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Isolation of a small molecule with anti-MRSA activity from a mangrove symbiont *Streptomyces* sp. PVRK-1 and its biomedical studies in Zebrafish embryos

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ABSTRACT

Objective: The aim of the present study was to isolate the anti-MRSA (Methicillin Resistant *Staphylococcus aureus*) molecule from the Mangrove symbiont *Streptomyces* and its biomedical studies in Zebrafish embryos. **Methods:** MRSA was isolated from the pus samples of Colachal hospitals and confirmed by amplification of *mecA* gene. Anti-MRSA molecule producing strain was identified by 16s rRNA gene sequencing. Anti-MRSA compound production was optimized by Solid State Fermentation (SSF) and the purification of the active molecule was carried out by TLC and RP-HPLC. The inhibitory concentration and LC_{50} were calculated using Statistical software SPSS. The Biomedical studies including the cardiac assay and organ toxicity assessment were carried out in Zebrafish. **Results:** The bioactive anti-MRSA small molecule A_2 was purified by TLC with Rf value of 0.37 with 1.389 retention time at RP-HPLC. The Inhibitory Concentration of the purified molecule A_2 was 30 μg/mL but, the inhibitory concentration of the MRSA in the infected embryo was 32-34 μg/mL for TLC purified molecule A_2 with LC_{50} mean value was 61.504 μg/mL. Zebrafish toxicity was assessed in 48-60 μg/mL by observing the physiological deformities and the heart beat rates (HBR) of embryos for anti MRSA molecule showed the mean of 41.33-41.67 HBR/15 seconds for 40 μg/mL and control was 42.33-42.67 for 15 seconds which significantly showed that the anti-MRSA molecule A_2 did not affected the HBR. **Conclusions:** Anti-MRSA molecule from *Streptomyces* sp PVRK-1 was isolated and biomedical studies in Zebrafish model assessed that the molecule was non toxic at the minimal inhibitory concentration of MRSA.

1. Introduction

The multi-drug resistant phenotype is a particular characteristic of the methicillin-resistant *Staphylococcus aureus* (MRSA) strains[1,2]. MRSA is a major pathogen associated with both nosocomial- and community-acquired infections (hospital-acquired [HA] MRSA and CA-MRSA, respectively)[3]. The homogeneous insusceptibility to all betalactams, characteristic of methicillin-resistant strains, together with the continuous accumulation and organization of many resistance genes, has made this species particularly difficult to treat. This antibiotic resistance is common for many classes of antibiotics such as aminoglycosides, macrolides, lincosamides, and fluoroquinolones[4]. In recent years, the widespread use of antibiotics has undoubtedly accelerated the evolution of *Staphylococcus aureus* (*S. aureus*), and led to the emergence of strains that have systematically acquired multiple resistance genes[5]. With the current emergence of multi-drug resistant isolates in hospitals on the one hand[1] and the dramatically increased incidence of hyper-virulent community-associated MRSA on the other[6,7], MRSA that has been able to evolve rapidly and create new clinical problems poses a substantial threat for the hospital environment, resulting in nosocomial infections[8]. The majority of MRSA strains have been associated with hospital-acquired colonization and infections[9]. 59.5% of *Staphylococcus aureus* strains causing infections in hospitals were MRSA, which is not only true for Europe and United States but for India as

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well. It was reported that vancomycin resistant *S. aureus* (VRSA) were severely found in the northern India with severe infections [10]. Natural products are potential new chemical structures possessing antimicrobial activity and it was reported that three of every four antibacterial agents are related in some way to natural products[11], and a majority of these discoveries have come from filamentous bacteria of the order *Actinomycetales* (actinomycetes). Actinomycetes are widely distributed in the natural environment and play an important role in the degradation of organic matter and production of bioactive compounds and bioactive molecules[12]. More than 9000 biologically active molecules have been isolated from actinomycetes, yielding more than 60 pharmaceutical agents used in the medical or agricultural fields[11]. It was also reported that in recent years the *S. aureus* was highly resistant to conventional and traditional antibiotic due the modification in the molecular physiology of the pathogens in clinical isolates in India[13]. Hence the search of novel therapeutic small molecule for drug resistant *S. aureus* is urgent. As part of our small molecule screening from mangroves we have identified an anti MRSA producing *Streptomyces* sp. PVRK-1. The strain was identified by 16S rRNA gene sequencing. The anti-MRSA molecule was purified by TLC and RP-HPLC. There are several experimental infection models have been set up to study human pathogens[14]. In the present study Zebrafish model have been used for toxicity evaluation of pharmaceutical agents[15,16]. Based on the above informations in this research work, we have identified a novel anti-MRSA producing mangrove symbiont *Streptomyces* sp. PVRK-1 and its biomedical studies were carried out in the Zebrafish embryos.

2. Materials and methods

2.1. Isolation and identification of microorganism

S. aureus was obtained from hand-swab samples of hospitals in Colachal, by personnel. The molecular characterization of *mecA* gene in the MRSA was already published in the laboratory[17] and the strain was used in the present study. The *Streptomyces* sp. PVRK-1 was collected from the Rhizosphere soil of *Rhizophora mucronata* (*R. mucronata*), Manakkudy estuary of Arabian Sea, Tamil Nadu, India (8 ° 6′ 12″ N 77 ° 28′ 57″ E) at 6 feet depth. Soil samples were serially diluted in sterile water and spread plated over the medium containing soluble starch 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, $FeSO_4$ 20 μM, agar 15 g, seawater from mangrove habitat 1 L, and 15 μg nalidixic acid were added to inhibit the growth of other bacteria and incubated at 28 ℃ for 3 days. The molecular identification of the *Streptomyces* sp. PVRK-1 was carried out based on the work in the laboratory[18] and submitted in the NCBI genebank (GenBank: FJ573228.1).

2.2. Antimicrobial assay

The isolated *Streptomyces* sp. PVRK-1 was further grown on the medium with starch 20 g, tryptone 5 g, yeast extract 5 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, distilled water 1 L. Antagonistic activity of the isolated strain *Streptomyces* sp. PVRK-1 was performed by double layer agar method [19]. The antibiotic production was optimized by Solid State Fermentation (SSF) supplemented with various nutritional sources and physical parameters by agar overlay method.

2.3. Culture conditions production and purification

A 100 mL flask containing 25 mL of the seed culture NDYE medium (Nitrate Defined Yeast Extract) was inoculated with *Streptomyces* sp. PVRK-1 at 30 ℃ for 3 days on a rotary shaker incubator for 140 rpm. The seed culture was transferred into 5 L conical flask containing 2.5 L of modified fermentation medium [20]. The fermentation medium consist of glucose 25 g, yeast extract 10 g, tryptone-100 12.5 g, $Fe_2(SO_4)_3 \cdot 4\ H_2O$ 20 μg, KBr 25 μg with pH-7. The temperature for antibiotic production in SSF was optimized at 37 ℃. The fermentation was carried out at 7 days with shaking at 180 rpm. After 7 days, the culture broth was centrifuged at 2000 rpm for 10 min at 4 ℃ and filtered using 0.2 μm membrane filters (Himedia). The cell free supernatant was added with 250 mL of hexane, chloroform, acetone and methanol, successively shaken well in a separation funnel for extraction. Thin layer chromatography was employed to separate the compounds present in the crude methanolic extracts. 10 μg of crude methanol extract was spotted on the TLC plate with the mobile phase methanol/n-butanol /acetic acid/water = 5:3:1:1. The spots were visualized by keeping the plates in Iodine chamber[21]. 250 mg of the TLC scrapped spots were dissolved in 1000 μL of methanol, vortexed for 5 min and centrifuged at 8000 rpm for 5 min. The supernatant was concentrated in the vaccuum concentrator 5301 (Eppendorf) at 30 ℃ for 4-6 hours. The absorbance microtitre of the TLC resolved spots of methanolic extract of *Streptomyces* sp. PVRK-1 were determined spectrophotometrically (Shimadzu). 1 mg of concentrated A_2 resolved in TLC was dissolved in 1 mL of HPLC mobile phase [Methanol: double distilled water (3:2)] and centrifuged at 8000 rpm for 5 min. 100 μL was made up to 1 mL with the HPLC mobile phase and the absorbance was scanned using UV-Vis spectrophotometer (Shimadzu). The HPLC mobile phase was used as blank. 10^{-1} dilution was prepared and 25 μL was injected in the HPLC (Shimadzu) system using reverse-phase C18 with an isocratic elution.

2.4. Minimal inhibitory concentration

TLC purified molecule A_2 was assayed to determine the MIC of the compound against MRSA by the microtiter broth dilution method[22]. Dilutions of the test compound dissolved in 1% DMSO were added to each well of the 96 well microtiter plate containing fixed volume of Nutrient agar broth. Each well was inoculated with bacteria (10^5 CFU mL^{-1}) and incubated at 37 ℃ for 24 h. The MIC was calculated at which no growth of bacteria was observed.

2.5. Breeding and maintenance of Zebrafish embryos

Zebrafishes were maintained in 30 L tanks at 28 ℃ with 14 h: 10 h light/dark cycle in Fish Culture Facility of International Centre for Nanobiotechnology (ICN), Centre for Marine Science and Technology. Following successful breeding, eggs fell through the mesh, and were subsequently collected from the bottom of tanks. Zebrafish were maintained according to Westerfield 1989[23]. Zebrafish embryos are raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$ and 0.16 mM $MgSO_4$).

2.6. Organogenesis effect of anti MRSA molecule A_2 in Zebrafish embryos

1 μL of the pathogenic culture MRSA were treated in the 2 dpf (days post fertilization) embryos in 48 well microtitre plates. 1 μL of the culture was inoculated in the wells of the titre plates and incubated for 24 hours to monitor pathological effects in the sterile laminar flow chamber. The TLC purified small molecule A_2 along with bacterial culture were studied for its pathological effect. Compounds were added to the wells in 1% DMSO vehicle and the DMSO alone was used as control. Heart Beat Rate (HBR) were studied in the developing embryo from 3-5 dpf for the anti MRSA molecule A_2. The embryos were anesthetized by 1% tricaine (Sigma) and the heart beat rates were recorded in digital camera (Canon Ixus) in the Light microscope (Motic). The heart beat rates were counted by Windows moviemaker. Anti MRSA molecule was tested at 10 - 100 μg/mL, and the highest, nonlethal concentration was reported. The LC_{50} values of anti MRSA molecule was calculated by probit analysis using SPSS software.

3. Results

3.1. Detection of mecA gene by PCR amplification

The *mecA* gene which is responsible for methicillin resistance in *S. aureus* confirmed the presence of MRSA in Colachal hospitals[17] and was the first report in Kanyakumari District, Tamilnadu, India. This confirmation of MRSA insisted to identify an antibiotic from the MRSA from the mangroves associated symbiont in Kanyakumari District and already anti MRSA molecule from *Rhizophora mucronata* Lamk was reported[17]. In present study we have screened around 60 *Streptomyces* for antimicrobial properties and 20 bioactive compound producing microbes were deposited in genebank (Figure 1)[18].

3.2. Determination of inhibitory concentration and production

The optimum production was standardized in the Solid State fermentation and analyzed for the activity by double layer agar method[22] (Figure 2-6). After supplementing the essential nutrients in the fermentation medium for anti MRSA compound production, the compound was extracted and the activity was found only in the methanolic extract. 600 mg of crude anti MRSA compound was obtained in the 2.5 L fermentation medium of *Streptomyces* sp. PVRK-1. UV-Vis spectrophotometric analysis was carried out to find the wavelength to be set in HPLC analysis and the absorption maximum of the active spot A_2 was 270 nm. The HPLC analysis showed that the retention time of TLC resolved molecule A_2 was 1.389 min. The TLC pattern of methanolic extract of *Streptomyces* sp. PVRK-1 was shown in Figure 7. The chromatogram of A_2 HPLC is shown in the Figure 8. The growth of the MRSA was entirely inhibited in 32-34 μg/mL of MRSA culture broth and 30 μg/mL was considered as the inhibitory concentration of the purified molecule A_2 in broth dilution method in which no bacterial growth was observed. The 30 μg/mL of methicillin as the inhibitory concentration of MSSA (Methicillin susceptible *Staphylococcus aureus*) was reported[17].

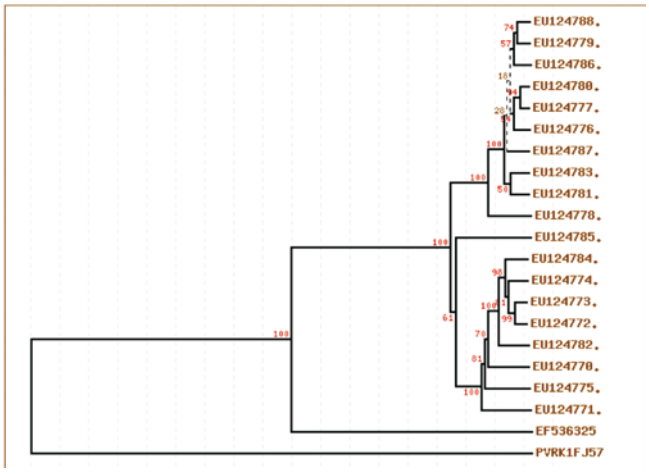

Figure 1. Phylogeny analysis of *Streptomyces* PVRK-1 with the other mangrove symbionts *Streptomyces* isolated from the Manakkudy mangrove.
EU124788.1 *Streptomyces* sp., EU124787.1 *Streptomyces* sp., EU124786.1 *Streptomyces roseus*, EU124785.1 *Streptomyces* sp., EU124784.1 *Streptomyces griseoruber*, EU124783.1 *Streptomyces bikiniensis*, EU124782.1 *Streptomyces* sp., EU124781.1 *Streptomyces showdoensis*, EU124780.1 *Streptomyces* sp., EU124779.1 *Streptomyces venezuelae*, EU124778.1 *Streptomyces* sp., EU124777.1 *Streptomyces roseoviridis*, EU124776.1 *Streptomyces* sp., EU124775.1 *Streptomyces caelestis*, EU124774.1 *Streptomyces caelestis*, EU124773.1 *Streptomyces caelestis*, EU124772.1 *Streptomyces caelestis*, EU124771.1 *Streptomyces olivoverticillatus*, EU124770.1 *Streptomyces olivoverticillatus*, EF536325.1 *Streptomyces* sp., FJ573228.1 *Streptomyces* sp. PVRK-1

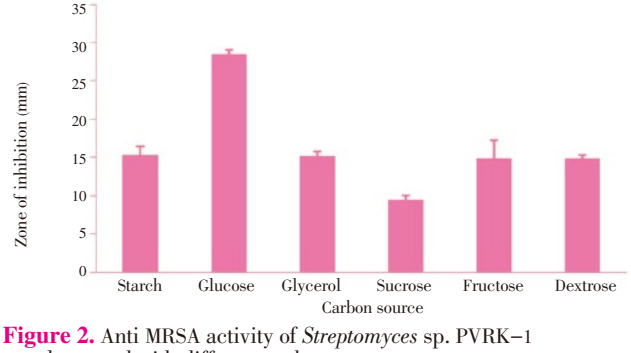

Figure 2. Anti MRSA activity of *Streptomyces* sp. PVRK-1 supplemented with different carbon sources.

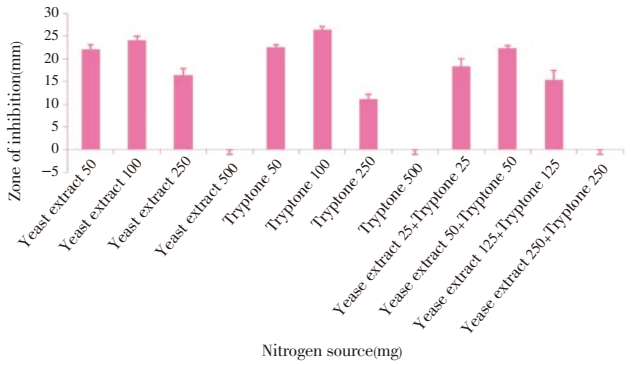

Figure 3. Anti MRSA activity of *Streptomyces* sp. PVRK-1 supplemented with different Nitrogen sources.

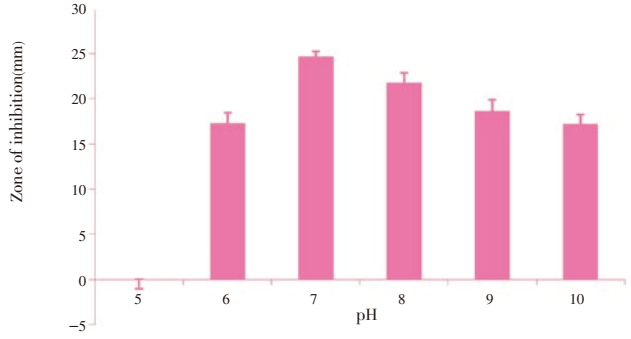

Figure 4. Anti MRSA activity of *Streptomyces* sp. PVRK-1 supplemented with different pH.

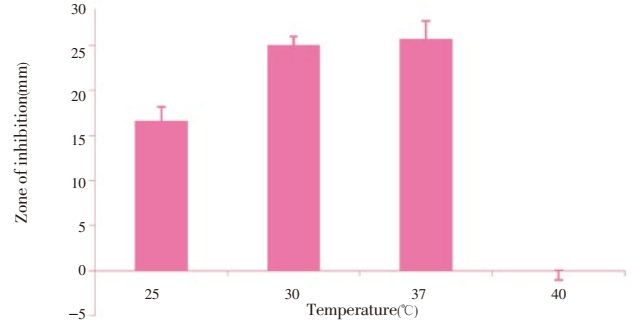

Figure 5. Anti MRSA activity of *Streptomyces* sp. PVRK-1 supplemented with different temperature.

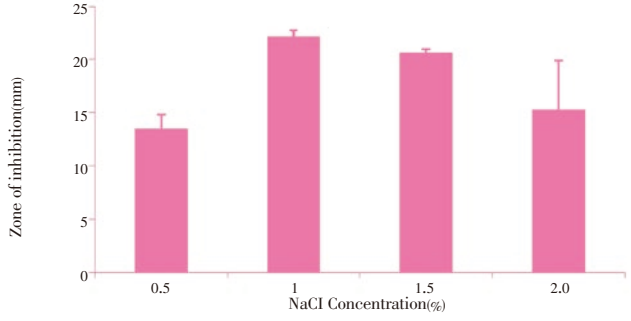

Figure 6. Anti MRSA activity of *Streptomyces* sp. PVRK-1 supplemented with different concentration of NaCl.

3.3. Compound toxicity assessment in Zebrafish embryos

The phenotypic deformities observed in 48-60 μg/mL of the molecule A_2 in 3 dpf of the developing embryo, showing the muscular deformities, pericardial edema, trunk flexure, protruded mouth and bulging (Figure 9). In the cardiac assay, Zebrafish embryos were exposed to compounds for 3 h before measurement of the Heart Beat Rates (HBR). HBR were recorded and analyzed in 3-5 dpf embryos for 40 μg/mL of anti MRSA molecule, the HBR value ranging from 41.33-41.67 HBR for 15 seconds in the molecule treated fish was similar 42.33-42.67 to control (Figure 10). There was no notable differences found in the HBR assay. The LC50 mean value of the purified small molecule A_2 is 61.504 μg/mL. The inhibitory concentration of the MRSA in the embryo was 32-34 μg/mL for the purified molecule in which the toxicity was not observed and MRSA was inhibited. All developmental deformities and the physiological effects were shown in Figure 9 A-K. The purified molecule A_2 as a potential antibiotic for methicillin resistance and the molecule was stable with less toxic effects in the Zebrafish embryos, was found in 48-60 μg and the inhibition of the MRSA in the infected embryos was in 32-34 μg/mL.

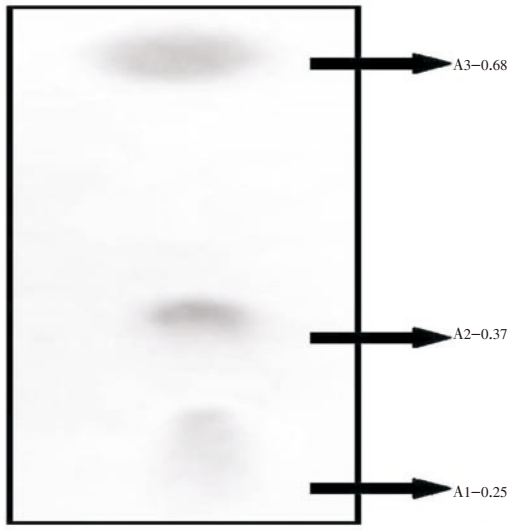

Figure 7. TLC analysis of methanol extract of anti MRSA molecule A_2

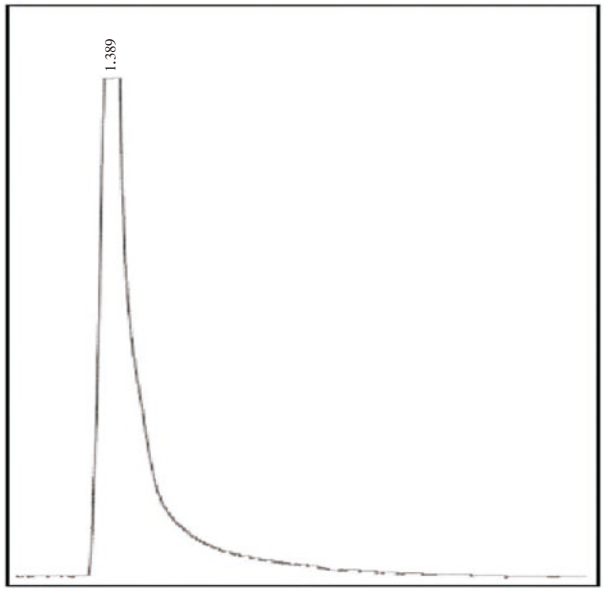

Figure 8. RP-HPLC analysis of TLC eluted anti MRSA molecule A_2.

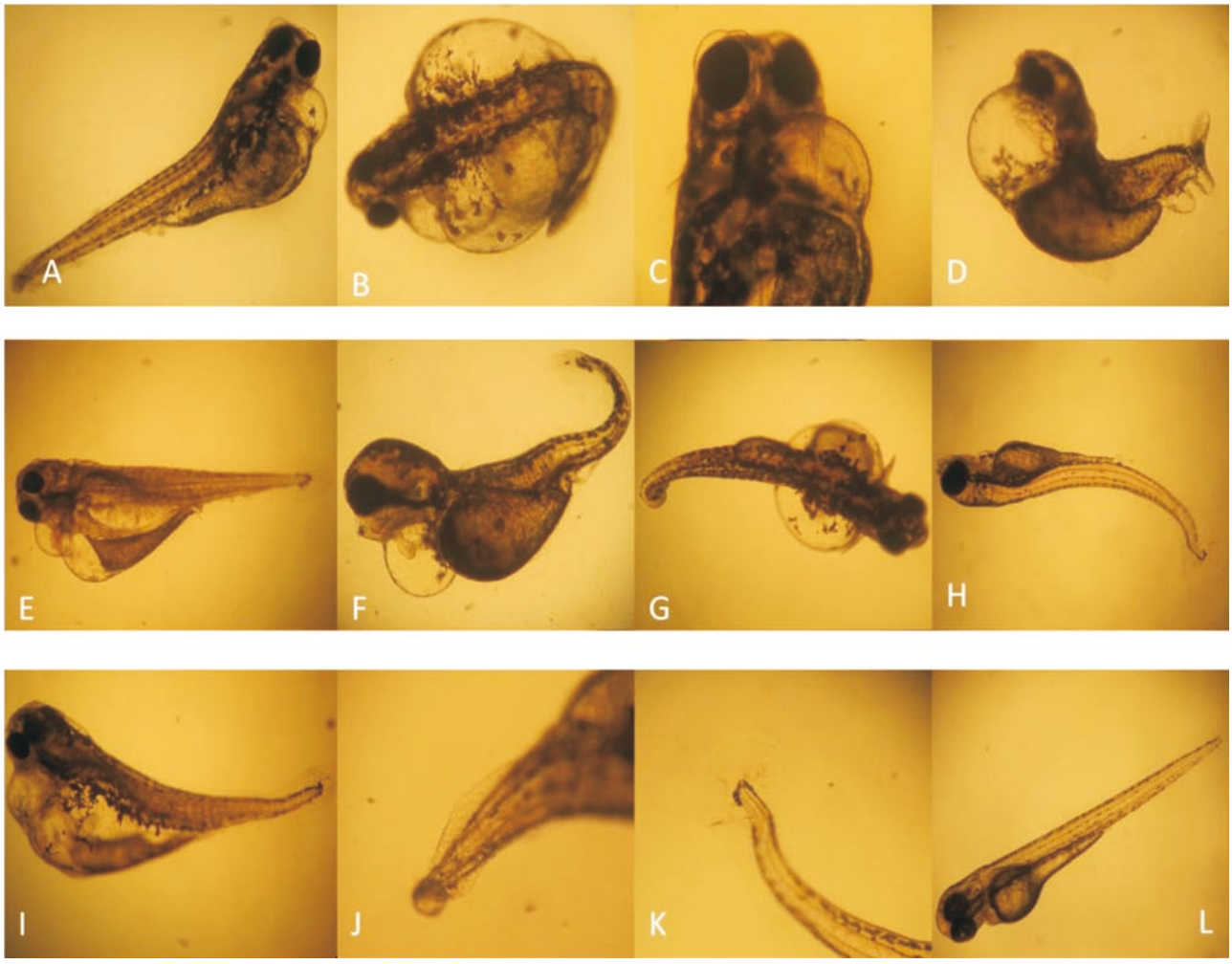

Figure 9. Organogenesis effect and compound toxicity in Zebrafish embryos.
A, B, C: Pericardial bulging, protruded mouth and abnormal eyes at 35-40 μg/mL in the 3dpf embryos; D: Pericardial bulging,Eye damage, trunk flexure, spinal cord flexure, tail damage 40-45 μg/mL at 3dpf; E, F: Pericardial bulging, gut deformities and trunk flexure; G, H, I: Damage in the cardiac, trunk and tail (45-50 μg/ml at 3dpf); J, K: Tail and caudal fin deformities at (40-50 -3 μg/ml in 3dpf embryos; L: control Zebrafish at 3dpf. (has mutation and small was observed in A, B, C, D, E & F)

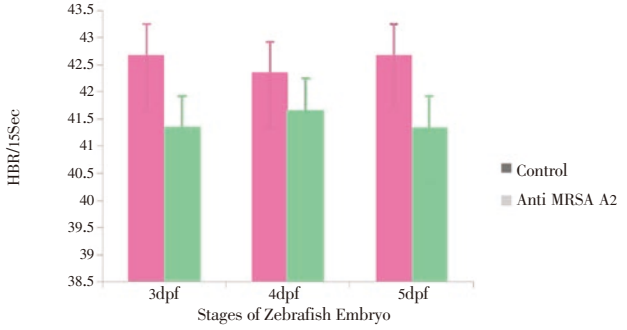

Figure 10. Cardiac assay of anti MRSA molecule A2 from *Streptomyces* sp. PVRK-1.

4. Discussion

The search for newer sources of antibiotics is a global challenge for preoccupying research institutions, pharmaceutical companies and academia, since many infectious agents are becoming resistant to synthetic drugs[24-28]. Recently published guidelines have recommended the use of vancomycin in the management of severe MRSA infection[29], but it was reported that the vancomycin resistant *S. aureus* was emerging in northern India[10]. So that the search of novel anti-MRSA molecules is needed globally. The *Streptomyces* sp. PVRK-1 was isolated from the manakkudy mangrove forest and the the phylogeny analysis was compared with the twenty *Streptomyces* isolated from Manakkudy mangrove sediments[18]. The *Streptomyces* sp. PVRK-1 showed unique differences when compared to the already submitted 20 *Streptomyces* from Manakkudy mangrove sediment. All the twenty *Streptomyces* from the mangrove comparable to the *Streptomyces* sp. PVRK-1 showed novel antimicrobial properties and reported[18]. Generally, terrestrial (soil) actinomycetes were the main stay for antibiotic discovery efforts in the previous five decades. In contrast, the marine environment, covering more than three-fourths of the world's surface, represents a vast and relatively untapped source of novel scaffolds with unique antimicrobial properties[30, 31]. In present

study we have screened a novel Anti-MRSA molecule producing *Streptomyces* sp. PVRK-1 from the Manakkudy, mangroves. Many small molecules identified from new marine actinomycete species have been shown to exhibit potent anti-MRSA activity with desirable cross activity against other bacterial pathogens[32,33]. Zebrafish is an ideal organism for small molecule studies. The ability to use the whole organism allows complex *in vivo* phenotypes to be assayed and combines animal testing with screening. During the last decade, this technology has been extended to the generation of high-value knowledge on safety risks of novel drugs[34]. Streptogramin antibiotic, etamycin, for the first time from a newly discovered marine actinomycete characterized its activity against a panel of HA- and CA-MRSA strains according to studies in murine infection models[35]. The inhibitory concentration of anti-MRSA molecule A_2 from *Streptomyces* sp. PVRK-1 was less than the inhibitory concentration of the etamycin accordign to Haste *et al*[35]. In the present work the anti MRSA molecule toxicity was assessed in the Zebrafish embryos. Similar studies were carried out in the anti MRSA compound from *Rhizophora mucronata*[18]. Embryos are easily treatable by waterborne exposure[36]. The Zebrafish model organism is increasingly used for assessing drug toxicity and safety and numerous studies confirm that mammalian and Zebrafish toxicity profiles are strikingly similar[37]. The anti-MRSA molecule A_2 did not showed any physiological deformities and organogenesis in the drug dose (*in vitro*) of the purified small molecule A_2 which is coincide with earlier reports[17]. Zebrafish development has been well characterized[38] and the heart beat rates were counted in the Purified Molecule A_2 treated embryos which did not show notable deviation in the HBR. Cardiotoxicity is a major problem that can result in drug withdrawal. In 2004, rofecoxib (Vioxx), Merck's blockbuster antiarthritic drug was removed from the market because of increased risk of heart attack and stroke[37]. However the function of the Zebrafish and mammalian heart are similar, the contractility, rhythmicity and morphology can be visually assessed[39] and there is no notable physiological toxic effects in the heart development, hence it could be concluded that the purified compound may not show any cardiotoxic effects when compare to the existing antibiotics. But the formation of pericardial edema, organogenesis and developmental deformities were observed in higher concentration (48-60 μg/mL) in the 3 dpf embryos. The pericardial bulging and small heart of the 3 dpf embryos showed the phenocopy of heart and soul mutation under the genetic screen[40]. Furthermore it was proved that small molecule concentramide also showed heart and soul mutation in the cardiogenesis of the Zebrafish embryos at 3 dpf by disrupting the heart patterning in the embryos[41] and such based mutations were also observed in the higher concentration of the anti MRSA molecule A_2 thus it can be concluded that the anti-MRSA small molecule A_2 was also affecting the cardiac patterning in higher concentration, but not in the inhibitory concentration. Also it can be concluded that the anti-MRSA molecule A_2 did not affecting the embryos in its drug dose concentration level in *in vitro* and *in vivo*. These basic preclinical studies suggest that the molecule is very novel and studies showed that the mangrove associated *Streptomyces* sp. PVRK-1 shows potential for the production of anti MRSA molecule due to a novel structure for antibiotic resistance. The biomedical and toxicity studies prove that the molecule is potent against antibiotic resistant *Staphylococcus aureus*, which did not, showed any toxic effects or organogenesis effects in the minimal inhibitory concentration. Hence this molecule could be further structurally characterized to get anti-MRSA drugs for the medicament of MRSA infected patients all over the world.

Conflict of interest statement

We declare that we have no conflict of interest.

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