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

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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.

Key words: *Metapenaeus monoceros,* Lectin, Antibiofilm, Agglutination, Immune molecule

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 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 ºC and the supernatant was transferred into fresh tubes and stored at −20 ºC until further purification.

2.2. Purification of *Mm*Lec 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.

135 2.3. Characterization of purified *Mm*Lec

136 2.3.1. Molecular weight determination of *Mm*Lec

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

145 2.3.2. High performance liquid chromatography (HPLC) and X-ray diffraction (XRD) of *Mm*Lec

146 HPLC separation was carried out using a reversed phase C_{18} column (7.8 mm x 30 cm).
147 Previously, equilibrated with TBS / $CaCl_2$ at a flow rate of 0.8 ml min^{-1}. HPLC system (Zorbax
148 Bio-series GF-250, DuPont, Willington, DE, USA), was used for the homogeneity analysis. In
149 order to determine the spatial distribution of atomic coordination and the arrangement of atoms,
150 an X-ray diffraction analysis (XRD, Scintag-SDS 2000) were performed on purified *Mm*Lec at
151 40 kV / 20 mA, using continuous scanning of 2θ mode [53]. The average grain size and shape of
152 the purified *Mm*Lec was determined using Scherrer's formula [53] as $d = 0.9\lambda / \beta\cos\theta$ [Where, d
153 is the mean diameter of purified *Mm*Lec, λ is the wavelength of the X-ray radiation source and β
154 is the angular FWHM of the XRD peak at diffraction angle (θ)].

155 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 6µm 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 20mM sodium dodecyl sulphate.

2.4. Functional analysis

2.4.1. Haemagglutination properties of *Mm*Lec

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. *Mm*Lec 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 hr at 25 ºC. 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 *Mm*Lec

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 ºC 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 ºC for 1 hr. 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 *Mm*Lec 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 ºC 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.

200 2.4.5. Antibiofilm properties of *Mm*Lec

201 The effect of purified *Mm*Lec on biofilm-forming Gram-negative (*Aeromonas hydrophila*
202 and *Vibrio parahaemolyticus*) and Gram-positive (*Staphylococcus aureus and Enterococcus*
203 *faecalis*) bacteria were tested using 24-well polystyrene plates containing glass pieces immersed
204 in Luria Bertani broth inoculated with the bacterial suspension of 1% inoculum from overnight
205 cultures (10^7 CFU ml^{-1}). Different concentrations (such as 50 and 100 $\mu g/ml^{-1}$) of *Mm*Lec were
206 introduced into the wells and incubated at 37 ºC for 48 hr. To examine the biofilm activity, the
207 media were discarded and weakly adherent cells were removed by thorough washing with
208 deionized water and allowed to air dry before staining. The biofilm were stained with 1 ml of
209 0.4% acridine orange (w/v) for 10 min. The biofilm inhibition in 3D view was observed by
210 confocal laser scanning microscopy (Carl Zeiss LSM 710, Germany). In addition, the experiment
211 was repeated with crytal violet dye for light microscopic studies. In control, *Mm*Lec was
212 replaced by BSA.

213 2.4.6. Anticancer activity

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

218 2.5. Statistical analysis

219 All treatments were conducted in triplicates (n=3). Data are shown as mean ± standard
220 error mean (S.E.M.). Statistical differences were analysed 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 *Mm*Lec

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 31.8372° 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 *Mm*Lec

The FTIR analysis of *Mm*Lec suggested the presence of a hydroxyl (OH) functional group stretching in the spectrum line from 3000 cm^{-1} to 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).

3.4. Functional analysis

3.4.1. Analysis of sugar-binding assay of *Mm*Lec

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 *Mm*Lec 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). 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

263 *Mm*Lec possess potential capability to act as an important coagulant agent in concentration
264 dependent manner.

265 3.4.3. *Mm*Lec features in enhancing PO activity

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

272 3.4.4. Effect of *Mm*Lec in encapsulation

273 The encapsulation assay was conducted to investigate the potential role of purified
274 protein *Mm*Lec in the phenomena of cellular encapsulation. The assay was conducted in a
275 concentration dependent manner using Sepharose CL-4B beads coated with *Mm*Lec (25, 50 and
276 100 μg/ml). After incubation with haemocytes at 25 ºC for 45 min, the reaction mixture was
277 monitored under light microscopy. The *Mm*Lec clearly enhanced the encapsulation at the highest
278 concentration (100μg/ml) (Fig. 7). TBS buffer control did not produce any effect on
279 encapsulation.

280 3.4.5. Antibiofilm characterization analysis of *Mm*Lec

281 The biofilm inhibition assay of *Mm*Lec was done using two concentrations (50 and 100
282 μg ml^{-1}) on various Gram-negative (*A. hydrophila*, *V. parahaemolyticus*) and Gram-positive (*S.*
283 *aureus*, and *E. faecalis*) bacteria at 24 h. Both concentrations of *Mm*Lec had considerable

284 inhibitory effect on growth of all bacterial species when compared to BSA added controls (Fig.
285 8). At 100 μg ml^{-1}, the effect was further enhanced on *Aeromonas* and *Vibrio sps* compared to
286 the other bacterial species. . The result strongly suggested that *Mm*Lec can be treated as an
287 effective tool for eradication of microbial pathogens widely distributed among shrimp culture
288 and aquaculture industry.

289 3.4.6. Cytotoxicity effect *Mm*Lec

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

293 **4. Discussion**

294 In recent few years, aquaculture has become one of the most advancing industrial sectors
295 globally. Aquaculture is also becoming an important economical factor in many countries
296 especially in Southeast-Asian including India [1, 2]. Among the vast numbers of aquaculture
297 organisms, invertebrate marine shrimp *M. monoceros* is an important commercial species widely
298 prominently in Indo-West Pacific region [5, 6]. Lectins are ubiquitously found in all living
299 creatures and are important components of the innate immunity system in aquatic invertebrate
300 animals, including shrimps [45, 56]. The presence of lectins has been known for almost a century
301 [57]. However, their function in many species including marine shrimp *M. monoceros* are not
302 satisfactory known yet. Therefore, the characterization of lectins from *M. monoceros* will enable
303 us to better understand the function and specificity of this important part of shrimp immune
304 system. .

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

328 [63] and Mitra and Das [64] who found that a single lectin protein can exhibit affinity towards
329 various sugar molecules.

330 Due to the high agglutination activity of *Mm*Lec towards human RBC and yeast cells, it
331 is likely that *Mm*Lec is an important agglutinin weapon to adhere the wide range of microbial
332 intruders. The results showed that *Mm*Lec had slightly higher agglutination activity towards
333 human erythrocytes compared to yeast cells. This may be due to the presence of sialic acid in
334 human erythrocytes possess sialic acid, which can combine with *Mm*Lec and thus, enhance the
335 agglutination. In addition, *Mm*Lec is efficient in identifying foreign antigens through its
336 pathogen associated molecular patterns (PAMPs) and PRRs thus, can eliminate pathogens
337 through stimulating phagocytosis. Sivakamavalli and Vaseeharan [58] reported related findings of a
338 lectin from green tiger prawn on human erythrocytes. Also, shrimp lectins from *Litopenaeus setiferus*
339 [65] and *Fenneropenaeus chinensis* [59] exhibited almost similar agglutination features on human
340 erythrocytes.

341 PO is an important cellular process and is well known for its interaction with immune
342 components of invertebrates including marine shrimp. It activates inactive ProPO through activation
343 of complement pathway of lectin and plays a notable immune-defensive role in many ways such as in
344 wound healing and removal of microbial intruders [58]. This was confirmed in the present study,
345 where increased concentrations of *Mm*Lec induced PO activity. This agrees with earlier studies of
346 Lee and Soderhall [66] where, lectin activated the PO cascade in freshwater Crayfish
347 *Pacifastacus leniusculus*. Junkunlo et al. [67] has also suggested that the activation of the PO
348 system is a result of lectin associates with PRPs and PAMPs creating a complex in LGBP
349 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 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.

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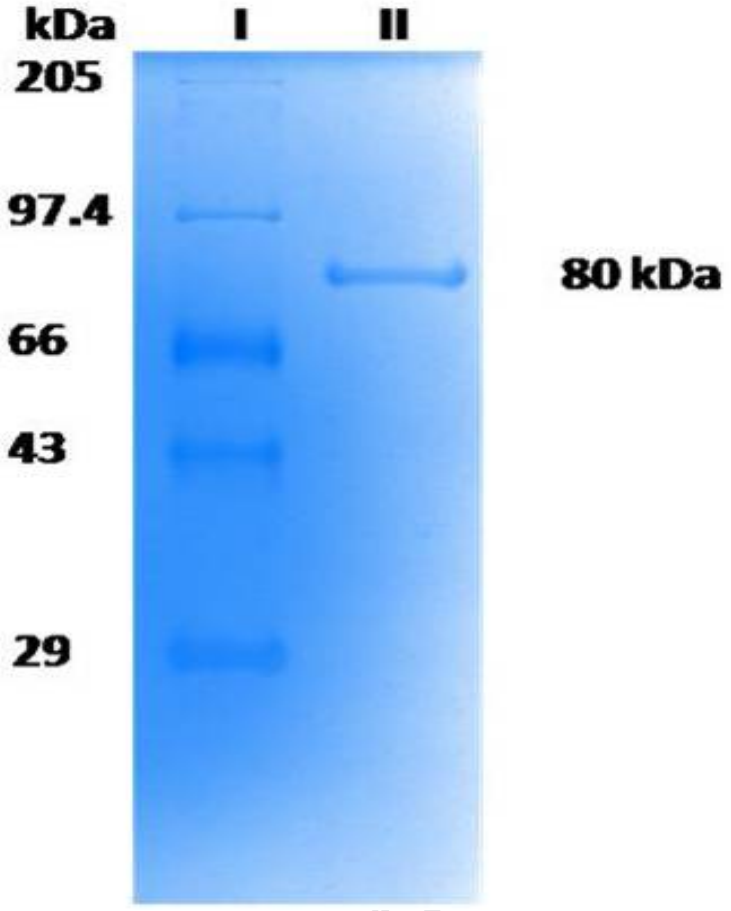
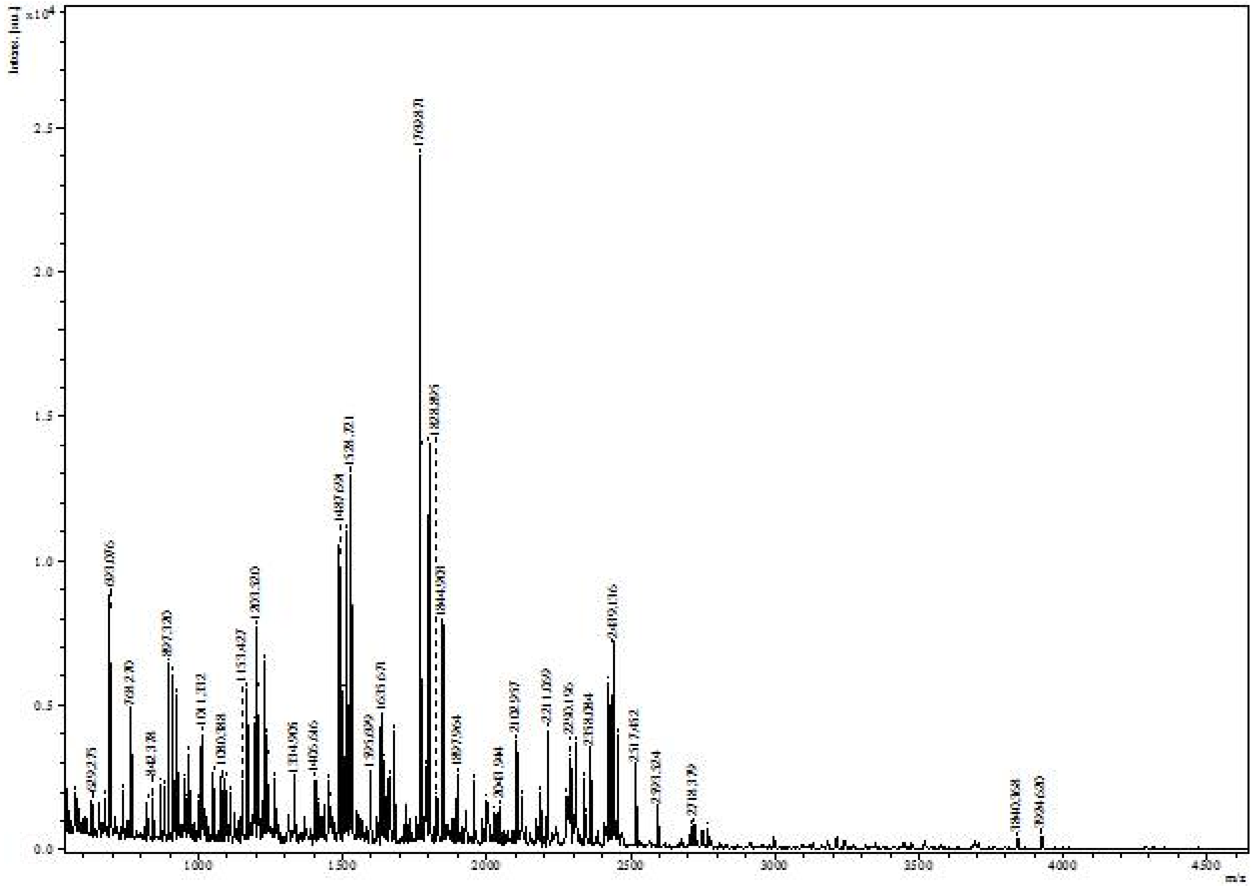
Table 1: Minimal inhibitory concentration corresponds to the lowest carbohydrate concentration able to neutralize haemagglutinating activity of *Mm*Lec. The values are expressed in millimolar and the highest carbohydrate concentration used was 250mM. Galactose could not inhibit the haemagglutination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

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





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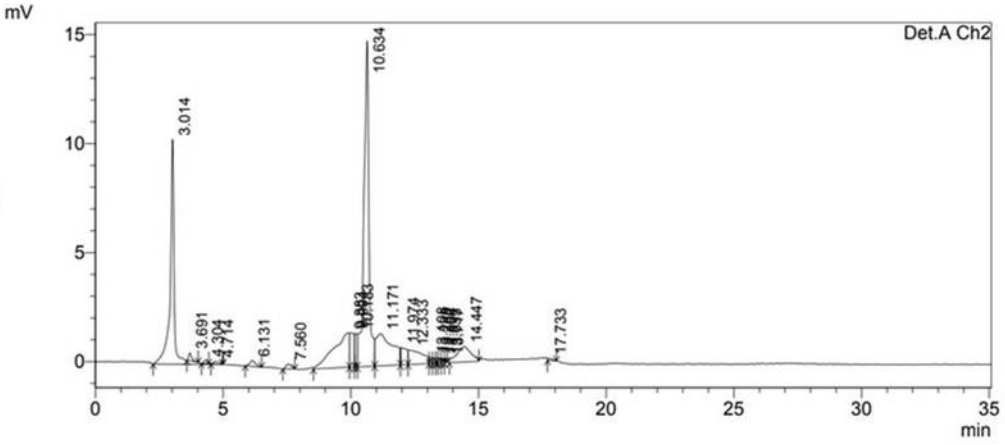
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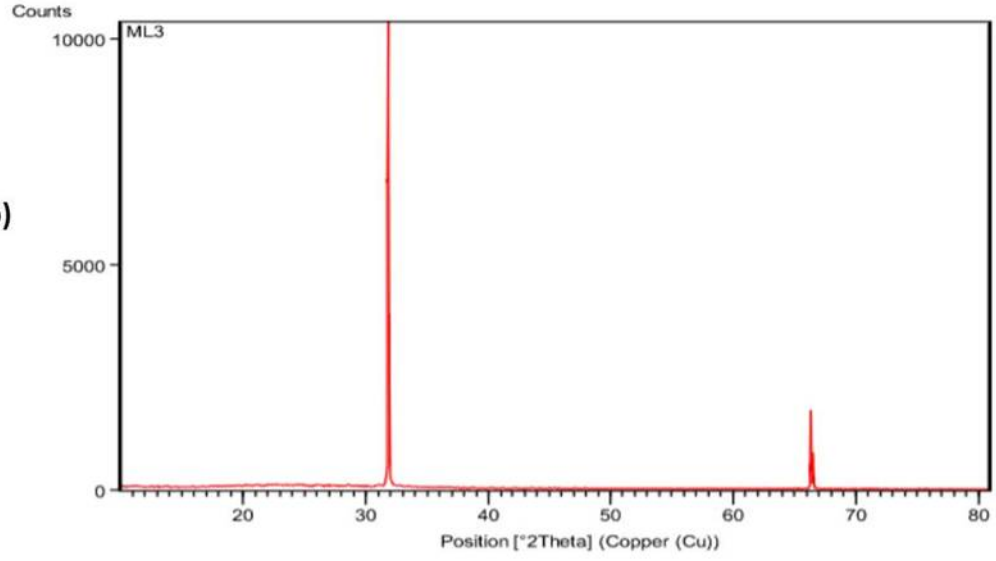
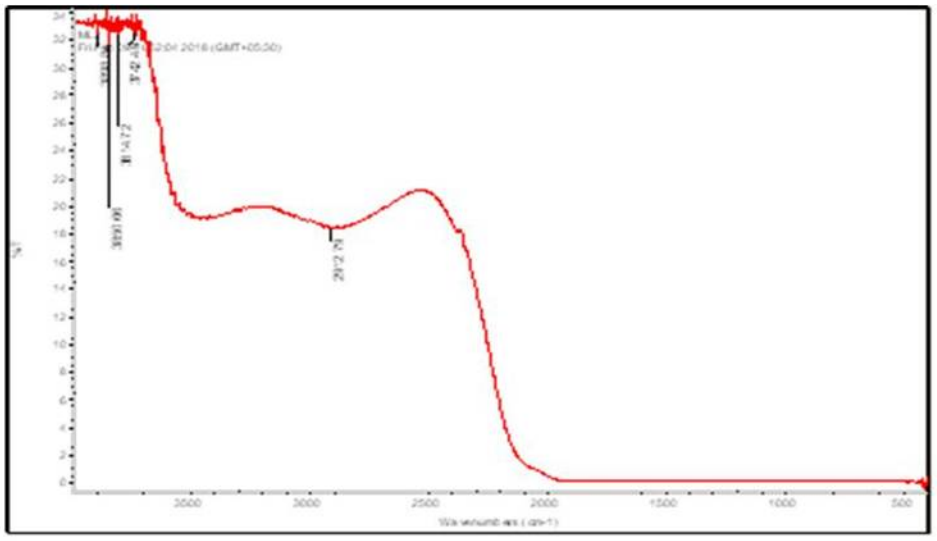
11.171
14.447
17.733
min

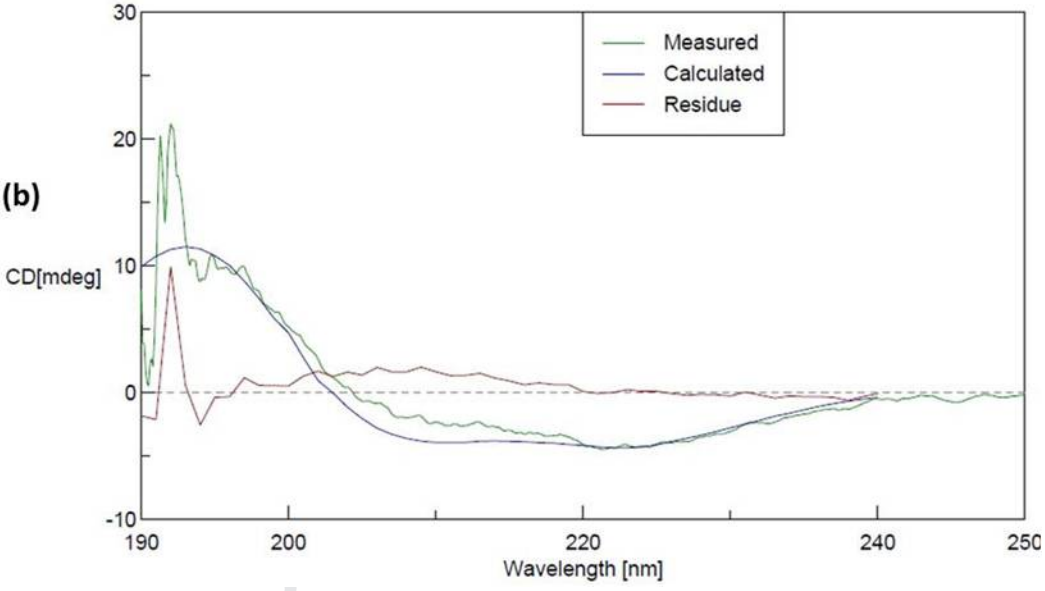
(b)


Counts
ML3
Position [°2Theta] (Copper (Cu))

(a)



(b)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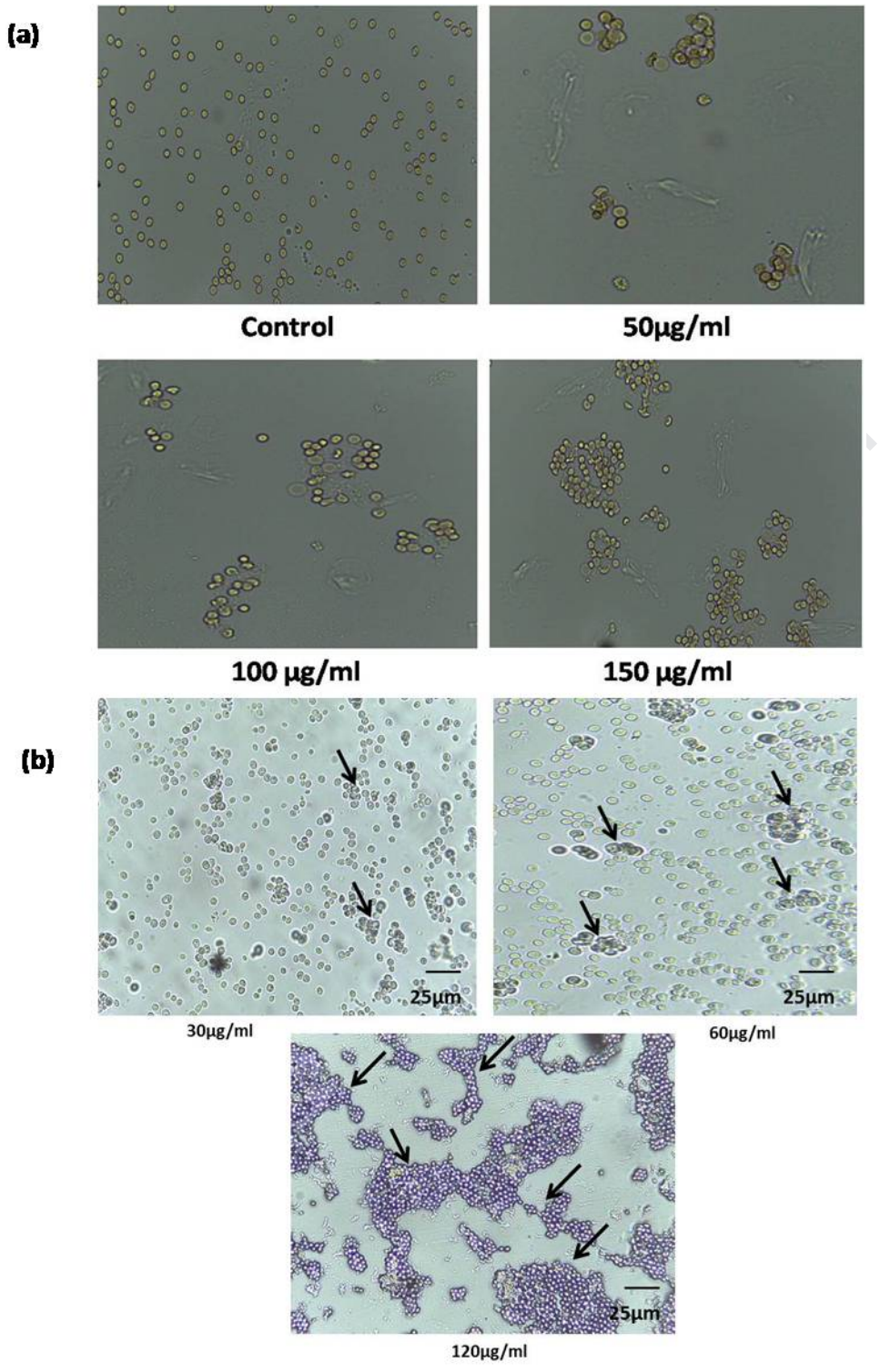
Wavelength [nm]


(a)
Control
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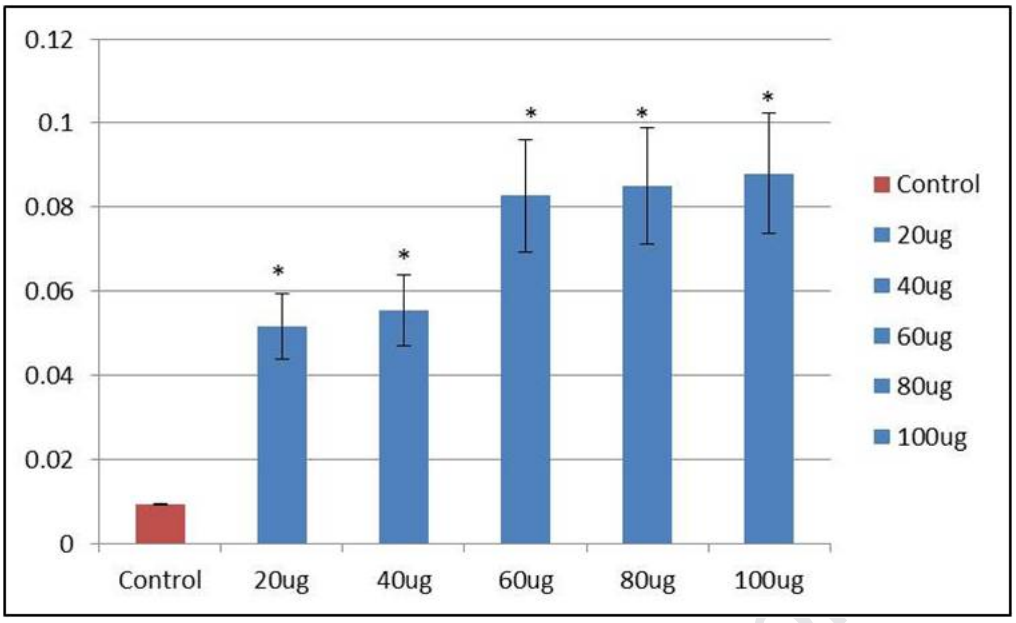
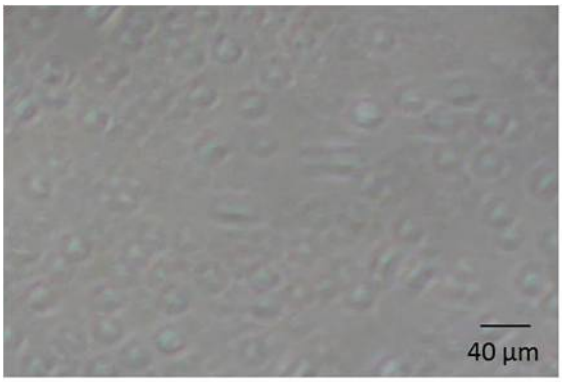
100 µg/ml
150 µg/ml
(b)
25µm
30µg/ml
25µm
60µg/ml
25µm
120µg/ml





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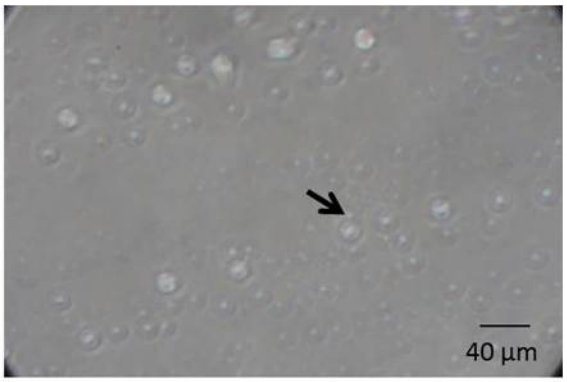

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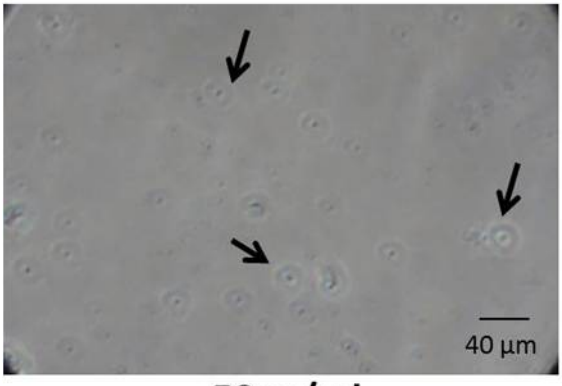

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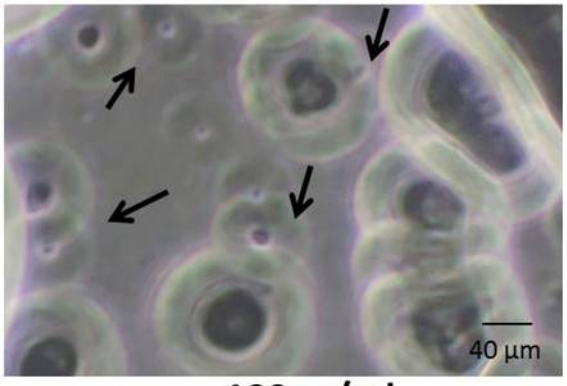

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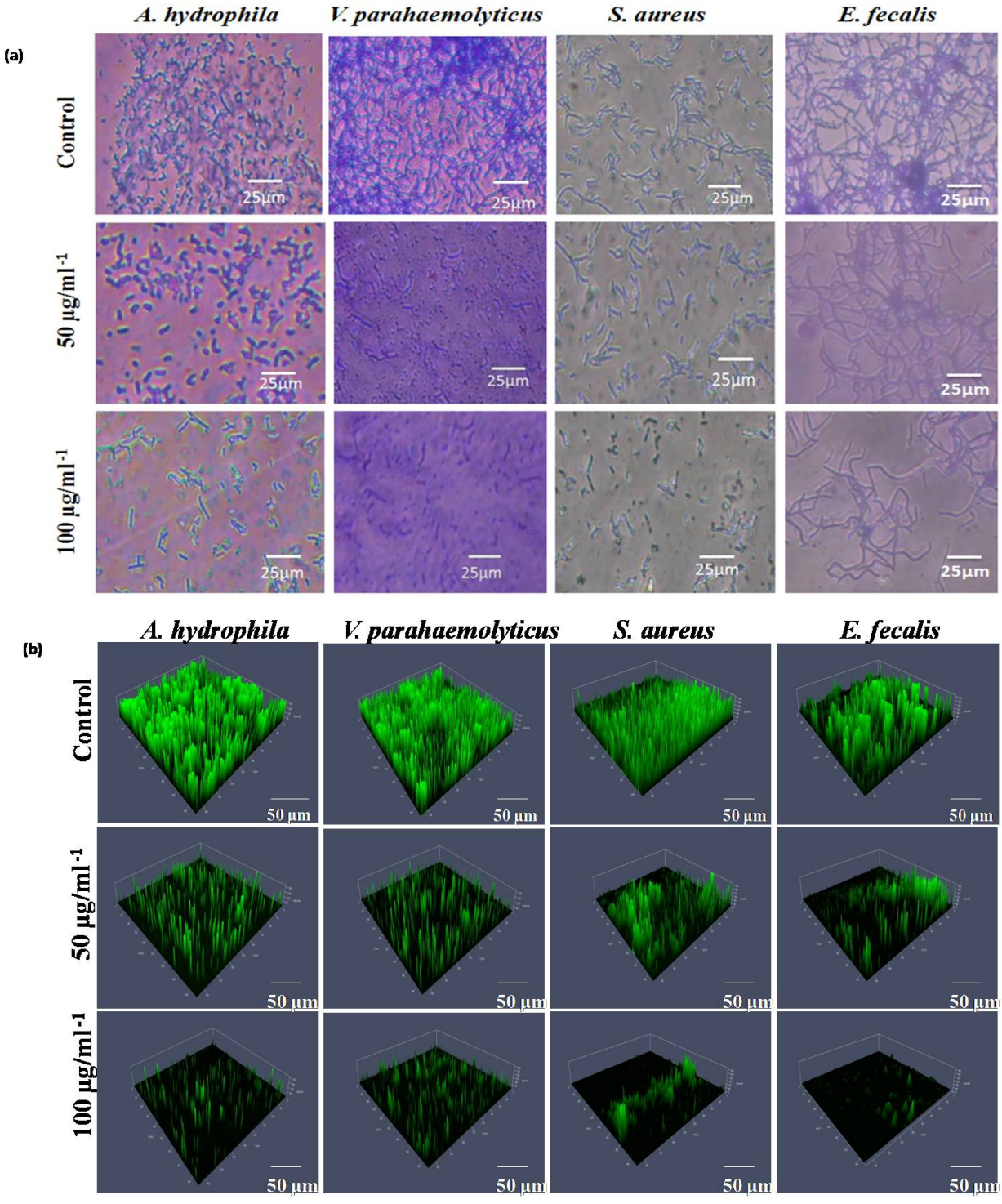
(a)
A. hydrophila
V. parahaemolyticus
S. aureus
E. fecalis
Control
50 µg/ml-1
100 µg/ml-1
25µm
(b)
A. hydrophila
V. parahaemolyticus
S. aureus
E. fecalis
Control
50 µg/ml-1
100 µg/ml-1
50 µm

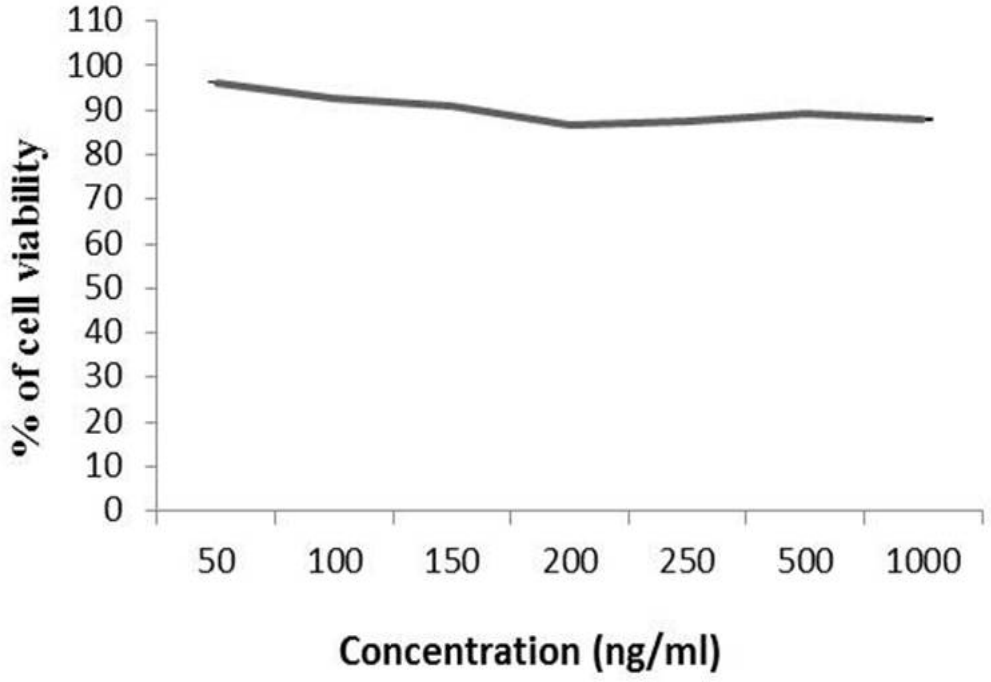
110
100
90
80
70
60
50
40
30
20
10
0
% of cell viability
50
100
150
200
250
500
1000
Concentration (ng/ml)

Figure Legends:

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 sulfate (SDS); Lane II indicates purified *Mm*Lec with molecular mass of 80 kDa and Lane I denotes Broad range molecular mass protein marker.

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 31.8372º 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.

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 193nm and a maximum at 191nm.

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.

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.*

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.*

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

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 250mM. Galactose could not inhibit the haemagglutination at the highest 250mM concentration. All other sugars could inhibit agglutination at a concentration of 3.125mM except N-acetyl galactosamine which inhibited agglutination at 6.25mM.

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

- An immune molecule *Mm*Lec (80 kDa) was isolated and purified from haemolymph of Speckled shrimp, *Metapenaeus monoceros*.
- The *Mm*Lec was reported to exhibit, haemagglutination and yeast agglutination, the phenoloxidase activity and encapsulation activity.
- The antimicrobial and antibiofilm properties of *Mm*Lec against certain aquatic pathogens were evaluated.