/$CaCl_2$ at a flow rate of 0.8 ml min^{-1}. HPLC system (Zorbax Bio-series GF-250, DuPont, Willington, DE, USA), was used for the homogeneity analysis. In order to determine the spatial distribution of atomic coordination and the arrangement of atoms, an X-ray diffraction analysis (XRD, Scintag-SDS 2000) were performed on purified *Mm*Lec at 40 kV/20 mA, using continuous scanning of 2θ mode [53]. The average grain size and shape of

the purified *Mm*Lec was determined using Scherrer's formula [53] as $d = 0.9\lambda/\beta\cos\theta$ [Where, d is the mean diameter of purified *Mm*Lec, λ is the wavelength of the X-ray radiation source and β is the angular FWHM of the XRD peak at diffraction angle (θ)].

2.3.3. Fourier-transform infrared (FTIR) and Circular Dichroism (CD) analysis

For FTIR spectroscopy studies, purified *Mm*Lec (1–50 μl) was placed in a thermostated cell fitted with CaF_2 windows (with 6 μm Teflon spacer for measurements in water). The spectra of biological molecules were recorded at a resolution of 4 cm^{-1} as described by Jayanthi et al. [50] with slight modifications. The Circular Dichroism (CD) studies the difference in absorption of left and right circularly polarized light. CD uses Jasco J-720 spectropolarimeter. Spectral scans were performed from 250 to 190 nm with a step resolution of 1 nm and a bandwidth of 1.0 nm and at a speed of 50 nm/min. Samples were measured at peptide concentrations of 30–40 μl in 20 mM Tris-HCl-20mM NaCl, pH 7.4, with or without 20 mM sodium dodecyl sulphate.

2.4. Functional analysis

2.4.1. Haemagglutination properties of MmLec

The haemagglutination assay (HA) was performed in microtiter plates according to the methodology of Correia and Coelho [54] with little changes. *Mm*Lec (50 μl) were serially diluted in PBS before addition of 50 μl 2% (v/v) suspension of human erythrocytes. In controls, purified *Mm*Lec was replaced by BSA. The titer was expressed as the highest dilution exhibiting haemagglutination. Specific haemagglutination was defined as the ratio between titer and protein concentration (mg/ml). These haemagglutinated titres of *Mm*Lec were visualized by light microscopy at the magnification of 40X (Leica DMIL).

In addition to Haemagglutination assay, Sugar binding assay was also done according to the methodology of Correia and Coelho [54] against eight sugar molecules by minimum inhibitory concentration (MIC) to find out the carbohydrate binding specificity of purified lectin.

2.4.2. MmLec yeast agglutination assay

To determine the ability of agglutination of *Mm*Lec to yeast cells (*Saccharomyces cerevisiae*), 50 μl of different concentrations of the purified *Mm*Lec was added to a U-shaped 96-well microtitre plate containing equal volume of Tris buffer. The same volume of a suspension of yeast (10^6 cells ml^{-1}) was then added to the wells and incubated for 4 h at 25 °C. In controls, purified *Mm*Lec was replaced by BSA. The pattern of agglutination was monitored with an inverted light microscope (40X) (Leica DMIL).

2.4.3. Phenoloxidase (PO) enhancing activity of MmLec

The ability of *Mm*Lec to activate prophenoloxidase was studied by measuring the formation of dopachromes from L-DOPA according to Iswarya et al. [55]. The different concentrations of purified *Mm*Lec (20, 40, 60, 80, 100 μg/ml) was mixed with equal volumes of laminarin (1 mg ml^{-1}) and incubated at 25 °C for 15 min. Then, 50 μl of the haemocyte lysate supernatant was introduced and incubated again for 45 min in the presence of 5 mM $CaCl_2$. Subsequently, 50 μl of L-DOPA (3 mg ml^{-1}) as an enzyme substrate was added and incubated at 25 °C for 1 h. In controls, *Mm*Lec was replaced by TBS/$CaCl_2$ buffer. After incubation, the formation of dopachrome was measured spectrophotometrically at 490 nm and expressed as unit/min/mg/protein.

2.4.4. Determination of MmLec encapsulation assay

To evaluate the encapsulation activity of *Mm*Lec, the haemocytic suspension was mixed with sepharose beads suspension and *Mm*Lec (25, 50 and 100 μg/ml) in V- bottomed microtitre plates and incubated at 25 °C for 45 min with intermediate mixing every 15 min. In control wells, *Mm*Lec was replaced by TBS buffer. The entire volume from each suspension was spread on sterilized glass slide and kept undisturbed for 10 min.

2.4.5. Antibiofilm properties of MmLec

The effect of purified *Mm*Lec on biofilm-forming Gram-negative (*Aeromonas hydrophila* and *Vibrio parahaemolyticus*) and Gram-positive (*Staphylococcus aureus and Enterococcus faecalis*) bacteria were tested using 24-well polystyrene plates containing glass pieces immersed in Luria Bertani broth inoculated with the bacterial suspension of 1% inoculum from overnight cultures (10^7 CFU ml^{-1}). Different concentrations (such as 50 and 100 μg/ml^{-1}) of *Mm*Lec were introduced into the wells and incubated at 37 °C for 48 h. To examine the biofilm activity, the media were discarded and weakly adherent cells were removed by thorough washing with deionized water and allowed to air dry before staining. The biofilm were stained with 1 ml of 0.4% acridine orange (w/v) for 10 min. The biofilm inhibition in 3D view was observed by confocal laser scanning microscopy (Carl Zeiss LSM 710, Germany). In addition, the experiment was repeated with crytal violet dye for light microscopic studies. In control, *Mm*Lec was replaced by BSA.

2.4.6. Anticancer activity

To evaluate the anticancerous activity of the *Mm*Lec, an MTT assay was performed against MDA-MB-231 breast cancer cell lines. Cells were seeded onto 96-well plates at a density of 1×10^5 cells per well. The MTT assay was performed in triplicate for *Mm*Lec at different concentrations.

2.5. Statistical analysis

All treatments were conducted in triplicates (n = 3). Data are shown as mean ± standard error mean (S.E.M.). Statistical differences were analyzed using one-way ANOVA using Tukey's Multiple Range Test using to estimate differences between treatments (SPSS ver. 11.5). Significance was accepted at $p > 0.05$.

3. Results

3.1. SDS-PAGE and MALDI-TOF/TOF analysis of MmLec

The mass of the extracted and purified *Mm*Lec fraction from speckled shrimp *M. monoceros* was determined in 10% SDS to nearly 80 kDa in both reduced and un-reduced situations (Fig. 1) and the molecular mass was confirmed by MALDI_TOF analysis (Fig. 2).

3.2. Determination of HPLC and XRD analysis

*Mm*Lec revealed two sharp and asymmetrical peaks at a retention time of 3.014 min and 10.634 min, when analyzed by reversed phase HPLC using C_{18} column (Fig. 3a). It indicates the homogeneousness, uniformity and integrity of purified protein *Mm*Lec.

Fig. 3b displays the XRD analysis of purified *Mm*Lec. The result indicated that *Mm*Lec possess a diffraction peak at 31.837$\mathring{2}$ along with a predicted and calculated lattice constant of 2.81085 Å. The result of XRD analysis suggested the crystalline nature of purified protein *Mm*Lec thus; confirmed their homogeneity and purity.

3.3. FTIR analysis of MmLec

The FTIR analysis of *Mm*Lec suggested the presence of a hydroxyl (OH) functional group stretching in the spectrum line from 3000 cm^{-1}–3500 cm^{-1} (Fig. 4a). Consequently, the CD spectrum line exhibited a characteristic α-helical conformation with a minimum at 193 nm and maximum at 191 nm in correspondence to β sheets of secondary structure (Fig. 4b).

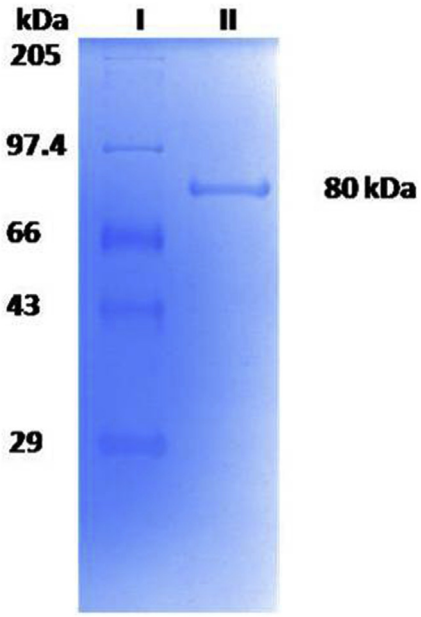

Fig. 1. Analysis of *Mm*Lec purified using mannose coupled Sepharose CL-4B affinity chromatography from *M. monoceros* heamolymph on Polyacrylamide gel electrophoresis (10%) in the presence of sodium dodecyl sulphate (SDS); Lane II indicates purified *Mm*Lec with molecular mass of 80 kDa and Lane I denotes Broad range molecular mass protein marker.

3.4. Functional analysis

3.4.1. Analysis of sugar-binding assay of MmLec

To know the potential capacity of purified *Mm*Lec to bind with specific sugar moieties, sugar binding specificity assay was directed using minimum inhibitory concentration (MIC). Among the eight different sugar molecules examined, six sugar molecules including arabinose, dextrose, rhamnose, N-acetyl glucosamine, fucose and mannose displayed strong inhibitory properties at an equal dosage i.e., 3.125 mM (Table 1). Other than this, it was also noticed that N-acetyl galactosamine was inhibitory but only at a dose of 6.25 mM. In contrast, galactose was not inhibitory. This suggests a broad spectrum binding ability of purified *Mm*Lec with a diversity of sugar molecules thus; it can be treated as a significant pattern recognition protein.

3.4.2. Role of MmLec in agglutination

To know the significant interference of purified *Mm*Lec in immune responses, agglutination properties of *Mm*Lec was assessed using human red blood cells (RBC) and fungal cells of yeast *S. cerevisiae*. The result shows that purified *Mm*Lec has the potential to agglutinate human erythrocytes. The activity increased with concentration of purified *Mm*Lec. The maximum agglutination was observed at 150 μg/ml followed by 100 and 50 μg/ml (Fig. 5a). No agglutination was observed when human erythrocyte cells were treated with BSA (control).

Furthermore, it was noticed that the purified *M. monoceros* lectin, agglutinated fungal cells of yeast *S. cerevisiae* at different concentrations. The highest rate of agglutination was found at a concentration of 120 μg/ml followed by 60 μg/ml and 30 μg/ml respectively (Fig. 5b).

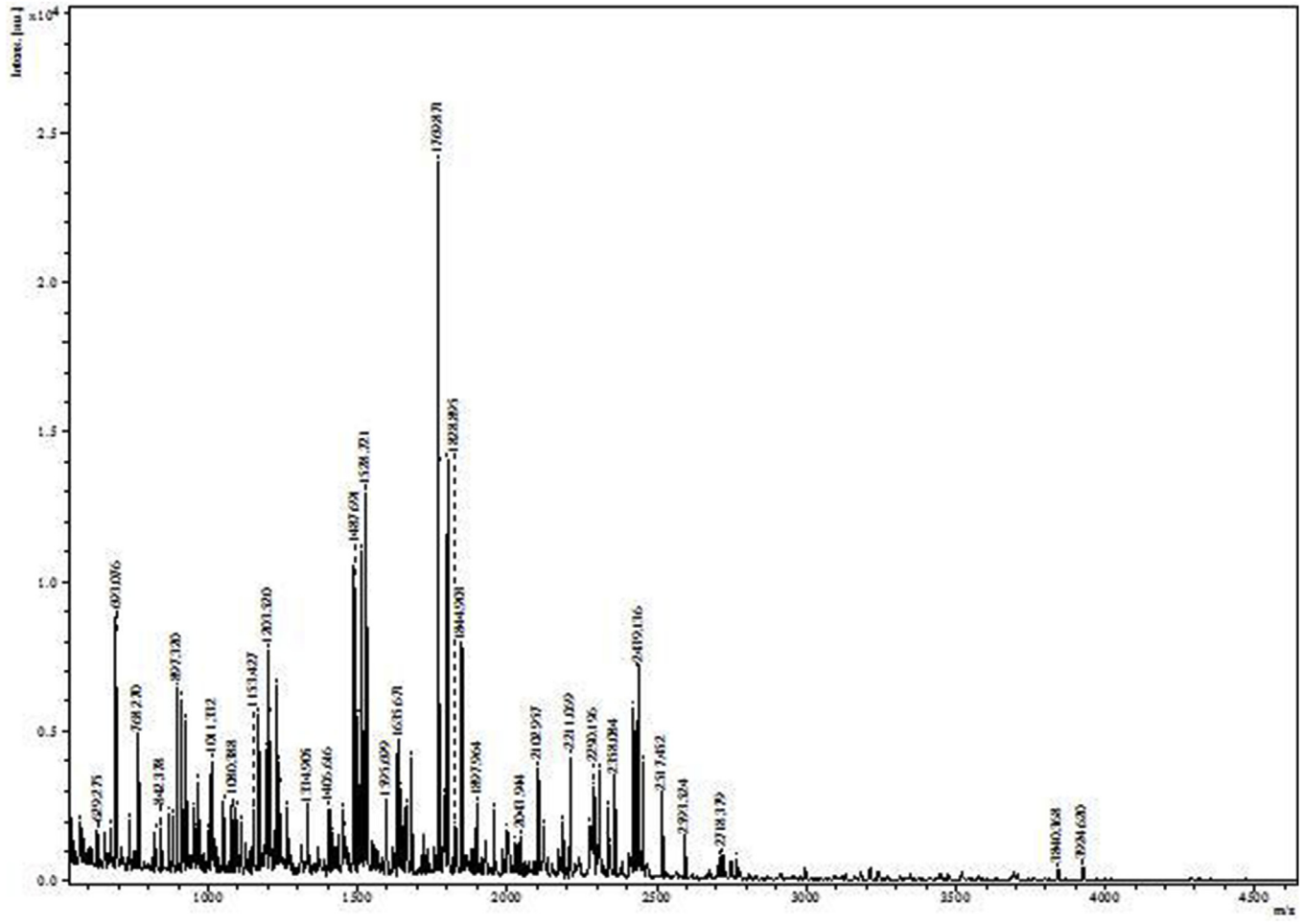

Fig. 2. MALDI-TOF/TOF analysis of 80 kDa *Mm*Lec from *M. monoceros* haemolymph.

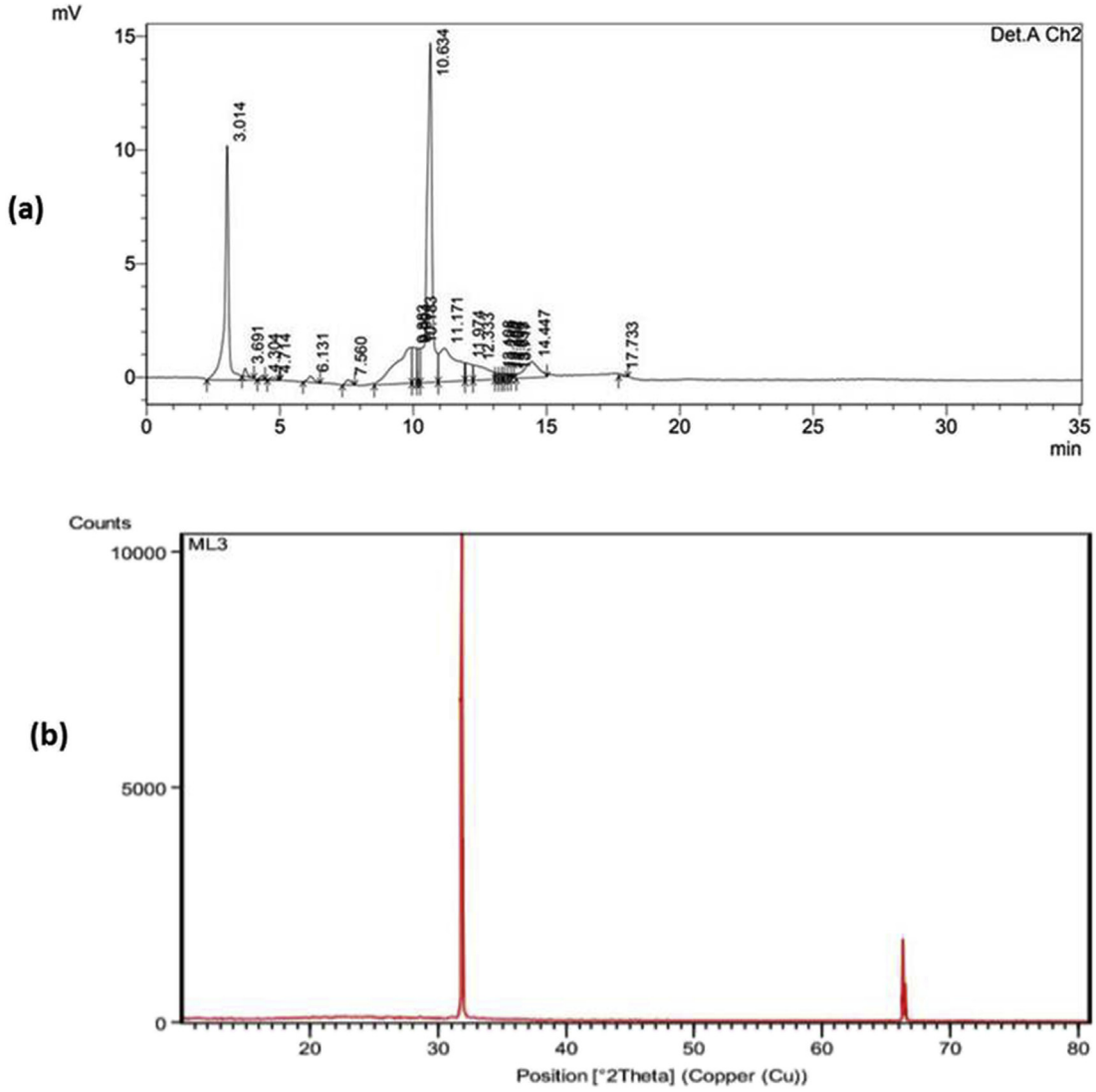

Fig. 3. (a) HPLC analysis of purified *Mm*Lec from *M. monoceros* haemolymph showing peaks at retention time of 3.014 min and 10.634 min. **(b)** XRD analysis of purified *Mm*Lec displayed one diffraction peak at 31.8372° which shows the purity and crystalline nature of *Mm*Lec protein. The lattice constant calculated from this pattern was found to be 2.81085 Å which explains the crystalline nature lattice arrangement of purified *Mm*Lec protein.

Similar to haemagglutination, no agglutination was found when *Mm*Lec was replaced with BSA. The result of agglutination assay, both in human erythrocytes and yeast cells clearly suggests that *Mm*Lec possess potential capability to act as an important coagulant agent in concentration dependent manner.

3.4.3. MmLec features in enhancing PO activity

The result of the phenoloxidase (PO) test suggested that purified *Mm*Lec/lamarin was able to enhance PO activity over the entire concentration range tested (20, 40, 60, 80, 100 μg) (Fig. 6). The activity was highest in the range 60–100 μg and lower at 20–40 μg. TBS buffer control did not produce any activity when treated with reaction mixture. The result of PO activity advocates the role of *Mm*Lec in triggering immune response of shrimp *M. monoceros* against the external pathogenic invaders.

3.4.4. Effect of MmLec in encapsulation

The encapsulation assay was conducted to investigate the potential role of purified protein *Mm*Lec in the phenomena of cellular encapsulation. The assay was conducted in a concentration dependent manner using Sepharose CL-4B beads coated with *Mm*Lec (25, 50 and 100 μg/ml). After incubation with haemocytes at 25 °C for 45 min, the reaction mixture was monitored under light microscopy. The *Mm*Lec clearly enhanced the encapsulation at the highest concentration (100μg/ml) (Fig. 7). TBS buffer control did not produce any effect on encapsulation.

3.4.5. Antibiofilm characterization analysis of MmLec

The biofilm inhibition assay of *Mm*Lec was done using two concentrations (50 and 100 μg/ml) on various Gram-negative (*A. hydrophila*, *V. parahaemolyticus*) and Gram-positive (*S. aureus*, and *E. faecalis*) bacteria at 24 h. Both concentrations of *Mm*Lec had considerable inhibitory effect on growth of all bacterial species when compared to BSA added controls (Fig. 8). At 100 μg/ml, the effect was further enhanced on *Aeromonas* and *Vibrio sps* compared to the other bacterial species. . The result strongly suggested that *Mm*Lec can be treated as an effective tool for eradication of microbial pathogens widely distributed among shrimp culture and aquaculture industry.

3.4.6. Cytotoxicity effect MmLec

The result of MTT assay are presented in Fig. 9 which indicates that, by increasing the concentration of purified *Mm*Lec generates a considerable and potential anticancer activity against the examined breast cancer cell line MDA-MB-231.

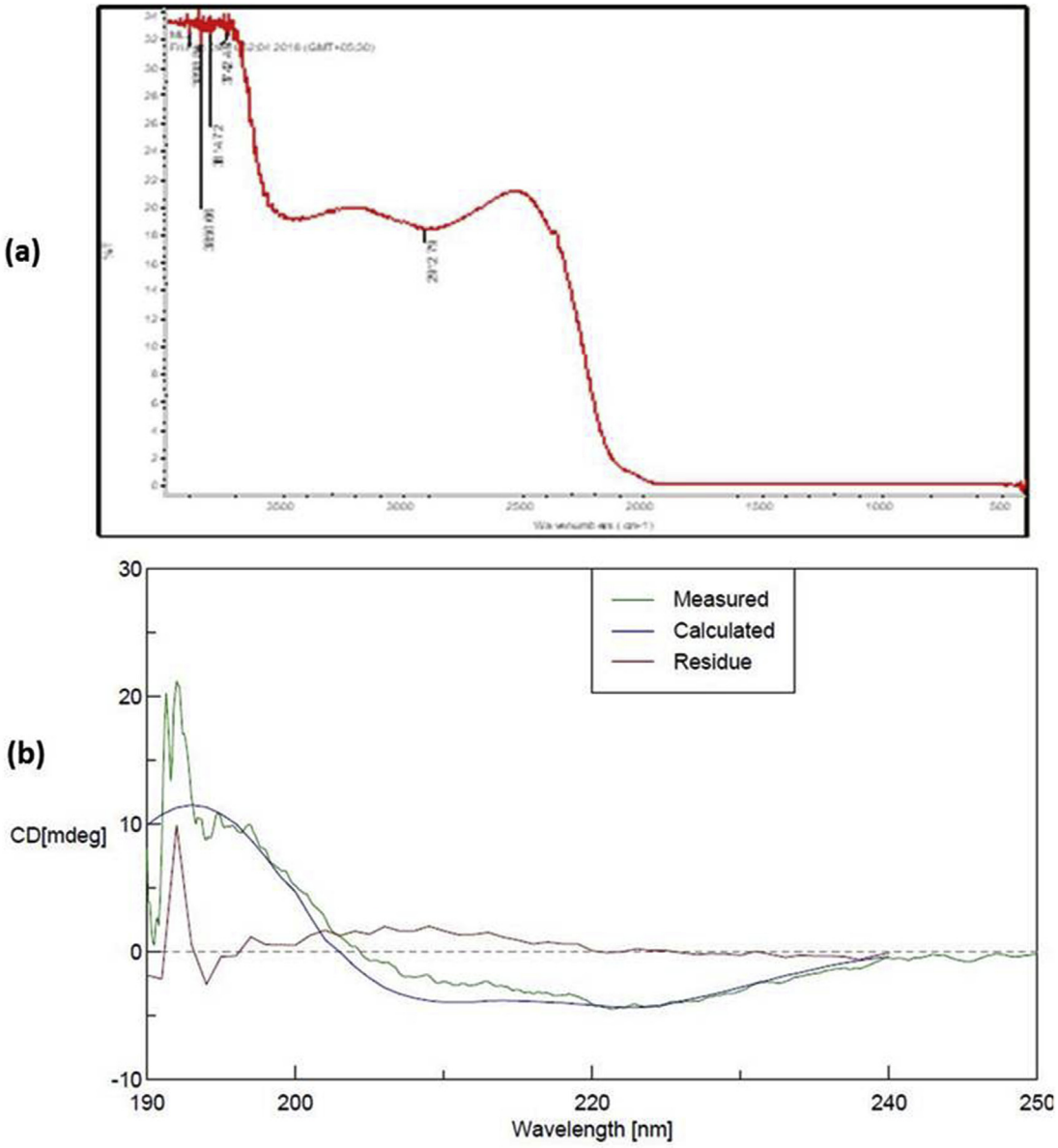

Fig. 4. (a) FTIR analysis for the identification of functional group of *Mm*Lec. The attributions of the main absorption characteristics of glycosidic structures are related to O–H stretching (3000–3500 cm^{-1}). **(b)** CD analysis for secondary structure determination of *Mm*Lec. The spectrum shows the characteristics of an α-helical conformation with minima at 193 nm and a maximum at 191 nm.

Table 1
Minimal inhibitory concentration corresponds to the lowest carbohydrate concentration able to neutralize haemagglutinating activity of *Mm*Lec. The values are expressed in millimolar and the highest carbohydrate concentration used was 250 mM. Galactose could not inhibit the haemagglutination at the highest 250 mM concentration. All other sugars could inhibit agglutination at a concentration of 3.125 mM except N-acetyl galactosamine which inhibited agglutination at 6.25 mM.

S. No.	Name of sugar	Minimum inhibitory Concentration of sugar (mM)
1	Arabinose	3.125
2	Dextrose	3.125
3	Rhamnose	3.125
4	Galactose	–
5	N-Acetyl Glucosamine	3.125
6	Fucose	3.125
7	Mannose	3.125
8	N-Acetyl Galactosamine	6.25

4. Discussion

In recent few years, aquaculture has become one of the most advancing industrial sectors globally. Aquaculture is also becoming an important economical factor in many countries especially in Southeast-Asian including India [1,2]. Among the vast numbers of aquaculture organisms, invertebrate marine shrimp *M. monoceros* is an important commercial species widely prominently in Indo-West Pacific region [5,6]. Lectins are ubiquitously found in all living creatures and are important components of the innate immunity system in aquatic invertebrate animals, including shrimps [45,56]. The presence of lectins has been known for almost a century [57]. However, their function in many species including marine shrimp *M. monoceros* are not satisfactory known yet. Therefore, the characterization of lectins from *M. monoceros* will enable us to better understand the function and specificity of this important part of shrimp immune system. .

In the present study, we isolated a protein from haemolymph of marine Speckled shrimp *M. monoceros*. Due to its greater binding

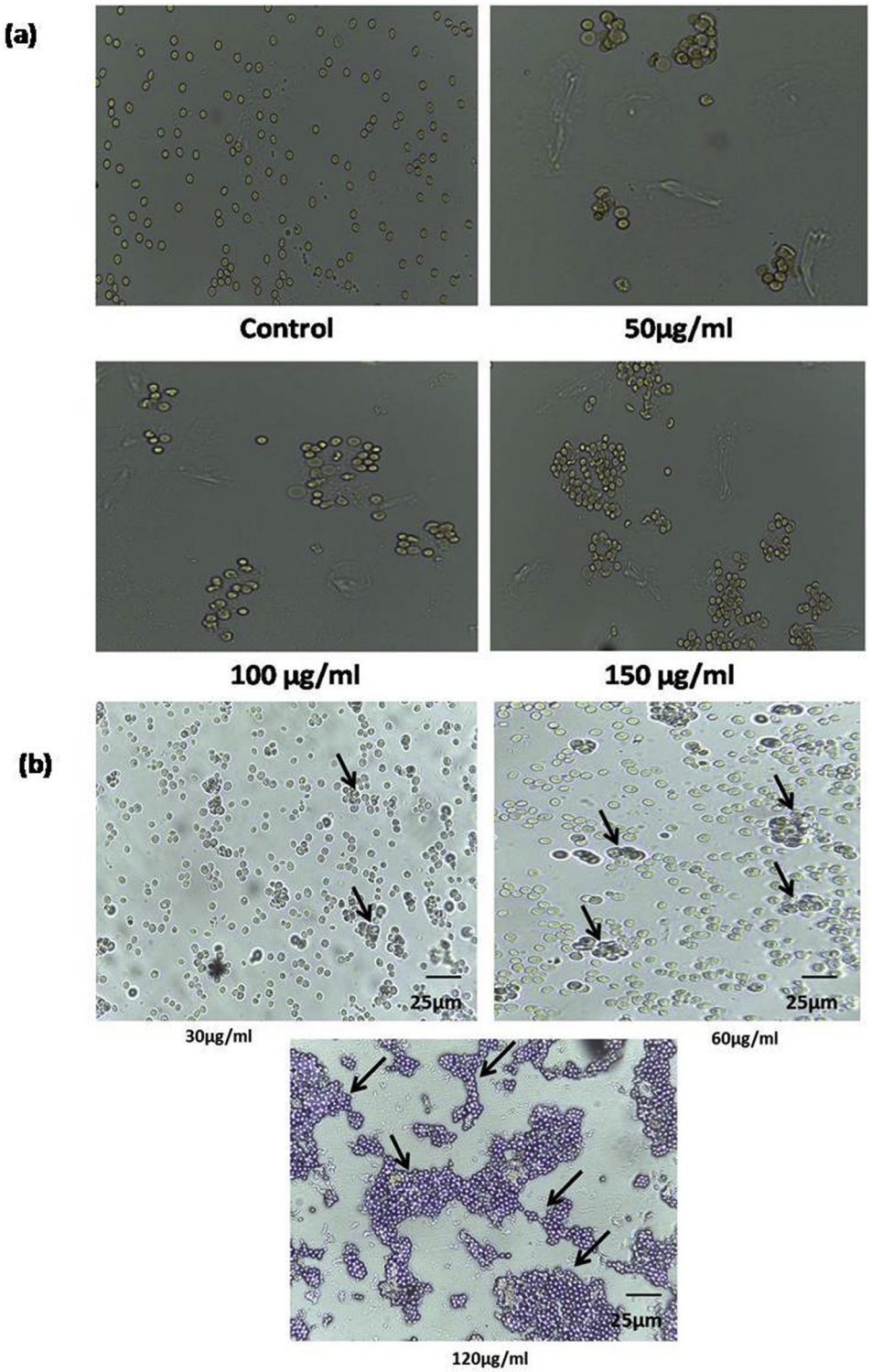

Fig. 5. (a) Haemagglutination assay of *Mm*Lec at different concentrations (50, 100 and 150 µg/ml) monitored by light microscope. **(b)** Yeast agglutination assay of *Mm*Lec at different concentrations (50, 100 and 150 µg/ml) monitored by light microscope. Agglutination activity was observed more at higher concentrations and is indicated by black arrows.

affinity towards variety of carbohydrate molecules as well as its other lectin specific functional characteristics including agglutination, encapsulation and PO activity, it was identified as a novel lectin *Mm*Lec. It has a great affinity and capacity to bind with a diversity of sugar molecules such as arabinose, mannose, fucose, dextrose, rhamnose, N-acetyl glucosamine and N-acetyl galactosamine. In last few years, studies have been reported the isolation of lectins from haemolymph/haemocytes of marine shrimps, crustaceans and fishes [22,23]. The isolated lectin protein *Mm*Lec had an apparent molecular mass of approximately 80 kDa on SDS-PAGE.

Lectins of various molecular weights have also been reported in other marine shrimp species including *Penaeus semisulcatus* (Mol. Wt = 118 kDa) [58], *Fenneropenaeus merguiensi* (31 kDa) [47], *Fenneropenaeus chinensis* (168 kDa) [59], *Penaeus japonicas* (452 kDa) [60],

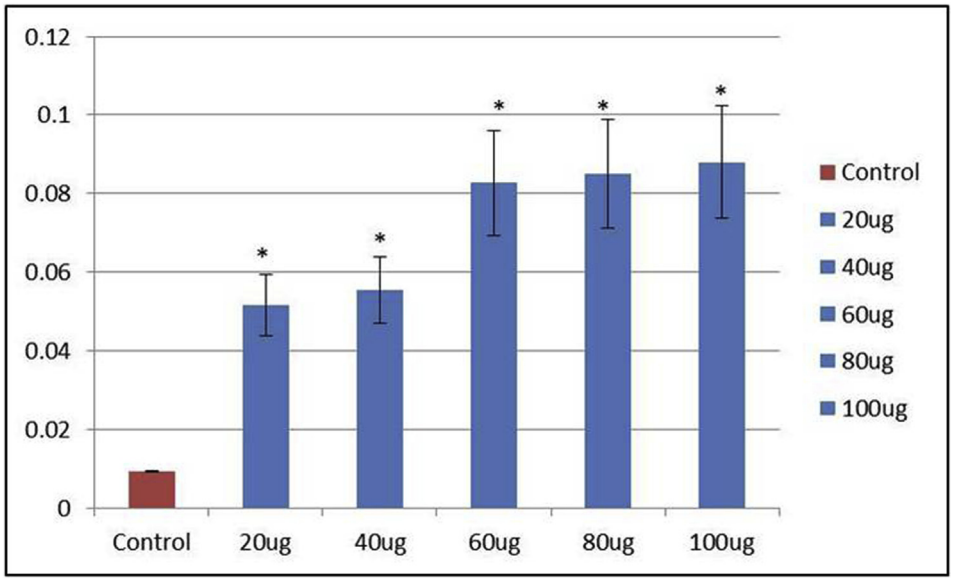

Fig. 6. Enhancement of PO activity by purified *Mm*Lec mixed with laminarin, and tested at different concentrations.

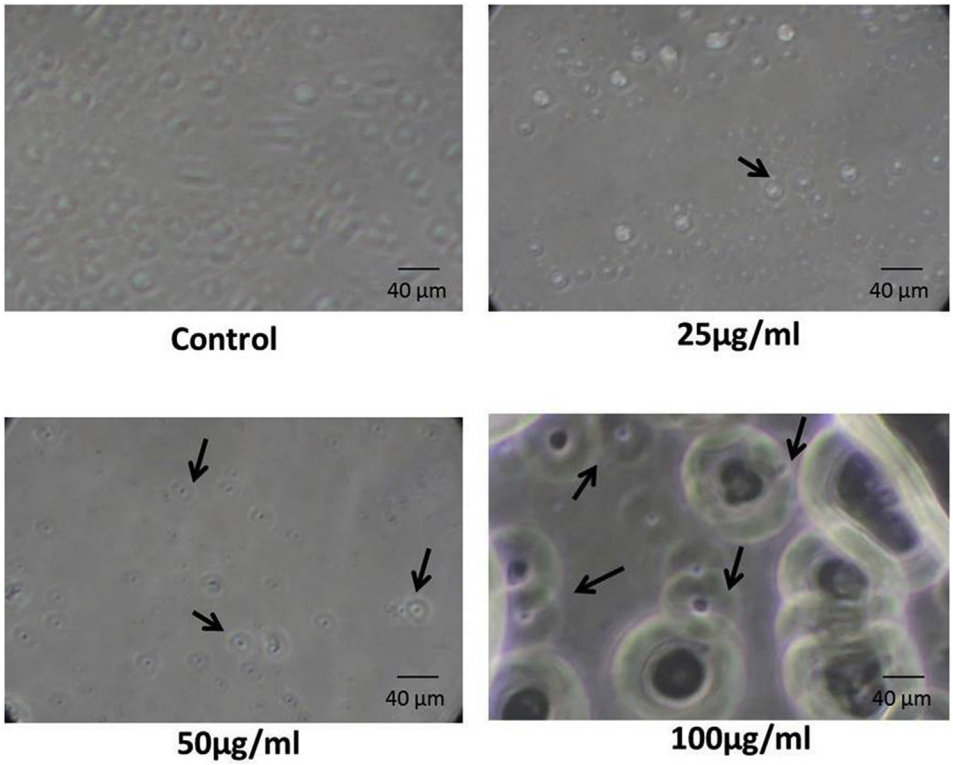

Fig. 7. Light microscopic image showing encapsulation of Sepharose CL-4B beads by purified *Mm*Lec from the haemolymph of *M. monoceros*.

Litopenaeus schmitti (220 kDa) [61]. Also, Donald and Wendy [62] reported a purified lectin with mol. wt. of approximately 66 kDa from catfish *Ictalurus furcatus* and *Ictalurid* catfish. They stated those lectins purified from different organisms are likely to have different molecular weights probably through different sizes of the amino acids and polypeptide chains. However, the result obtained through HPLC, XRD as well as FTIR analysis revealed about the purity, homogeneity and crystalline organization of *Mm*Lec. Jayanthi et al. [50] and Ishwarya et al. [55] have reported the similar findings in their previous investigations thus; our findings are in agreement with their earlier studies.

The analysis of purified *Mm*Lec was multivalent showing higher affinity to arabinose, dextrose, rhamnose, N-acetyl glucosamine, fucose and mannose, a lower affinity to N-acetyl galactosamine, and no activity towards galactose. This agrees with previous results by Silva et al. [63] and Mitra and Das [64] who found that a single lectin protein can exhibit affinity towards various sugar molecules.

Due to the high agglutination activity of *Mm*Lec towards human RBC and yeast cells, it is likely that *Mm*Lec is an important agglutinin weapon to adhere the wide range of microbial intruders. The results showed that *Mm*Lec had slightly higher agglutination activity towards human erythrocytes compared to yeast cells. This may be due to the presence of sialic acid in human erythrocytes possess sialic acid, which can combine with *Mm*Lec and thus, enhance the agglutination. In

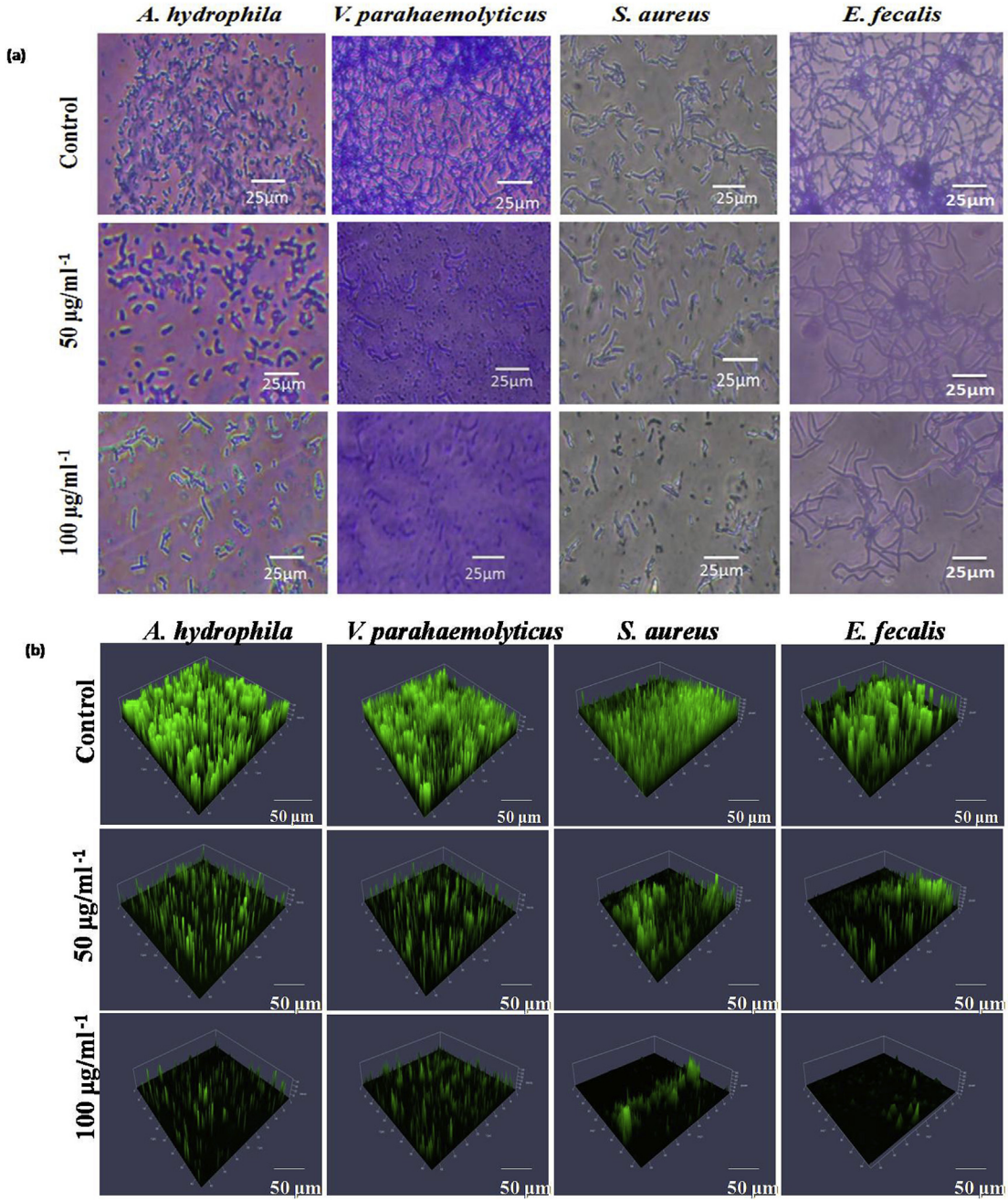

Fig. 8. (a) Light microscopic analysis and **(b)** CLSM images showing the antibiofilm activity of *Mm*Lec at two different concentrations against *A. hydrophila, V. parahaemolyticus, S. aureus* and *E. faecalis.*

addition, *Mm*Lec is efficient in identifying foreign antigens through its pathogen associated molecular patterns (PAMPs) and PRRs thus, can eliminate pathogens through stimulating phagocytosis. Sivakamavalli and Vaseeharan [58] reported related findings of a lectin from green tiger prawn on human erythrocytes. Also, shrimp lectins from *Litopenaeus setiferus* [65] and *Fenneropenaeus chinensis* [59] exhibited almost similar agglutination features on human erythrocytes.

PO is an important cellular process and is well known for its interaction with immune components of invertebrates including marine shrimp. It activates inactive ProPO through activation of complement pathway of lectin and plays a notable immune-defensive role in many ways such as in wound healing and removal of microbial intruders [58]. This was confirmed in the present study, where increased concentrations of *Mm*Lec induced PO activity. This agrees with earlier studies of Lee and Soderhall [66] where, lectin activated the PO cascade in freshwater Crayfish *Pacifastacus leniusculus*. Junkunlo et al. [67] has also suggested that the activation of the PO system is a result of lectin associates with PRPs and PAMPs creating a complex in LGBP fashion on the surface of invaders including bacteria, viruses and fungi etc.

On the other hand, encapsulation is another important cellular phenomenon which is exclusively present in invertebrate animals and is actively involved in combating a variety of foreign hazardous elements. In contrast to phagocytosis which destroys large foreign lethal components, encapsulation aims in creating multi-layered sheaths around and over the microbial intruders leading to destructions inside the encapsulated cascade. In current study, *Mm*Lec was able to stimulate encapsulation of Sepharose beads by haemocytes. Therefore; *Mm*Lec can act by eliminating and abolition of foreign pathogens from shrimp aquaculture. In several previous studies, lectins with wide range immunological functions with enhanced encapsulation process have been

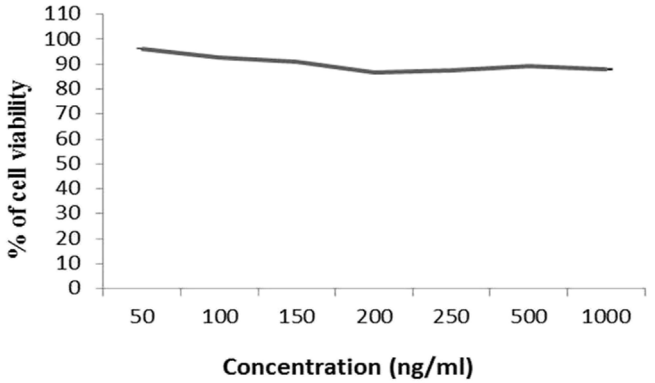

Fig. 9. Cytotoxic activity of *Mm*Lec against breast cancer cell lines MDA-MB-231.

reported in many invertebrates including marine white shrimp *L. vannamei* [68–70].

The purified *Mm*Lec was was able to reduce the biofilm formation generated by those bacterial species tested. *Mm*Lec therefore has a great potential to inhibit and accumulate undesirable pathogenic products by preventing the proliferation and colonization as well as interrupting their biofilm architectural cascade. Jayanthi et al. [50], Sivakamavalli et al. [71] and Anjugam et al. [72] have also reported the similar activities of various lectins purified from other shrimp species. They also postulated that lectins, showing antibiofilm properties can be treated as a potent antimicrobial therapeutic tool.

The present study also showed that *Mm*Lec has clear anti-cancerous and cytotoxicity effect when tested on the breast cancer cell line MDA-MB-231. Kwak et al. [73] have described the function of lactose specific sugar molecules as an anti-tumor agent in skin mucus of eel. However, Kumar et al. [74] have also reported about toxic effects of biopolymer on MDA-MB-231 breast cancer cell lines. Thus, our findings are in accordance with the findings of earlier reports. Therefore, on the basis of our findings as well as various earlier reports, we could perhaps proposed that the purified *Mm*Lec interferes significantly with the immune response and can play major role in enhancing antimicrobial resistance in shrimps along with wide range of aquatic animals.

5. Conclusion

Conclusively, it is stated that the purified *Mm*Lec through affinity chromatography revealed an apparent molecular mass of 80 kDa on SDS-PAGE under reducing circumstances. HPLC, XRD and FTIR analysis displayed the purity, homogeneity as well as crystalline nature of *Mm*Lec. The active involvement of *Mm*Lec in immune system as well as its immune protective role in Speckled shrimp was established by agglutination, phenoloxidase and encapsulation augmenting properties. Consequently, to ascertain antibiofilm properties, the purified *Mm*Lec was projected against various Gram-negative and positive bacteria. Based on all these outcomes, we concluded that *Mm*Lec are an important component of immune system which can be treated as a potential antimicrobial and therapeutic tool to boost the immune mechanism of shrimp as well as to prevent the aquaculture industry from deadly microbes.

Acknowledgement

This research was supported by the DST-SERB project ECR/2015/000554 to E. P. The authors are grateful to Ms. Surya and Ms. Anamika for their technical support. The authors thank Prof. Dr. K. Karthikeyan Sivashanmugam, VIT, for his support during the present study.

References

[1] FAO, The State of World Fisheries and Aquaculture, FAO, Rome, Italy, 2014, p. 223.
[2] Food and Agriculture Organization of the United Nations, FAO, Inland water resources and aquaculture service, fishery resources division, FAO, Fish Circ 886 (1) (2002) 1–163.
[3] P. Edwards, Traditional asian aquaculture, in: G. Burnell, G. Allan (Eds.), New Technologies in Aquaculture, Improving Production, Efficiency, Quantity and Environmental Management, Woodhead Publishing Limited, Oxford, U.K, 2009, pp. 1029–1063.
[4] B. Rosenberry, World Shrimp Farming, Shrimp News International, San Diego, California, 2001.
[5] G.S. Rao, Length weight relationship and other dimensional relationship of *Metapenaeus monoceros* (Fabricus, 1798) from the Kakinada coast, Indian J. Fish. 35 (3) (1988) 211–215.
[6] M. Madhupratap, K.N.V. Nair, T.C. Gopalakrishnan, P. Haridas, K.K.C. Nair, P. Venugopal, et al., Arabian sea oceanography and fisheries of west coast of India, Curr. Sci. 81 (4) (2001) 355–361.
[7] A.J. De Freitas, Selection of nursery areas by six southeast African Penaeidae, Estuar. Coast Shelf Sci. 23 (1986) 901–908.
[8] P. Rönnbäck, A. Macia, G. Amqvist, L. Schultz, M. Troell, Do penaeid shrimps have a preference for mangrove habitats? Distribution pattern analysis on Inhaca Island, Southern Mozambique, Estuar. Coast Shelf Sci. 55 (2002) 427–436.
[9] P.A. Karakoltsidis, A. Zotos, M. Constantinides, Composition of the commercially important mediterranean finfish, crustaceans and molluscs, J. Food Compos. Anal. 8 (1995) 258–273.
[10] C.T. Achuthankutty, S.S.R. Nair, K.L. Kumari, Growth of juvenile shrimp, *Metapenaeus monoceros* fed with squid and mussel, Indian J. Mar. Sci. 22 (1993) 283–286.
[11] S. Tourtip, S. Wongtripop, G.D. Stentiford, K.S. Bateman, S. Sriurairatana, J. Chavadej, et al., *Enterocytozoon hepatopenaei* sp. nov. (Microsporida: enterocytozoonidae), a parasite of the black tiger shrimp *Penaeus monodon* (Decapoda: penaeidae): fine structure and phylogenetic relationships, J. Invertebr. Pathol. 102 (2009) 21–29.
[12] Tangprasittipap, J. Srisala, S. Chouwdee, M. Somboon, N. Chuchird, C. Limsuwan, et al., The microsporidian *Enterocytozoon hepatopenaei* is not the cause of white feces syndrome in whiteleg shrimp *Penaeus* (*Litopenaeus*) *vannamei*, BMC Vet. Res. 9 (2013) 139.
[13] T.W. Flegel, Historic emergence, impact and current status of shrimp pathogens in Asia, J. Invertebr. Pathol. 110 (2012) 166–173.
[14] S.N. Chen, C.F. Lo, S.M. Liu, G.H. Kou, The first identification of *Penaeus monodon baculovirus* (*MBV*) in the cultured sand shrimp *Metapenaeus ensis*, Bull. Eur. Fish Pathol. 9 (1989) 62–64.
[15] C.F. Lo, T. Aoki, J.R. Bonami, T.W. Flegel, J.H. Leu, D.V. Lightner, et al., Nimaviridae, in: A.M.Q. King, M.J. Adams, E.B. Carstens, E.J. Lefkowitz (Eds.), Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses, Elsevier, New York, 2012, pp. 229–234.
[16] L. Tran, L. Nunan, R.M. Redman, L.L. Mohney, C.R. Pantoja, K. Fitzsimmons, et al., Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp, Dis. Aquat. Org. 105 (2013) 45–55.
[17] S. Thitamadee, A. Prachumwat, J. Srisala, P. Jaroenlak, P.V. Salachan, K. Sritunyalucksana, et al., Review of current disease threats for cultivated penaeid shrimp in Asia, Aquaculture 452 (2016) 69–87.
[18] M.R.F. Marques, M.A. Barracco, Lectins, as non-self-recognition factors, in crustaceans, Aquaculture 191 (2000) 23–44.
[19] J.A. Hoffmann, J.M. Reichhart, *Drosophila* innate immunity: an evolutionary perspective, Nat. Immunol. 3 (2002) 121–126.
[20] R. Medzhitov, C.A.J. Janeway, Decoding the patterns of self and nonself by the innate immune system, Science 296 (2002) 298–300.
[21] M.W. Johansson, P. Keyser, K. Sritunyalucksana, K. Söderhäll, Crustacean haemocytes and haematopoiesis, Aquaculture 191 (2000) 45–52.
[22] Y. Kumagai, S. Akira, Identification and functions of pattern-recognition receptors, J. Allergy Clin. Immunol. 125 (2010) 985–992.
[23] H. Schulenburg, C. Boehnisch, N.K. Michiels, How do invertebrates generate a highly specific innate immune response? Mol. Immunol. 44 (2007) 3338–3344.
[24] N. Sharon, H. Lis, Lectins, second ed., Kluwer Academic Publishers, Dordrecht, The Netherlands, 2003, pp. 105–174.
[25] S. Dutta, B. Sinham, B. Bhattacharya, B. Chatterjee, S. Mazumder, Characterization of a galactose binding serum lectin from the Indian catfish, *Clarias batrachus*: possible involvement of fish lectins in differential recognition of pathogens, Comp. Biochem. Physiol., C 14 (2005) 76–84.
[26] C.A. Janeway, R. Medzhitov, Innate immune recognition, Annu. Rev. Immunol. 20 (2002) 197–216.
[27] S. Tasumi, T. Ohira, I. Kawazoe, H. Suetake, Y. Suzuki, K. Aida, Primary structure and characteristics of a lectin from skin mucus of the Japanese eel (*Anguilla japonica*), J. Biol. Chem. 277 (2002) 27305–27311.
[28] H. Tateno, T. Ogawa, K. Muramoto, H. Kamiya, M. Saneyoshi, Rhamnose-binding lectins from steelhead trout (*Onchorhynchus mykiss*) eggs recognize bacterial lipopolysaccharides and lipoteichoic acid, Biosci. Biotechnol. Biochem. 66 (2002) 604–612.
[29] G.K. Christophides, E. Zdobnov, C. Barillas-Mury, E. Birney, S. Blandin, C. Blass, et al., Immunity-related genes and gene families in *Anopheles gambiae*, Science 298 (5591) (2002) 159–165.
[30] X.Q. Yu, M.R. Kanost, Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *Manduca sexta*, is induced in response to gram-negative bacteria, J. Biol.

Chem. 275 (48) (2000) 37373–37381.
[31] X.Q. Yu, M.E. Tracy, E. Ling, F.R. Scholz, T. Trenczek, A novel C-type immulectin-3 from *Manduca sexta* is translocated from hemolymph into the cytoplasm of hemocytes, Insect Biochem. Mol. Biol. 35 (4) (2005) 285–295.
[32] S. Tunkijjanukij, J.A. Olafsen, Sialic acid-binding lectin with antibacterial activity from the horse mussel: further characterization and immunolocalization, Dev. Comp. Immunol. 22 (2) (1998) 139–150.
[33] Z.Y. Zhao, Z.X. Yin, X.P. Xu, S.P. Weng, X.Y. Rao, Z.X. Dai, et al., A novel C-type lectin from the shrimp *Litopenaeus vannamei* possesses anti-white spot syndrome virus activity, J. Virol. 83 (1) (2009) 347–356.
[34] A.N. Zelensky, J.E. Gready, The C-type lectin-like domain superfamily, FEBS J. 272 (2005) 6179–6217.
[35] M.D. Adams, S.E. Celniker, R.A. Holt, C.A. Evans, J.D. Gocayne, P.G. Amanatides, The genome sequence of *Drosophila melanogaster*, Science 287 (2000) 2185–2195.
[36] X.W. Wang, W.T. Xu, X.W. Zhang, X.F. Zhao, X.Q. Yu, J.X. Wang, A C-type lectin is involved in the innate immune response of Chinese white shrimp, Fish Shellfish Immunol. 27 (2009) 556–562.
[37] M. Kakiuchi, N. Okino, N. Sueyoshi, S. Ichinose, A. Omori, S.I. Kawabata, et al., Purification, characterization, and cDNA cloning of α-Nacetylgalactosamine specific lectin from starfish, *Asterina pectinifera*, Glycobiology 12 (2002) 85–94.
[38] S.I. Kawabata, Nonself-recognizing lectins in invertebrate innate immunity, Protein, Nucleic Acid and Enzyme, 45 2000, pp. 679–689.
[39] S.M.A. Kawsar, Y. Fujii, R. Matsumoto, T. Ichikawa, H. Tateno, J. Hirabayashi, et al., Isolation, purification, characterization and glycan-binding profile of a D-galactoside specific lectin from the marine sponge, *Halichondria okadai*, Comp. Biochem. Physiol. B 150 (2008) 349–357.
[40] S.M.A. Kawsar, T. Takeuchi, K.I. Kasai, Y. Fujii, R. Matsumoto, H. Yasumitsu, et al., Glycan-binding profile of a D-galactose binding lectin purified from the annelid *Perinereis nuntia*, Ver. Vallata. Comp. Biochem. Physiol. B 152 (2009) 382–389.
[41] Kenjo, M. Takahashi, M. Matsushita, Y. Endo, M. Nakata, T. Mizuochi, et al., Cloning and characterization of novel ficolins from the solitary ascidian, *Halocynthia roretzi*, J. Biol. Chem. 276 (2001) 19959–19965.
[42] T. Luo, H. Yang, F. Li, X. Zhang, X. Xu, Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon*, Dev. Comp. Immunol. 30 (2006) 607–617.
[43] T. Naganuma, T. Ogawa, J. Hirabayashi, K. Kasai, H. Kamiya, K. Muramoto, Isolation, characterization and molecular evolution of a novel pearl shell lectin from a marine bivalve, *Pteria penguin*, Mol. Divers. 10 (2006) (2006) 607–618.
[44] J. Sun, L. Wang, B. Wang, Z. Guo, M. Liu, K. Jiang, et al., Purification and characterisation of a natural lectin from the serum of the shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 23 (2007) 292–299.
[45] H.Z. Guo, P.F. Zou, J.P. Fu, Z. Guo, B.K. Zhu, P. Nie, et al., Characterization of two Ctype lectin-like domain (CTLD)-containing proteins from the cDNA library of Chinese mitten crab *Eriocheir sinensis*, Fish Shellfish Immunol. 30 (2010) 515–524.
[46] M. Kondo, H. Matsuyamam, T. Yano, The opsonic effect of lectin on phagocytosis by hemocytes of Kuruma Prawn, *Penaeus japonicas*, Fish Pathol. 27 (1992) 217–222.
[47] W. Rittidach, N. Paijit, P. Utarabhand, Purification and characterization of a lectin from the banana shrimp *Fenneropenaeus merguiensis* hemolymph, Biochim. Biophys. Acta 1770 (2007) 106–114.
[48] T. Luo, X. Zhang, Z. Shao, X. Xu, PmAV, a novel gene involved in virus resistance of shrimp *Penaeus monodon*, FEBS Lett. 551 (2003) 53–57.
[49] T.H. Ma, S.H. Tiu, J.G. He, S.M. Chan, Molecular cloning of a C-type lectin (LvLT) from the shrimp *Litopenaeus vannamei*: early gene down-regulation after WSSV infection, Fish Shellfish Immunol. 23 (2007) 430–437.
[50] S. Jayanthi, R. Ishwarya, M. Anjugam, A. Iswarya, S. Karthikeyan, B. Vaseeharan, Purification, characterization and functional analysis of the immune molecule lectin from the haemolymph of blue swimmer crab *Portunus pelagicus* and their antibiofilm properties, Fish Shellfish Immunol. 62 (2017) 237–247.
[51] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of *bacteriophage* T4, Nature 227 (1970) 680–685.
[52] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[53] P. Scherrer, Bestimmung der Grosse und der Inneren Struktur von Kolloidteilchen Mittels Rontgenstrahlen, Nachrichten von der Gesellschaft der Wissenschaften, Gottingen. Mathematisch-Physikalische Klasse, (1918), pp. 98–100 2.
[54] M.T.S. Correia, L.C.B.B. Coelho, Purification of a glucose/mannose specific lectin, isoform 1, from seeds of *Cratylia mollis* mart. (Camaratu Bean), Appl. Biochem. Biotechnol. 55 (1995) 261–273.
[55] R. Ishwarya, B. Vaseeharan, A. Iswarya, S. Karthikeyan, Haemolytic and antibiofilm properties of haemocyanin purified from the haemolymph of indian white shrimp *Fenneropenaeus indicus*, Fish Shellfish Immunol. 59 (2016) 447–455.
[56] Y.C. Liu, F.H. Li, B. Dong, B. Wang, W. Luan, X.J. Zhang, et al., Molecular cloning, characterization and expression analysis of a putative C-type lectin (Fclectin) gene in Chinese shrimp *Fenneropenaeus chinensis*, Mol. Immunol. 44 (2007) 598–607.
[57] K. Drickamer, Evolution of Ca^{2+}-dependent animal lectins, Prog. Nucleic Acid Res. Mol. Biol. 45 (1993) 207–232.
[58] J. Sivakamavalli, B. Vaseeharan, Purification, characterization and functional role of lectin from green tiger shrimp *Penaeus semisulcatus*, Int. J. Biol. Macromol. 67 (2014) 64–70.
[59] J. Sun, L. Wang, B. Wang, Z. Guo, M. Liu, K. Jiang, et al., Purification and characterization of a natural lectin from the plasma of the shrimp *Fenneropenaeus chinensis*, Fish Shellfish Immunol. 25 (2008) 290–297.
[60] H. Yang, T. Luo, F. Li, S. Li, X. Xu, Purification and characterization of a calcium independent lectin (*Pj*Lec) from the haemolymph of the shrimp *Penaeus japonicas*, Fish Shellfish Immunol. 22 (2007) 88–97.
[61] M.R. Cominettia, M.R.F. Marquesb, D.M. Lorenzinib, S.E. Löfgrena, S. Daffrec, M.A. Barracco, Characterization and partial purification of a lectin from the hemolymph of the white shrimp *Litopenaeus schmitti*, Dev. Comp. Immunol. 26 (2002) 715–721.
[62] D.O. Donald, M.R. Wendy, Purification, characterization and seasonal variation of mannose binding C-type lectin in Ictalurid catfish, Aquaculture 321 (2011) 191–196.
[63] C.D.C. da Silva, M.C. Coriolano, M.A. da Silva Lino, C.M. de Melo, R. de Souza Bezerra, E.V. de Carvalho, et al., Purification and characterization of a mannose recognition lectin from *Oreochromis niloticus* (tilapia fish): cytokine production in mice splenocytes, Appl. Biochem. Biotechnol. 166 (2012) 424–435.
[64] S. Mitra, H.R. Das, A novel mannose-binding lectin from plasma of *Labeo rohita*, Fish Physiol. Biochem. 25 (2002) 121–129.
[65] R. Zenteno, L. Vazquez, C. Sierra, A. Pereyra, M.C. Slomianny, S. Bouquelet, et al., Chemical characterization of the lectin from the freshwater prawn *Macrobrachium rosenbergii* (De Man) by MALDI-TOF, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 127 (2000) 243–250.
[66] S.Y. Lee, K. Soderhall, Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*, J. Immunol. 166 (2001) 7319–7326.
[67] K. Junkunlo, A. Prachumwat, A. Tangprasittipap, S. Senapin, S. Borwornpinyo, T.W. Flegel, et al., A novel lectin domain-containing protein (LvCTLD) associated with response of the whiteleg shrimp *Penaeus (Litopenaeus) vannamei* to yellow head virus (YHV), Dev. Comp. Immunol. 37 (2012) 334–341.
[68] H. Lis, N. Sharon, Lectins: carbohydrate-specific proteins that mediate cellular recognition, Chem. Rev. 98 (1998) 637–674.
[69] L. Wang, L. Wang, D. Zhang, F. Li, M. Wang, M. Huang, et al., A novel C type lectin from crab *Eriocheir sinensis* functions as pattern recognition receptor enhancing cellular encapsulation, Fish Shellfish Immunol. 34 (2013) 832–842.
[70] L. Cerenius, P. Jiravanichpaisal, H. Liu, I. Söderhäll, Crustacean immunity, Invertebrate Immunity vol. 708, Springer, US, 2010, pp. 239–259.
[71] J. Sivakamavalli, R. Nirosha, B. Vaseeharan, Purification and characterization of a Cysteine-Rich 14-kDa antibacterial peptide from the granular hemocytes of mangrove crab *Episesarma tetragonum* and its Antibiofilm Activity, Appl. Biochem. Biotechnol. 176 (4) (2015) 1084–1101.
[72] M. Anjugam, A. Iswarya, B. Vaseeharan, Multifunctional role of β-1,3 glucan binding protein purified from the haemocytes of blue swimmer crab *Portunus pelagicus* and in vitro antibacterial activity of its reaction product, Fish Shellfish Immunol. 48 (2016) 196–205.
[73] C.H. Kwak, S.H. Lee, S.K. Lee, S.H. Ha, S.J. Suh, K.M. Kwon, et al., Induction of apoptosis and antitumor activity of eel skin mucus, containing lactose-binding molecules, on human leukemic K562 cells, Mar. Drugs 13 (2015) 3936–3949.
[74] P. Kumar, S. Senthamilselvi, M. Govindaraju, R. Sankar, Unraveling the caspase-mediated mechanism for phloroglucinol-encapsulated starch biopolymer against the breast cancer cell line MDA-MB-231, RSC Adv. 4 (2014) 46157–46164.