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Shrimp lectin (Md-*Lec*) conjugated copper sulfide nanoparticles enhance the elimination of aquatic pathogens in infected Nile tilapia (*Oreochromis niloticus*)†

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Lectins are known for their ability to bind to cell surface glycans, and are useful to develop a glycan-targeted drug delivery system. This study aimed to evaluate the capacity of pectin capped copper sulfide nanoparticles (pCuS NPs) to modulate the antibacterial activity of a lectin, Md-*Lec*, purified from the shrimp, *Metapenaeus dobsoni*. Fluorescence spectroscopy revealed that Md-*Lec* has the ability to form a complex with pCuS NPs. Haemagglutination assay showed that the carbohydrate binding site of the lectin was preserved even after complexing with pCuS. The minimum inhibitory concentrations (MICs) obtained for Md-*Lec* and pCuS NPs against the tested aquatic pathogens were 50 μg ml^{-1} and 12.5 μM, respectively. Interestingly, the MIC of Md-*Lec*–pCuS NPs complex was four fold lower than that of pCuS, which was attributed to the bacterial cell surface glycan recognization activity of Md-*Lec*. Zone of inhibition assay showed that the zone size was highest for the lectin conjugated nanoparticles. Mechanistic study revealed that Md-*Lec*–pCuS NPs affect the bacterial membrane integrity and produce a large volume of reactive oxygen species to kill the bacteria. The practical aspect of using this lectin– pCuS NPs complex was evaluated by treating bacteria infected Nile tilapia (*Oreochromis niloticus*). The bacterial load was much less in the lectin–pCus NPs complex treated fish; moreover, the fish fully recovered from the infection. It was concluded that the conjugate of antibacterial lectin and NPs is more effective than the individual components.

1. Introduction

As the aquaculture industry has headed to new heights in a short span of time, the problems related to fish farming, including infectious diseases, are also increasing.[1] In order to combat this scenario, there has been extensive and unethical use of antibiotics by farmers which has eventually led to the emergence of multiple antibiotic resistances.[2] In order to resolve this situation, scientists have been looking for alternatives that have minimal side effects. The introduction of nanoparticle-conjugated drugs has increased the opportunities and possibilities to treat and address a lot of infectious diseases.[3,4] Metal nanoparticles such as Au, Ag, Cu, Pt and Pd and metal oxide nanoparticles such as ZnO, TiO$_2$, Bi$_2$O$_3$, CuO,

and Fe$_2$O$_3$ in particular are used as they have promising antibacterial properties.[5–9] Functionalized NPs are expected to be more active than NPs alone. For example, *Butea monosperma* lectin functionalized AgNPs were better than AgNPs in inhibiting the biofilms formed by *E. coli*.[10] *Parkia platycephala* seed lectin enhances the antibiotic activity of gentamicin against the multi-drug resistant *E. coli* and *S. aureus*.[11]

Lectins are glycoproteins that possess one or more Carbohydrate Recognition Domains (CRD) which can reversibly and specifically bind to certain sugar molecules.[12–15] Lectins are present ubiquitously in nature, that is in all living beings, from bacteria and viruses to higher animals, fish and plants.[16,17] Lectins are classified into different groups, on the basis of their binding and structural properties. They are C-type lectins, galectins, rhamnose binding lectins, fucose binding lectins, intelectins, lily-type lectins *etc.*, and among these, C-type lectins comprise the majority of the marine lectins.[18,19] Previously, the important roles and therapeutic potential of different types of fish lectins were described by Preetham *et al.*[20] Herein, Md-*Lec*, a C-type lectin purified from the haemolymph of the shrimp, *Metapenaeus dobsoni*, was evaluated for its antibacterial activity against common aquatic pathogens.[21] This study aims to

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analyse the complex formation ability of Md-*Lec* with pectin capped copper sulfide nanoparticles (pCuS NPs), and further to study the extent of the antibacterial activity of the complex *in vitro* and *in vivo*. The results indicate that the Md-*Lec*–pCuS NPs complex has a better or enhanced antibacterial activity than the pCuS NPs alone. *In vivo* testing revealed that Md-*Lec*–pCuS NPs are highly effective in reducing the bacterial load in the infected Nile tilapia. This study concludes that lectin-NPs conjugates can be considered as a useful alternative to antibiotics for the treatment of bacterial infection in aquatic animals.

2. Materials and methods

2.1 Purification of Md-*Lec*

The shrimp lectin, Md-*Lec* was previously purified by affinity chromatography using mannose-sepharose CL 4B[22] from the haemolymph of the Kadal shrimp, *Metapenaeus dobsoni*, by our group.[21] The mannose acted as a ligand to which the lectin in the haemolymph samples could bind, and later the lectin was eluted by passing through elution buffer (10 mM Tris HCl, 140 mM NaCl, 3 mM EDTA, pH 8.0). Then the eluted fractions were tested for purity and molecular mass by SDS-PAGE.

2.2 Interaction of CuS NPs with Md-*Lec*

The CuS NPs were prepared using the procedure reported in Subramaniyan *et al.*[23] Briefly, pectin (50 mg) and copper chloride (1 mM) were dissolved in 50 ml double distilled water. To this mixture, 200 μl ammonium hydroxide was added, which was followed by 400 μl hydrazine hydride. In 3 h, the solution color changed from blue to a wine red color, suggesting the formation of copper nanoparticles. The addition of 1 mM sodium sulphide to the copper nanoparticles changed the color from wine red to olive green in 3 h, suggesting the formation of pectin stabilized copper sulfide nanoparticles (pCuS NPs). The obtained pCuS NPs was purged with nitrogen gas for 15 min and then used in the study. The interaction between lectin and pCuS NPs was studied using a Jasco-FP8200 spectrophotometer. Typically, small aliquots of 1 mM pCuS NPs were added to 0.1 $OD_{280\ nm}$ of Md-*Lec* and allowed to equilibrate for 2 min. Then, the fluorescence emission spectra were measured with an excitation wavelength of 280 nm. The addition of NPs drastically affected lectin fluorescence. A blank experiment was performed by titrating Md-*Lec* with small aliquots of buffer. The addition of buffer did not affect the lectin fluorescence significantly.

2.3 Lectin activity assay

To find out whether there was any alteration in the activity of Md-*Lec* after complexing with the pCuS NPs, haemagglutination assay was performed according to the method described by Correia and Coelho[24] with slight modifications. Briefly, 50 μl of sample preparations of 50 μg ml^{-1} concentration were added to PBS before addition of 50 μl 2% (v/v) suspension of human erythrocytes in a 96 well plate. In the control, the sample was replaced by BSA.

2.4 Antibacterial studies

2.4.1 *In vitro* antibacterial activity by rezasurin microtitre assay (REMA). The minimum inhibitory concentration of pCuS NPs and the complex, Md-*Lec*–pCuS NPs against three aquatic pathogens, *Aeromonas hydrophila* (ATCC 7966), *Enterococcus fecalis* (ATCC 29212) and *Streptococcus iniae* (ATCC 29178) were determined by REMA method.[23,25] Briefly, 100 μl pCuS NPs (100 μM) was added to first well of the 96-well plate and then serially diluted. Next, each well was mixed with Md-*Lec* (25 μg ml^{-1}). To this mixture about 1×10^5 CFU ml^{-1} bacterial cells were seeded into each well and incubated at 37 °C with shaking for 24 h. The OD_{660} was monitored with a microtiter plate reader (Thermo Scientific multi-scan EX). To observe the cell viability, 30 μl of resazurin solution (0.01% w/v) was added to each well and incubated for 2 h at 37 °C.

2.4.2 Zone of inhibition assay (ZOI). The antibacterial efficacy of Md-*Lec*–pCuS NPs complex was further confirmed by the zone of inhibition (ZOI) assay.[26] Active bacterial cells were swabbed uniformly on LB agar plates using sterile cotton swabs. Sterile filter paper discs loaded with the samples on the agar and incubated at 37 °C for 24 h. After incubation, the zone size was measured with a ruler and reported.

2.4.3 Membrane integrity study. The outer membrane (OM) permeability of Gram-negative pathogens was determined by fluorescence probe uptake assay using 1-*N*-phenylnaphthyl-amine (NPN), whereas, the membrane integrity of Gram-positive pathogens was judged using propidium iodide (PI).[27] Briefly, cells grown at mid-log phase were treated with the NPs for 2 h. The treated cells were collected, washed twice with phosphate-buffered saline (PBS) buffer and then, exposed to 0.5 mM NPN or PI. After 2 h exposure, the fluorescence was measured with a JASCO spectrofluorometer FP-8200. The excitation wavelengths for NPN and PI were 350 and 543 nm, respectively. The emission wavelengths for NPN and PI were 420 and 600 nm, respectively.

2.4.4 Live/dead cell imaging. The extent of antibacterial activity exhibited by the Md-*Lec*–pCuS NPs complex was evaluated by dual staining using propidium iodide (PI) and acridine orange (AO).[28] Briefly, cells were allowed to grow to mid-log phase in the presence of Md-*Lec*–pCuS NPs complex and then the cells were collected and washed twice with PBS buffer and resuspended in an equal volume of the buffer. AO/PI at a concentration of 0.5 mM was added to the bacterial culture. Bacterial cells without Md-*Lec*–pCuS NPs complex were maintained as control. Fluorescence microscopy images were recorded using a NIKON Eclipse Fluorescence Microscope after 2 h of incubation . An FITC (green) filter was used for AO and a PI (red) filter was used for visualizing live and dead cells, respectively.

2.4.5 Lipid peroxidation assay. The malondialdehyde (MDA) generated during membrane damage of bacteria caused by reactive oxygen species was determined. Thiobarbituric acid (TBA) assay was used to determine the MDA in the culture media.[29] Briefly, 1 ml of the bacterial cultures treated with Md-*Lec*–pCuS NPs complex at their MIC was collected and mixed with 10% trichloroacetic acid. Then, 0.67% of TBA was added and incubated at 95 °C for 1 h. The reaction mixture was cooled

down to room temperature and then centrifuged at 6000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm and the percent of MDA production was determined. Untreated cells and cells treated with hydrogen peroxide served as negative and positive control, respectively.

2.5 *In vivo* antibacterial studies

All experiments were performed in compliance with the guidelines as prescribed by the Institutional Animal Ethics Committee (SOST/PhD001/2019) of Kerala University of Fisheries and Ocean Studies, India. Optimizations of bacterial dosage for establishment of infection were done previously.[40] Infection was done intramuscularly and treatment was given from the opposite side to that of the infection.[23] The *in vivo* bacterial infection and the treatment with Md-*Lec*–pCuS NPs ($1\times$ MIC) were performed in Nile tilapia that was divided into 3 groups; group I: bacteria infected control, group II: bacterial infected + treated with Md-*Lec*–pCuS NPs, group III: control, uninfected fish. Each group consisted of 10 fish. In a typical experiment, 10 µl of 0.1 OD_{660} culture of *A. hydrophila* was used for infection. The infection dose was optimized previously. The fish were fed normally and after 3 h of infection with bacteria, 10 µl of samples were injected as described above. The fish were monitored for mortality due to bacterial infection for a period of 24 h. At regular time points, (3 h, 6 h, 12 h) one fish from each group was removed, anesthetized and sacrificed. Approximately 100 mg of muscle tissue was dissected and homogenized in sterile PBS. The homogenate was serially diluted using sterile PBS and plated on sterile LB agar plates. The plates were incubated for 24 h at 37 °C. After 24 h, bacterial colonies were counted and reported. All of the experiments were performed in triplicate.

2.6 Statistical analysis

All experiments were performed in triplicate ($n = 3$). Data is presented as mean $\pm$ standard deviation (S.D.) of control and treated samples. The data were subjected to one way analysis of variance (ANOVA) and the significance of differences between mean were calculated by Tukey's HSD test and the significance accepted at $P < 0.05$.

3. Results and discussion

Being a lectin, Md-*Lec* has the ability to bind to the sugar moieties present on membrane surfaces.[21,30] Meanwhile, the pCuS nanoparticles were reported to have an antibacterial property.[31,32] Thus, it was assumed that the antibacterial properties of the Md-*Lec* and pCuS nanoparticles would be enhanced through conjugation.

3.1 Antimicrobial activity of Md-*Lec*

Infections caused by aquatic pathogens are the leading reason for the mortality of fish in the aquaculture industry.[1] Herein, we evaluated the antimicrobial activity of Md-*Lec* against the common fish pathogens, *A. hydrophila*, *E. fecalis* and *S. iniae*. Md-*Lec* was isolated from the haemolymph of *Metapenaeus*

dobsoni using affinity chromatography and their purity was judged by SDS-PAGE.[21]

3.2 Interaction of purified Md-*Lec* with CuS nanoparticles

Of late, nanoparticles have been widely considered for antimicrobial and drug delivery applications. The copper sulphide nanoparticles used in this study were prepared from the precursor copper chloride and sodium sulphide, where pectin was used as stabilizing agent. The morphology and size of the prepared NPs were determined from transmission electron microscopy (Fig. S1†). It is clear from Fig. S1,† the pCuS NPs are spherical in shape with size range from 3 to 14 nm, which is consistent with previous reports. Having observed the antibacterial activity of Md-*Lec*, we set out to evaluate the antibacterial activity of Md-*Lec* in combination with pCuS NPs. Therefore, the complex formation ability of pCuS NPs and Md-*Lec* was judged by fluorescence spectroscopy. The tryptophan emission spectra of Md-*Lec* were observed between 300–450 nm with an emission maximum at 342 nm (Fig. 1).[33,34] The titration of Md-*Lec* with pCuS NPs affected the fluorescence intensity, suggesting a possible interaction between Md-*Lec* and pCuS NPs. The intrinsic fluorescence of Md-*Lec* was quenched to 22.8% at 100 µM pCuS NPs, which could be attributed to the energy transfer between Md-*Lec* and pCuS NPs. The fluorescence quenching data was analyzed by Stern–Volmer plot, which gives the information about the mechanism of quenching [Fig. S2†].

$$F_0/F_c = 1 + K_{sv}[\text{pCuS NPs}] = 1 + K_q\tau_0[\text{pCuS NPs}] \quad (1)$$

$$K_q = K_{sv}/\tau_0 \quad (2)$$

where F_0 and F_c are the relative fluorescence intensities of Md-*Lec* at 342 nm in the absence and presence of pCuS NPs, respectively, K_{sv} is the Stern–Volmer fluorescence quenching constant, K_q is the biomolecular quenching constant, and τ_0 is the average fluorescence lifetime of lectin (10^{-9} s). The Stern–Volmer plot shows good linearity with respect to increasing concentration of pCuS NPs with a K_{sv} value of 3.37 (±0.08) $\times 10^3$ M^{-1}. The values of K_{sv} were well comparable with those for the interaction of lectins with nanoparticles.[41] The K_q value calculated from K_{sv} was in the order of 10^{12} L mol^{-1}, which is higher the maximum value (10^{10} L mol^{-1}) obtained for any diffusion controlled quenching process. The higher K_q value obtained in this study is attributed to a static quenching mechanism and a non-fluorescent complex is likely formed between Md-*Lec* and pCuS NPs. Further, the binding affinity (K_b) was determined by analyzing the fluorescence quenching data by modified Stern–Volmer equation.[42] The K_b was found be in the order of 10^3 M^{-1}, which is comparable to that for the binding of lectins with hydrophobic ligands and plant growth hormones, such as porphyrin, 2,6-toluidinylnapthalenesulphonic acid, adenine, auxins and cytokinins ($K_b = 10^3$ to 10^6 M^{-1}).[43]

3.3 Lectin activity assay

The sugar binding site of Md-*Lec* is important for recognizing bacterial cell surface glycans. Therefore, the sugar binding
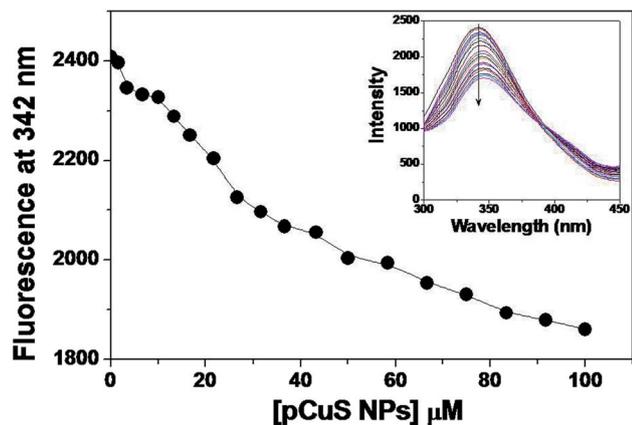
Fig. 1 Binding study. Titration. The addition of pCuS to Md-Lec quenches the intrinsic fluorescence as evidenced by the decrease in the fluorescence at 342 nm. Excitation wavelength = 280 nm. Inset shows the emission spectra obtained from the titration. The arrow indicates the quenching with respect to increasing concentration (0–100 mm) of pCuS NPs.

activity of Md-Lec complexed with pCuS NPs was evaluated by haemagglutintion assay. In the control well, a negative result was observed as button-like formation at the bottom due to the settling of erythrocytes. In the wells containing Md-Lec alone and Md-Lec–pCuS NPs, a positive result was observed as a fluffy cotton-like formation at the bottom due to clumping of the erythrocytes (Fig. 2). The formation of clumps or aggregates (haemagglutination) was caused by the Md-Lec as it can bind to the carbohydrate molecules present on the cell surfaces of the erythrocytes. Even after conjugating with the nanoparticles, the Md-Lec could retain its haemagglutination activity and it was obvious that the carbohydrate binding site of lectin was conserved. This also reveals that the immunological activity of lectin is retained in the Md-Lec–pCuS NPs complex. As this property is retained, this lectin-nanoconjugate can be used for specific identification and binding to pathogens or tumour cells.[35]

3.4 Antibacterial activity of the Md-Lec–pCuS NPs complex

Having learnt that the complex formation between Md-Lec and pCuS NPs could occur without affecting the cell surface sugar
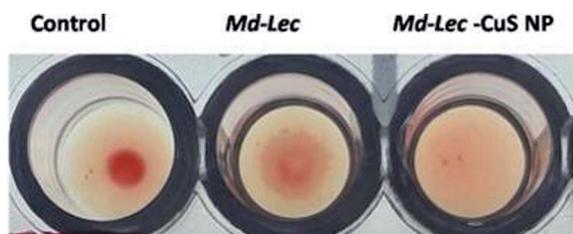

Fig. 2 Lectin activity assay. Negative result is observed as a button-like formation and the positive result is observed as a fluffy cotton-like formation at the bottom. C – control, i – Md-Lec (50 µg ml⁻¹), ii – Md-Lec–pCuS NPs (25 µg ml⁻¹), respectively.
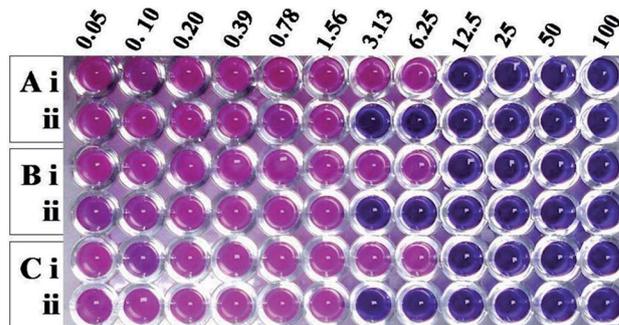

Fig. 3 Antibacterial activity of (i) pCuS NPs and (ii) the complex, Md-Lec–pCuS NPs. 25 µg ml⁻¹ Md-Lec was used in all the wells, which corresponds to 0.5× MIC of Md-Lec. (A) A. hydrophila, (B) E. fecalis and (C) S. iniae.

recognition site, we expanded the investigation of Md-Lec–pCuS NPs complex against A. hydrophila, E. fecalis and S. iniae. Fig. 4 shows the REMA of pCuS NPs with and without Md-Lec. In REMA, the respiring viable cells reduce resazurin to resorufin and appear pink in color, whereas the cells lacking respiration appear blue in color.[23] It is noted from Fig. 3, the MIC of pCuS NPs is 12.5 µM against the tested pathogens as they appear blue in colour, suggesting that the cells are inactive; below that, the cells are able to respire and convert the resazurin to resorufin. Strikingly, the MIC of pCuS NPs in the presence of 25 µg ml⁻¹ Md-Lec was drastically reduced from 12.5 µM to 3.13 µM. The four fold reduction in the MIC of pCuS was attributed to Md-Lec functionalization, because Md-Lec has the ability to bind to the bacterial cell surface, as indicated by the bacterial agglutination study.

3.5 Zone of inhibition assay (ZOI)

The ability of the test samples to inhibit the growth of bacteria was evident from the zone of inhibition (ZOI) assay. In zone of inhibition assay, filter paper discs loaded with Md-Lec, pCuS NPs and Md-Lec–pCuS NPs were used against A. hydrophila, E. fecalis and S. iniae. The test sample diffuses out from the filter paper discs into the media, such that the bacteria susceptible to the test sample are inhibited from growing around the disc and the bacteria which are more susceptible will exhibit a wider zone of clearance. From Fig. 4, it is evident that the ZOI was smaller for lectin and nanoparticles. Interestingly, the ZOI was largest around the filter paper disc loaded with Md-Lec–pCuS NPs (25 µg ml⁻¹) confirming the intensified antibacterial activity of Md-Lec when conjugated with pCuS NPs. However, the zone size differs with respect to the tested bacterium, which can be attributed to the difference in the bacterial membrane composition.

3.6 Effect of Md-Lec–pCuS NPs complex on membrane integrity

The bacterial membrane serves as a first line of defense for the bacteria's survival. The effect of Md-Lec–pCuS NPs complex was investigated using the fluorescence probe, NPN for A. hydrophila
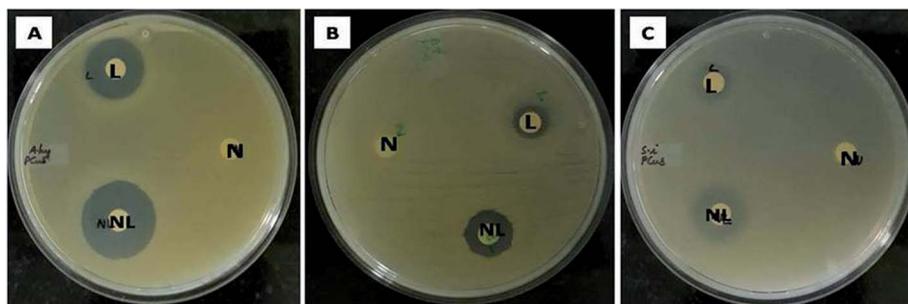
Fig. 4 Evaluation of zone of inhibition of Md-*Lec*–pCuS NPs against (A) *A. hydrophila*, (B) *E. fecalis* and (C) *S. iniae*. L – 50 μg ml^{-1} Md-*Lec*, N – 3.13 μm pCuS NPs, NL – Md-*Lec*–pCuS NPs, which is composed of 50 μg ml^{-1} Md-*Lec* and 3.13 μm pCuS NPs. It is noted the larger zone was observed in NL, suggesting that the antibacterial activity of Md-*Lec* is enhanced when combined with pCuS nanoparticles.

and *E. fecalis*, and PI for *S. iniae*. These probes enter into cells with compromised membranes, showing strong fluorescence. The bacterial cells were treated with 0.5× and 1× MIC for 2 h, and then stained with the fluorescent probe. After staining, the fluorescence of the cells was measured and data is shown in Fig. 5. The untreated cells were used as negative control, 3.13 μM CTAB, a membrane damaging agent, was used as positive control for Gram negative bacteria, and 3.13 μM Triton X 100

was used as positive control for Gram positive bacteria. It is noted from Fig. 5A, the cells treated at 1× MIC show strong fluorescence, as good as the positive control, suggesting the loss of membrane integrity. The extent of membrane damage by Md-*Lec*–pCuS NPs was further analyzed by scanning electron microscopy. The SEM of *S. iniae* was used to represent the membrane damaging activity of Md-*Lec*–pCuS NPs. Fig. 5B and C show that the untreated cells and the cells treated at 0.5× MIC
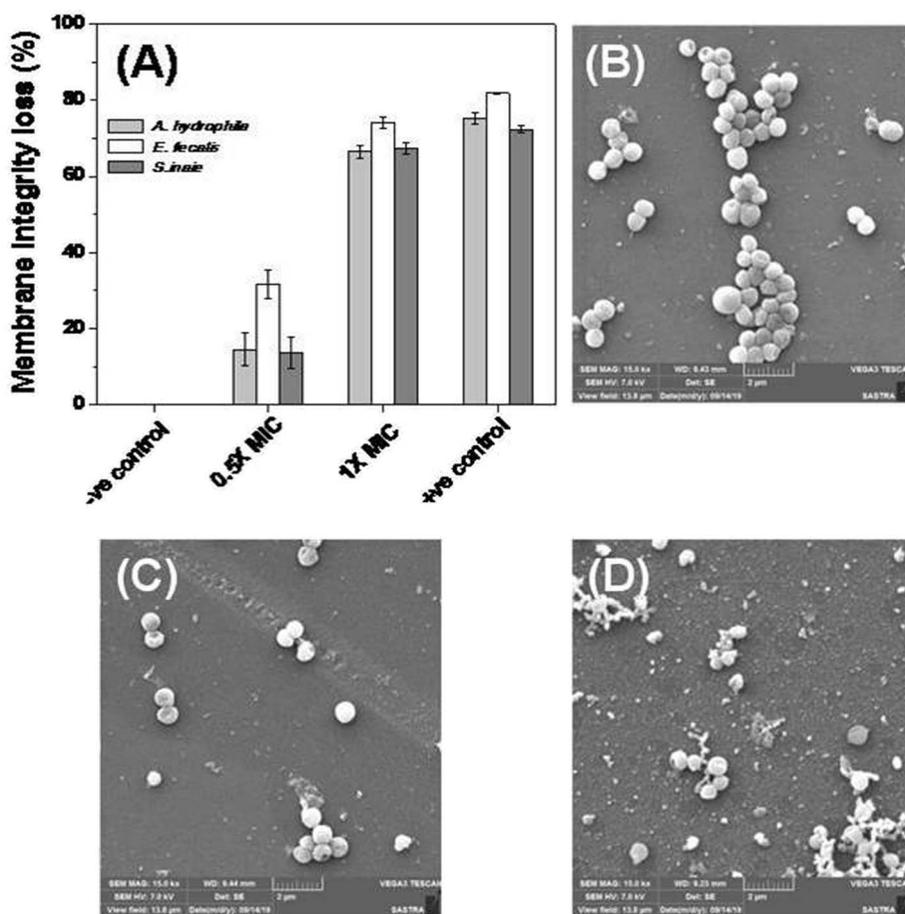

Fig. 5 Membrane integrity study. (A) Loss of membrane integrity of treated cells evaluated by fluorescence probe. (B) Representative scanning electron microscopy image of untreated *S. iniae*, (C) treated with 0.5× MIC and (D) treated with 1× MIC of Md-*Lec*–pCuS NPs.

of Md-*Lec*–pCuS NPs are spherical in morphology with intact cell wall. Interestingly, the cells treated at 1× MIC of Md-*Lec*–pCuS NPs exhibited cell wall damage, as evident from the damaged and shrunken cells (Fig. 5D). The maximum damage

to membrane at 1× MIC was considered as an important mechanism for cell death induced by Md-*Lec*–pCuS NPs.

Further evidence for Md-*Lec*–pCuS NPs induced cell death was analyzed by live and dead staining using fluorescence microscopy. In this study, acridine orange (AO), and propidium iodide (PI) were used as a dual probe. AO has the ability to cross the intact membrane and stains the nucleic acid to give green fluorescence from live cells, whereas PI can only cross the compromised membrane and shows red fluorescence from dead cells. The fish specific pathogens were treated with 1× MIC of Md-*Lec*–pCuS NPs for 2 h and stained with the dual probe, AO and PI. Fig. 6 shows the fluorescence microscopy image of untreated cells and cells treated with 1× MIC Md-*Lec*–pCuS NPs. The green fluorescent cells observed in Fig. 6A suggest the cells are stained only with AO, thus, these cells are considered as live cells. Whereas the cells treated with 1× MIC show red fluorescence. Similarly, the cells treated with membrane damaging agent, Triton X 100 also show red fluorescence. Of particular note, the treated cells visualized with a green filter showed no green cells, suggesting all the cells were dead in the treated group. This result suggests that Md-*Lec*–pCuS NPs damaged the bacterial membrane, which resulted in cell death, supporting the result obtained from the membrane integrity study.
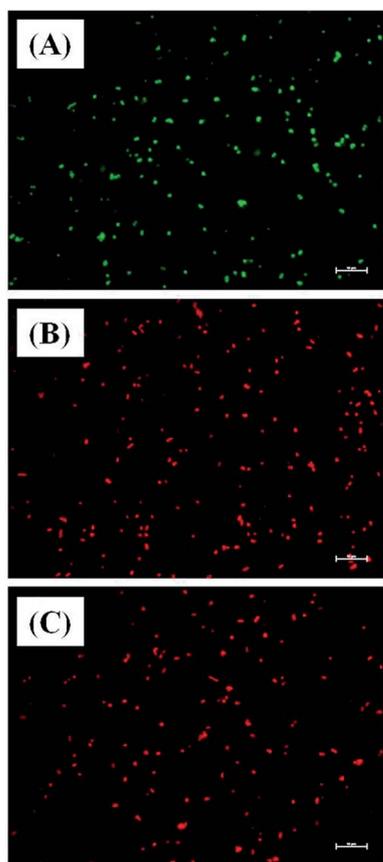
3.7 Lipid peroxidation assay

The lipid peroxidation was estimated for evaluating the oxidative damage caused to the bacterial membrane. The reactive oxygen species produced leads to the formation of lipid peroxide radical which is then converted into malondialdehyde. The formation of the lipid peroxidation product was estimated by thiobarbituric acid reactive substances assay (TBARS assay). It is noted from Fig. 7, that the LPO production was high when the lectin was conjugated with nanoparticles. The increased production of malondialdehyde as a result of membrane damage caused by reactive oxygen species confirmed the enhanced antibacterial activity of the lectin conjugated nanoparticles.[36–38] Noteworthy was that the activity of the conjugate is different against the different bacteria tested (Fig. 7). pCuS NPs conjugated with lectin (25 μg ml^{-1}) exhibited higher LPO

Fig. 6 Representative live and dead staining of *S. iniae*. (A) Untreated *S. iniae*, (B) *S. iniae* treated with 3.13 μm Md-*Lec*–pCuS NPs and (C) *S. iniae* treated with 3.13 μm Triton X 100. The cells are stained with AO and PI. Live cells show green fluorescence and dead cells shows red fluorescence. Scale bar: 10 μm.
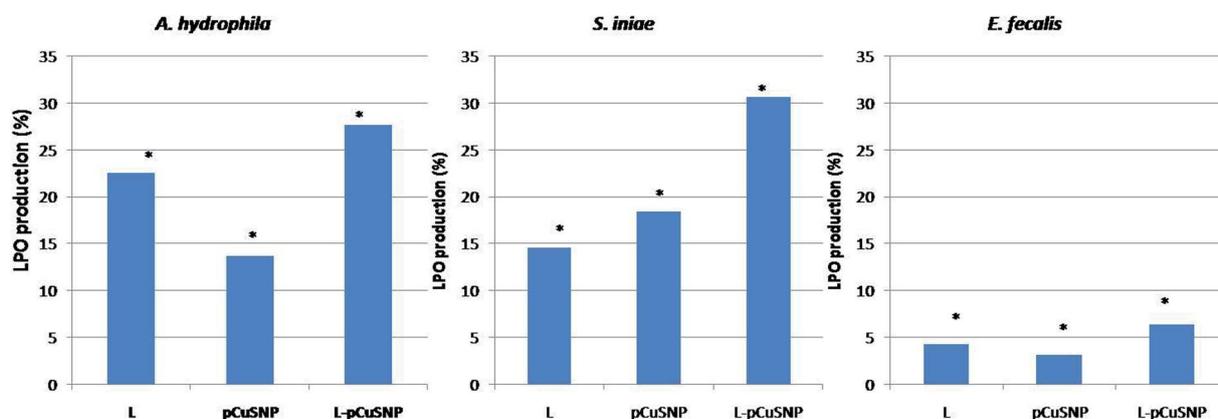

Fig. 7 Evaluation of lipid peroxidase production of Md-*Lec* conjugated with pCuS NPs against (i) *Aeromonas hydrophila* (ii) *S. iniae* and (iii) *E. fecalis*. The lipid peroxidase production of Md-*Lec* is enhanced when combined with CuS NPs and is more active against *S. iniae*.
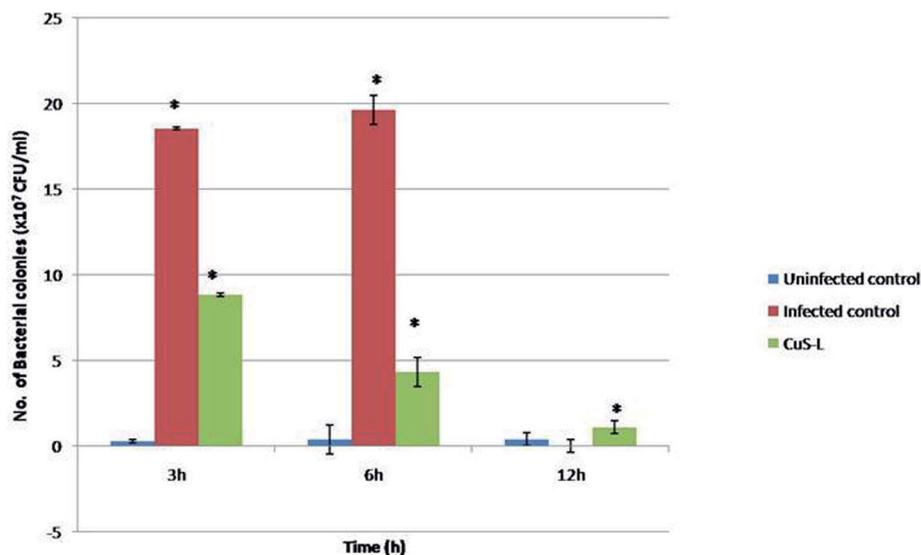
Fig. 8 *In vivo* assay for antibacterial activity. In the fish treated with Md-*Lec*–pCuS NPs, the number of viable bacterial cells present is reported to be declining with increasing time.

activity against *S. iniae* than *A. hydrophila* and comparatively lesser activity against *E. fecalis*. The difference in the LPO activity can be attributed to the differential membrane composition of the tested bacterium. These results go in parallel with the LPO activity reported earlier.[23,33]

3.8 *In vivo* antibacterial studies

The *in vivo* antibacterial studies show that the pathogenic bacterial load was decreased drastically when they were injected with Md-*Lec*–pCuS NPs (Fig. 8). Each treatment group consisted of 10 fish each and all of them, except the last group that served as uninfected control, were intramuscularly infected with a dose of 10 μl of 0.1 OD_{660} cultures of *A. hydrophila* which was optimized previously. After 3 h of infection, the second group was injected with 10 μl of Md-*Lec*–pCuS NPs (3.13 μM). The fish in the infected control group died within 12 h while the fish in the other two groups survived. The bacterial load in the muscle tissue of the fish was analyzed at different time points by sacrificing each one of them. The muscle tissue (100 mg) was homogenized and serially diluted, and then plated in LB agar plates (10^{-4} dilution) and incubated (25 °C for 24 h) to obtain a viable bacterial count. The infected control group showed the highest number of colonies at 3 h which increased at 6 h and finally all of the fish in the group died by 12 h. Meanwhile the treated group showed a rapid decline in the viable bacterial count with the progression of time. This study suggests that treatment of fish with Md-*Lec*–pCuS NPs could inhibit the proliferation of infectious bacteria, thus preventing the fish from suffering and spreading the disease.[31,39]

4. Conclusion

In the present study, the antibacterial activity of the complex formed between Md-*Lec* and pCuS NPs was reported by REMA,

and zone of inhibition assay. The mechanism of action was judged by lipid peroxidase assay, and membrane integrity study. The *in vivo* efficacy of the Md-*Lec*–pCuS NPs complex was demonstrated through treating Nile tilapia infected with bacteria. The results revealed that the nanoparticles and lectin, when administered individually, are proved to have a limited activity, while their combination is reported to have a very high antibacterial activity. This sheds light on the possibilities of these combinations to be used as promising therapeutic agents after further proper investigations.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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