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Cell wall distracting anti-Methicillin-resistant *Staphylococcus aureus* compound PVI331 from a marine sponge associated *Streptomyces*

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ABSTRACT

Isolation of unexplored frontier molecules are needed to treat multidrug resistant pathogens especially Methicillin resistant *Staphylococcus aureus* (MRSA). A marine sponge endosymbiotic *Streptomyces albus* ICN33 produces an anti-MRSA metabolite is reported. The crude extract exhibited anti-MRSA activity and the active principle was isolated through fermentation and chromatographic techniques. A compound PVI331 with a molecular mass of 506 Da have been determined by high resolution mass spectrometry. LC–MS based dereplication analysis had revealed that the detected compound PVI331 as unknown. The antibacterial assay of the compound PVI331 showed remarkable antagonistic activity against MRSA and *Escherichia coli*. Minimum inhibitory concentrations were found to be 1 μg/ml against MRSA. Sub-inhibitory concentration of the compound PVI331 reduced the biofilm formation of *Staphylococcus aureus* ATCC25923 and increased the cell surface hydrophobicity index. Scanning electron microscopic observation of the sub-inhibitory concentration exposure revealed a wrinkled membrane surface and slight cellular damage shows the cell wall distracting property of the compound. Zebrafish embryo based toxicity assays exhibited 48 ± 2 μg/ml of LC_{50} value and 30 μg/ml of compound as maximal non-lethal concentration which had demonstrated the positive relationship in safety index. This study highlighted the anti-MRSA property of *Streptomyces albus* ICN33 from a marine sponge.

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Introduction

The increasing threat of multidrug resistant pathogens and the continuing evolution of resistance made there is a pressing need to develop new antibacterial agents (Bologa et al., 2013). Infections due to bacterial biofilms have noteworthy financial costs and morbidity. The multifaceted genetic and molecular basis of biofilm formation in *Staphylococcus aureus* is difficult to treat (Aparna and Yadav, 2008). All medical devices or tissue engineering constructs are susceptible to microbial colonization thus increases the risk for biofilm formation and subsequent infection (Castelli et al., 2006). Small secreted molecules can elegantly mediate the disassembly of biofilms (Oppenheimer-Shaanan et al., 2013). Recent discovery of a new class of Teixobactin antibiotic from an uncultivable bacterium had taken a look back to the pharmaceutical industries in antibiotic research (Ling et al., 2015). Such an isolation of unexplored frontier molecules are needed to treat multidrug resistant pathogens especially MRSA.

Marine animals fight daily for survival and this underwater warfare is waged with antagonistic small molecules which have recognized as the potential chemical weapons to kill bacteria. Sponges are filter feeders, having numerous tiny pores on their surface, which allow entry of water through a series of canals where symbiotic microorganisms and organic particles are filtered out and eaten (van Soest et al., 2012). It is hypothesized that symbiotic marine microorganism harbored by sponges are the original producers of these bioactive compounds (Proksch et al., 2002; Radjasa et al., 2007; Zhang et al., 2005; Newman and Hill, 2006). Searching for diverse and novel chemotype frameworks in a particular project can be desirable because different structural scaffolds offer opportunities in terms of chemical accessibility and possibilities for subsequent lead structure optimization (Grabowski et al., 2008).

Many drugs come with adverse effects and antibiotics are no exception. Preclinical safety evaluation of the lead molecules is crucial. Zebrafish has been the focus of several forward genetic screening studies and has emerged as a major model organism for biomedical research (Rennekamp and Peterson, 2015). The National Institutes for Health (NIH) has ranked the zebrafish as the third most important experimental organism and U.S. Food and Drug Administration (FDA) announced zebrafish as a preclinical trial subjects and serves as gatekeeper if adverse effects found as a red flag and alerts us to take a closer look for toxicity (FDA, 2013). In the present study, we aimed to isolate anti-MRSA compound producing *Streptomyces* from a marine sponge of south west coast of Tamilnadu and its antibiofilm efficacy.

Materials and methods

Marine sponge collection, isolation and identification of producer strain

The marine sponge was collected as by catch in the fishing nets at Colachel coast, Kanyakumari District, Tamil Nadu (latitude 08° 10′ 27.4″ N and longitude 77° 14′ 53.09″ E) and thoroughly washed with sterile sea water to remove ectobiotic fauna. The sponge was identified as *Acanthella elongata* (class *Demospongiae*, order *Halichondrida*, family *Hymeniacidonidae*) as per the morphological and spicule patterns (Hooper and Van Soest, 2002). 3 cm^2 size of sponge tissue was homogenized in sterile sea water and spread plated over the SYP-SW medium containing soluble starch 10 g, yeast extract 4 g, peptone 2 g, agar 18 g and 1 l of 0.22 μm filtered natural seawater and incubated at 28 °C up to 21 days (Kennedy et al., 2009). 15 mg nalidixic acid and 50 mg cycloheximide was included to minimize fast growing bacterial and fungal growth. To enable the sponge specific symbiotic bacteria isolation the media was supplemented with 0.1% of sponge extract which is prepared and 0.22 μm filter sterilized in PBS. On seventh day of incubation, a white aerial mycelium producing strain ICN33 was isolated. Scanning electron microscopic spore morphology, spore chain morphology, characteristic growth on different media, biochemical and physiological characterization were carried out as described previously (Iniyan et al., 2015). 16S rRNA gene sequencing with universal primers and phylogeny analysis using type strains were carried out in EzTaxon-e server and MEGA 6.0 (Kim et al., 2012; Tamura et al., 2013).

Fermentation and fractionation of antagonistic metabolites

Streptomyces albus ICN33 from a well sporulated mycelium was inoculated over the broth 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, distilled water 1 l and incubated at 28 °C up to 14 days in a shaker. After 14 days cells were harvested and homogenized using micropestle and spore suspension was prepared. 50 μl of the spore suspension containing approximately 1×10^7 CFU ml^{-1} of the strain ICN33 was swabbed on 50 agar plates containing AIM medium and incubated for 5 days at 28 °C. At the end of this Agar Plate Fermentation (APF), the grown mycelial cake was cut into small pieces and the organic compounds were extracted using double the volume of ethyl acetate for 24 h and the solvent phase was removed. The mycelial agar pieces were again extracted with same solvent once and equal volume of methanol for one hour each. The organic crude extracts were filtered using Whattman No. 1 filter paper and the solvents were evaporated to dryness in vacuum concentrator (Eppendorf 5301) at 45 °C.

A clean dried glass column (2.5 cm × 60 cm width and length) containing silica gel (60–120 mesh) was packed with hexane. About 1.5 g of the crude ethyl acetate extract was adsorbed in silica gel powder and the compound was carefully added into column with the help of a glass funnel. The fractions were eluted from the column using Hexane and chloroform (1:1), chloroform and ethyl acetate (5:0, 4:1, 3:2, 2:3, 1:4, 0:5), ethyl acetate: methanol (5:0, 4:1, 3:2, 2:3, 1:4, 0:5) and methanol alone with 20 ml of each ratios and totally 30 fractions were made. All the fractions were subjected to antimicrobial assay using disc diffusion assay. The fractions showing antibacterial activity were further fractioned using Thin Layer Chromatography (TLC) using TLC silica gel 60 F_{254} aluminum sheets (Merck) with the mobile phase of chloroform:ethyl acetate:methanol (8:2:1) and the active spot was identified using bioautographic evaluation of 2% solution of

2,3,5-triphenyl-tetrazolium chloride (TTC) spray detection. A single band showing inhibition zone was scrapped from the replicate sheets and isolated.

RP-HPLC and LC–MS analysis

The reverse phase high performance liquid chromatographic (RP-HPLC) analysis of active compound from ICN33 was carried out on a HPLC system (Waters) using C_{18} column with Photo Diode Array (PDA) detector. The solvent system Acetonitrile and Water (HPLC grade) was used in the ratio of 65:35. The mixture was premixed and sonicated in the sonicator for 15 min. 20 μl of the sample was injected on HPLC using Reverse Phase C_{18} with an isocratic elution with a flow rate of 1 ml/min. Each peak fractions were collected individually and assayed. Liquid chromatography–mass spectrometry (LC–MS) with Electro Spray Ionization (ESI) (Thermo Exactive) was carried out to determine the molecular weight and possible structure of antibacterially active substance. The results were detected in the positive ion mode and *m/z* 50–1000 of the scanning mass range in full scan MS. The spectra were analyzed from the library data using NIST Chemistry WebBook and KNApSAcK v1.200.03 (Nakamura et al., 2014).

Antibacterial assay

Staphylococcus aureus ATCC25923, MRSA ATCC33591, *Staphylococcus aureus* MTCC7433, a beta lactamase producing *mecA* positive clinical isolate *Staphylococcus aureus* MRSA BL, *Escherichia coli*, *Klebsiella pneumonia* MTCC9238 and *Pseudomonas aeruginosa* MTCC3542 were used for the antibacterial experiments. Minimal Inhibitory Concentration (MIC) was determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines using broth microdilution method with 5×10^5 CFU/ml of test pathogens and the glycopeptides antibiotic vancomycin (HiMedia) was used as control antibiotic. *In vitro* time kill experiments were performed as per our previous method with some modifications (Iniyan et al., 2015). Briefly, overnight grown cells were harvested and regrown in fresh medium for 4 h at 37 °C and suspended in 0.9% NaCl. A 5×10^5 CFU/ml of inoculums was exposed to 1× MIC and 4× MIC of antibacterial compound PVI331 or 4× MIC of vancomycin at 37 °C. 100 μl samples were withdrawn for the determination of bacterial counts colony-forming units (CFUs) at 0, 2, 4, 8 and 24 h in triplicate LB agar plates at 37 °C.

Antibiofilm assay in polystyrene plates

The effect of active compound PVI331 on biofilm formation was carried out in the 96-well polystyrene plates in the presence of 1% DMSO and various concentrations of antimicrobial agent (1/4× MIC, 1/2× MIC, 2× MIC and 4× MIC of PVI331 or 2× MIC of vancomycin). Briefly, overnight culture of the biofilm producing *Staphylococcus aureus* ATCC25923 was inoculated with 100 μl of fresh Luria Broth in the presence and absence (negative control) of antimicrobial agents. The plates were incubated for 24 h at 37 °C. After incubation, the plates were washed with sterile Phosphate Buffered Saline (PBS) to remove planktonic cells and allowed to air dry before being stained. The biofilms were stained with 0.4% crystal violet solution (w/v) for 5 min. Subsequently the unstained dye was discarded, and the wells were rinsed twice with deionized water and then allowed to dry. The stained areas were visualized microscopically (Coslab) and photomicrograph was taken. Duplicate plates were stained with 2% acridine orange and imaged in fluorescent microscope (Leica). Finally, 200 μl of absolute ethanol was added in each well. The optical density was determined at 570 nm and percentage of biofilm inhibition was calculated using the following formula:

$$\text{Percentage of inhibition} = \left[\frac{\text{Control OD570 nm} - \text{Test OD570 nm}}{\text{Control OD570 nm}}\right] \times 100.$$

The biofilm inhibitory concentration (BIC) was determined as the lowest concentration that produced visible disruption of biofilm formation and a significant reduction in readings when compared with that of the control wells at OD 570 nm (VERSAmax, Molecular Devices). Wells containing medium and extract without bacterial inoculums were used as blanks. Thus, the BIC was determined by both spectro-photometric quantification and also by microscopic visualization.

Influence of PVI331 on cell-surface hydrophobicity

Bacterial adherence to hydrocarbon (BATH) assay was performed to measure cell hydrophobicity. 100 μl of cell suspension from *Staphylococcus aureus* ATCC 25923 containing 5×10^5 CFU/ml was grown in 900 μl tryptic soy broth at 37 °C overnight, and the cells were harvested after centrifugation at 4000 × *g* for 5 min. The pellets were washed and resuspended in 2 ml of 0.9% sodium chloride supplemented with the 0 (negative control), 1/4× MIC, 1/2× MIC, 2× MIC and 4× MIC of antibacterial compound PVI331 or 2× MIC of vancomycin and incubated at 37 °C for 24 h. After incubation initial optical density (OD initial) of the cell suspension at 600 nm was determined. Toluene (250 μl) was added to each cell suspension and vortexed for 2 min and allowed to equilibrate at room temperature for 30 min. Toluene phase was separated from the aqueous phase and the absorbance of the aqueous phase (OD final) was taken at 600 nm. The hydrophobicity index (HPBI) was calculated as: (OD initial − OD final/OD initial) × 100%. The changes in cell surface hydrophobicity of treated cells were calculated (Chusri et al., 2012).

Scanning electron microscopic observations

Staphylococcus aureus ATCC 25923 cells were harvested and pretreated with the active agent PVI331 at its sub-MIC concentration and Vancomycin at its sub-MIC concentration for 1 h to find the morphological changes. Controls were prepared without antibacterial agents. The cells were then air dried and coated with gold and examined for the scanning electron microscopy (FESEM-SUPRA55-Carl Zeiss, Germany) as described previously (Kockro et al., 2000).

Toxicity assessment in zebrafish embryos

Zebrafish were maintained according to Westerfield (1989) in fish culture facility of International Centre for Nanobiotechnology, M. S. University. 2 days post fertilization (dpf) stage

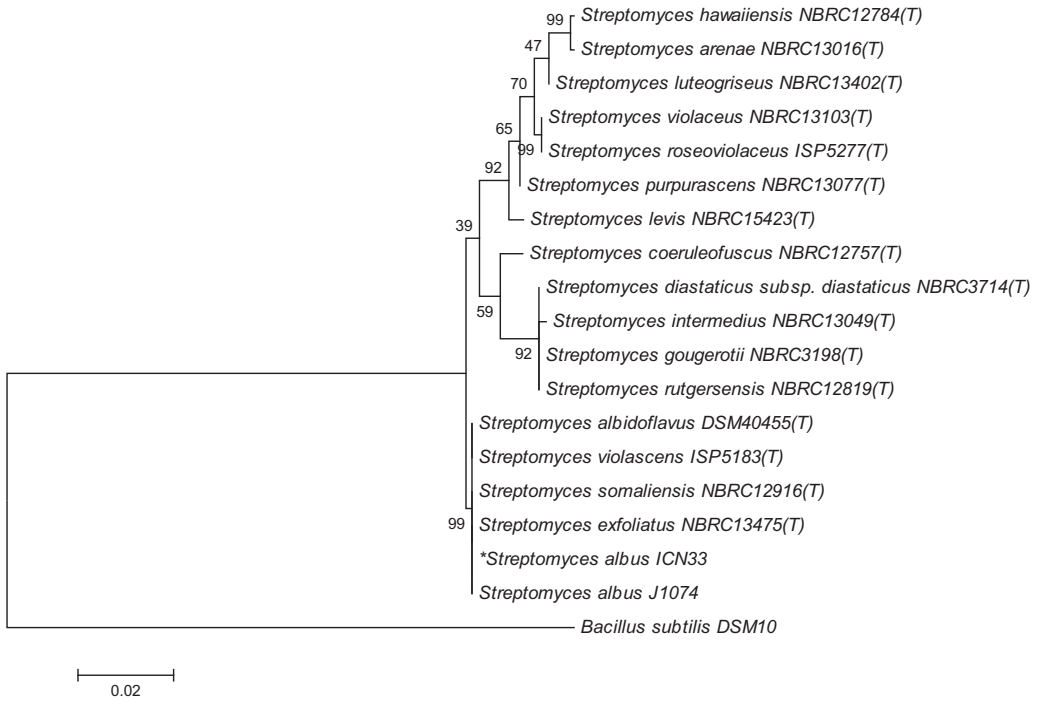

Fig. 1 – Maximum-likelihood phylogenetic representation of actinomycetes type strains with validly published prokaryotic names from EzTaxon server based on the 16S rRNA gene sequences. Bootstrap values are indicated at the nodes with 500 replications. 16S rRNA gene sequence of *Bacillus subtilis* (GenBank accession number AJ276351) served as an outgroup to root the tree.

embryos were exposed to different concentrations of PVI331 compound (1 μg/ml, 2 μg/ml, 4 μg/ml, 8 μg/ml, 16 μg/ml, 20 μg/ml, 30 μg/ml, 40 μg/ml and up to 80 μg/ml) in a 48 well plate containing 10 embryos per well with 1% DMSO in Embryo Rearing Solution (ERS) as previously described (Kannan et al., 2014). Control embryos were also maintained in the presence of 1% DMSO in ERS. Three independent experiments were conducted to find the compound effect assessment. The following consequent changes in the developing embryos were noted during the next 72 h. LC_{50} analysis, heart beat rate assay (HBR) and any abnormalities in the organ development (brain, eye, heart, ear, somite, notochord, trunk, tail and fin) in the light microscope (Coslab) fixed with an attached digital camera (Nikon). LC_{50} values were calculated as per the OECD regulations and the HBR assay was performed as described previously (Kannan and Vincent, 2012; Kalaiarasi et al., 2016).

Results and discussions

Strain identification and compound isolation

16S rRNA gene sequencing analysis revealed the strain belongs to *Streptomyces albus* ICN33 which was identified to be 99.9% similarity with *Streptomyces albus* strain J1074 (NR102949). Fig. 1 shows the phylogenetic relationship of this strain with its evolutionary related taxa. The white aerial mycelium producing strain was found to produce spiny spore surface (Fig. 2) and the mycelia were arranged in a spiral spore chain. The presence of LL-Diaminopimelic acid and absence of characteristic sugars in the TLC analysis confirmed that the strain belongs to the type I cell wall chemotype which is a characteristic of *Streptomyces*. Characteristic growth, biochemical and physiological characteristics of the strain ICN33 is depicted in Table 1. The strain ICN33 had shown different growth characteristics on International Streptomyces Project (ISP) media and nutrient agar (Supplementary Table S1). Marine derived actinobacteria produce structurally diverse small molecules with a broad array of biological activities, several of which compounds are being now developed as antibacterial and anti-tumor agents (Claverías et al., 2015; Mizuno et al., 2015; Mullowney et al., 2015). Especially, marine sponge associated actinobacteria is reported to produce many novel taxa and novel antibiotics (Sun et al., 2015; Viegelmann et al., 2014; Manivasagan et al., 2014). The producer actinobacteria of the present study, *Streptomyces albus* is one of the most widely distributed streptomycetes and has been isolated from diverse biotopes including sponges, marine sediments and insects (Viegelmann et al., 2014; Ian et al., 2014; Fan et al., 2011; Barke et al., 2010; Schoenian et al., 2011; Hanshew et al., 2014).

The strain ICN33 produced anti-MRSA substance in the agar plates and was extracted with ethyl acetate with a yield about 1.8 g/l media used. Column fractionation in silica gel chromatography yielded 6 active fractions. Bioassay guided purification through TLC bioautography of each active column fraction found out an active fraction at the R_f value of 0.45. Further HPLC purification with acetonitrile and water in the ratio of 65:35 yielded the anti-MRSA active substance at the retention time of 29.210 and 29.339 at 246.5 nm UV absorbance. LC–MS

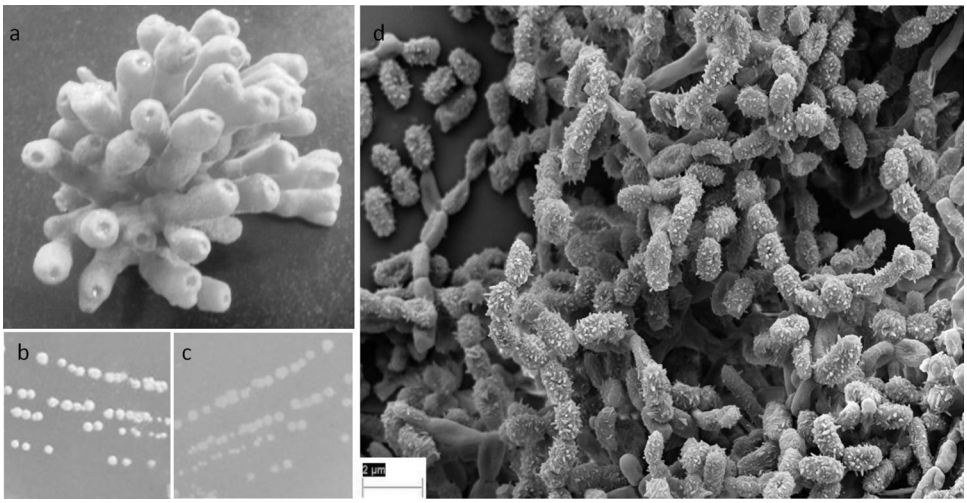

Fig. 2 – Morphology of the isolation source and ICN33 strain. (a) marine sponge, (b) ariel mycelial view the antagonistic strain ICN33, (c) substrate mycelial view the antagonistic strain ICN33, (d) spore surface morphology show spiny spores in Scanning Electron Microscopic observation of ICN33.

analysis determined the pseudomolecular ion at 507.2714 as $[M+H]^+$ which is the top highest peak and the molecular mass was identified as 506.2636 Da. The second and third highest peaks were found at *m*/z 437.1931and *m*/z 482.1684 (Fig. 3). Database search had revealed three compounds originated from *Streptomyces* were found to be closest relatives. The most similarity was found to the manumycin class antibiotic Manumycin G isolated from *Streptomyces* sp. strain WB-8376 with exact mass of 506.2053 g/mol. It was observed to be active against *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus* whereas weakly active against *Proteus mirabilis*, *K. pneumonia* and *Candida albicans* (Shu et al., 1994). The next matches were found to be Esterastin and the macrolide antibiotic Antimycin A4b with exact mass of 506.3355 g/mol and 506.2264 g/mol respectively (Umezawa et al., 1978; Shiomi et al., 2005). The high resolution molecular mass of PVI331 and its signature ions, fragment ions and antibacterial spectrum has not matched with these known compounds. Hence, the compound PVI331 evidenced a potential chemical worthy for further investigation. Recently novel frigocyclinone, dimethyl dehydro rabelomycin and six new angucyclinone derivative antibiotics had reported from a Caribbean sponge associated *Streptomyces* sp. M71 (Vicente et al., 2015).

Recently, *Streptomyces albus* J1074 is evidenced as heterologous gene expression host for biosynthetic engineering, mutasynthesis and production optimization (Fu et al., 2015) and *Streptomyces albus* is reported to produce an anti-MRSA active compound Tetarimycin A (Kallifidas et al., 2012). Furthermore, the strain *Streptomyces albus* ATCC21838 is known to produce salinomycin (Yurkovich et al., 2012) and the draft genome sequence of another type strain *Streptomyces albus* NBRC 13014 had reported the lack of salinomycin biosynthetic gene cluster (Komaki et al., 2015). It is evident that the antibiotic production and biosynthetic gene cluster likely harbors strain specific not species specific (Seipke, 2015). This shows that the chemical fingerprints of each strain can vary significantly among bacteria of the same species which supports the strain ICN33 might produced a new compound PVI331.

Antibacterial activity of ICN33 extract

The strain ICN33 had shown antibacterial activity against *Staphylococcus aureus* ATCC 25923, MRSA ATCC 33591, *Staphylococcus aureus* MTCC7433, MRSA BL and *E. coli*. ICN33 is not active against *K. pneumonia* MTCC9238 and *P. aeruginosa* MTCC3542. Minimum inhibitory concentration, that is, the minimum concentration of the molecule required to inhibit the growth of the bacteria was determined for the purified compound PVI331. The glycopetide antibiotic vancomycin was used in this study to compare the results. The compound PVI331 showed good activity against MRSA. Moreover, the molecule was found to be more active toward *Staphylococcus aureus* than *E. coli*. For example, the range of MIC value was 1–2 μg/mL against *Staphylococcus aureus* whereas MIC values were 4 μg/mL for *E. coli*. The compound showed rapid bactericidal activity against both the clinical isolate MRSA BL and the reference strain *Staphylococcus aureus* ATCC25923. The rate of bacterial killing after exposure to the higher concentrations of PVI331 at four times MIC had decreased the cell counts at 2 h and 4 h (Fig. 4).

Biofilm inhibition

Notably, antibiotic therapy typically cures the signs caused by planktonic cells released from the biofilm, but fails to kill the biofilms (Sritharan and Sritharan, 2004). Hence, the development of these anti-MRSA agents alone do not effective to the unique bacterial groups, *i.e.* biofilm producers. It is reported that the *Staphylococcus aureus* is one of the biofilm forming human pathogens that causes an array of diseases (de Castro Melo et al., 2013; Tang et al., 2013). Qualitatively, biofilm formation of *Staphylococcus aureus* ATCC25923 in the presence of antibacterial compound PVI331 was observed by light and fluorescent microscopic observations at the magnifications

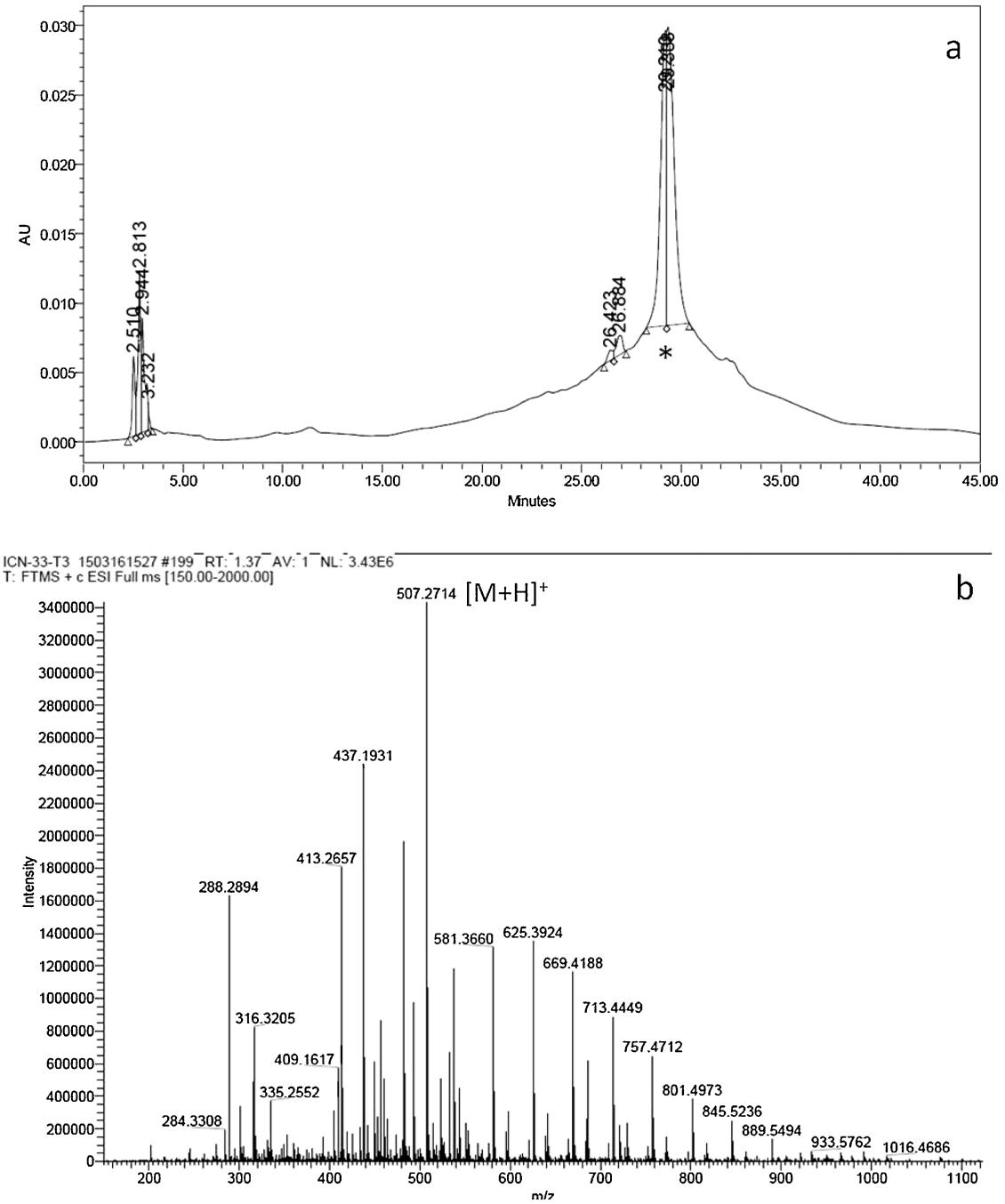

Fig. 3 – (a) HPLC chromatogram of antibacterial fraction PVI331 from ICN33. (b) LC–MS based mass analysis of antibacterial compound PVI331.

×400 and quantitatively analyzed by spectrophotometry at OD_{570} nm. Concentration-dependent decrease in biofilm formation of *Staphylococcus aureus* ATCC25923 was obtained upon treatment with PVI331. Control plates had shown well developed biofilm formation of *Staphylococcus aureus* whereas; upon treatment with compound PVI331 poor biofilm formation was observed (Fig. 5). The compound PVI331 showed biofilm inhibition at its lower drug concentration of 0.25 μg/ml itself. The compound PVI331 showed a prominent biofilm inhibition of 92.17 ± 1.67% at 4 μg/ml concentration. The anti-MRSA antibiotic Vancomycin at 8 μg/mL also showed inhibition of 32.58 ± 2.52% but our compound PVI331 inhibited the biofilm formation significantly (Fig. 6).

Cell surface hydrophobicity and morphological changes

The effect of subMIC concentrations of antibacterial compound PVI331 on cell-surface hydrophobicity is depicted in Fig. 6. The BATH assay of strain *Staphylococcus aureus* ATCC 25923 revealed significant increases on cell surface

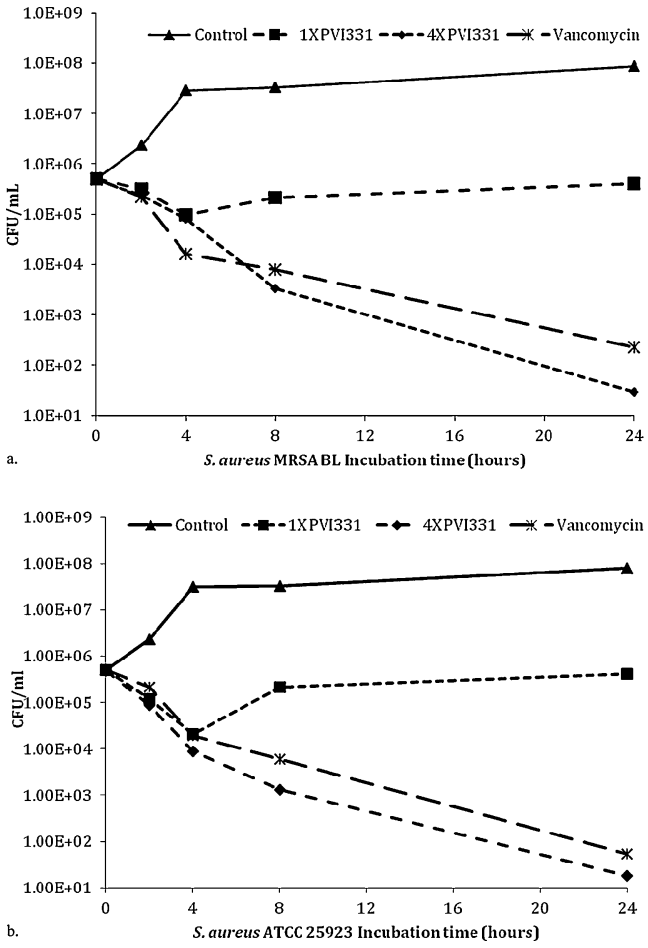

Fig. 4 – Anti-MRSA kinetics of the compound PVI331 (1× MIC and 4× MIC concentrations) from *Streptomyces albus* ICN33. The time dependent killing is shown for the β-lactamase producing MRSA BL (a) and *S. aureus* ATCC 25923 (b).

hydrophobicity even at one fourth concentration MIC of PVI331 exposure. At this concentration 82.21 ± 2.79% hydrophobicity was observed and the control showed 21.43 ± 2.79% hydrophobicity which is statistically significant ($P < 0.05$). Morphological observations of *Staphylococcus aureus* ATCC25923 upon treatment with subMIC concentration at 0.25 μg/ml demonstrated distorted cell surface morphology as wrinkled membrane surface and slight cellular damage when compared to control cells (Fig. 7). In contrast, cells treated with subMIC of Vancomycin at 0.5 μg/ml treatment had shown membrane disruption, lysis and release of cell contents. Earlier studies reported that the microbial cell surface properties like charge and hydrophobicity play a crucial role in bacterium-host cell interactions (Swiatlo et al., 2002). The peptidoglycan matrix of cell wall is essential for survival, and in MRSA it is densely functionalized with wall teichoic acids which are necessary for β-lactam resistance (Brown et al., 2012). The cell wall active antibiotic Teixobactin demonstrated its activity by inhibiting precursor of peptidoglycan and wall teichoic acid (Ling et al., 2015). The cell wall distracting potential of compound PVI331 need to be further investigated for its therapeutic target.

Table 1 – Cultural characteristics of *Streptomyces albus* ICN33.

Characteristics	*Streptomyces albus* ICN33
Morphology	
Aerial mycelium color	Gray
Substrate mycelium color	White
Pigmentation in the medium	Brown
Melanin pigment	Absence
Metabolite Exudation	Absence
Shape of the aerial hyphae	Open spiral (S)
Series	Gray
Spore chain	Highly branched, Straight
Spore surface	Spiny
Physiology	
Growth temperatures	20 °C–45 °C
Optimum temperature	28 °C
pH tolerance	6–9
Optimum pH	7
NaCl tolerance	0–5%
Optimum NaCl concentration	1%
Carbon source utilization	
Glucose, maltose, sucrose, lactose, Starch,	Positive
Mannose, dextrose, arabinose	Positive
Fructose, cellulose	Negative
Enzyme activity	
Catalase, oxidase	Positive
Protease, lipase	Negative
Urease, nitrate reductase	Negative
Antibiotic susceptibility	
Amoxycilin (30 μg/disc)	R
Amphotericin B (100units/disc)	R
Ampicilin (10 μg/disc)	6 mm
Cefazolin (30 μg/disc)	R
Chloramphenicol (30 μg/disc)	36 mm
Clindamycin (2 μg/disc)	32 mm
Erythromycin (15 μg/disc)	10 mm
Fluconazole (10 μg/disc)	R
Nystatin (100 units/disc)	R
Oxacillin (1 μg/disc)	R
Penicilin G (10 units/disc)	R
Streptomycin (10 μg/disc)	46 mm
Tetracycline (30 μg/disc)	22 mm
Vancomycin (30 μg/disc)	R
Antibacterial activity	
S. aureus ATCC25923	+++
MRSA ATCC33591	++
S. aureus MTCC7433	++
S. aureus MRSA BL	++
E. coli	++
Klebsiella pneumonia MTCC9238	–
Pseudomonas aeruginosa MTCC3542	–

Note: +++, >20 mm inhibition zone in double layer assay; ++, 10–20 mm inhibition zone in double layer assay; –, no zone of inhibition; R, resistance.

Compound safety assessment in zebrafish embryos

Heart beat measurement is one of the key components in assessing cardiac function because changes in heart beat can be the causes of hidden heart failure conditions (Luca et al., 2014). The heart beat pattern of embryonic zebrafish exhibits

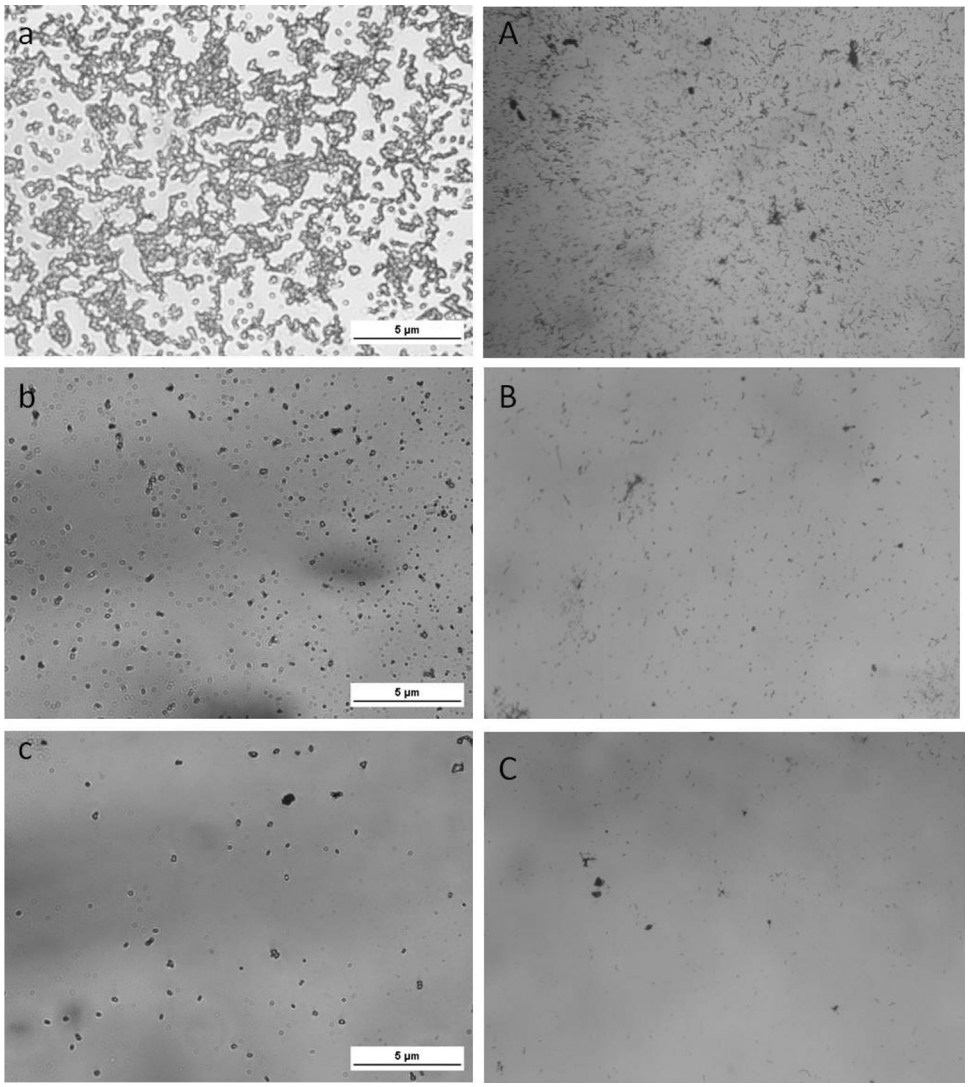

Fig. 5 – Biofilm inhibition assay using fluorescent (a–c) and light microscopic (A–C) images (×400). Figure shows the antibiofilm activity of the compound PVI331 from *Streptomyces albus* ICN33 against *S. aureus* ATCC 25923. (a, A) Untreated control. (b, B) 4 µg/ml of vancomycin treated well. (c, C) 4 µg/ml of PVI331 treated well.

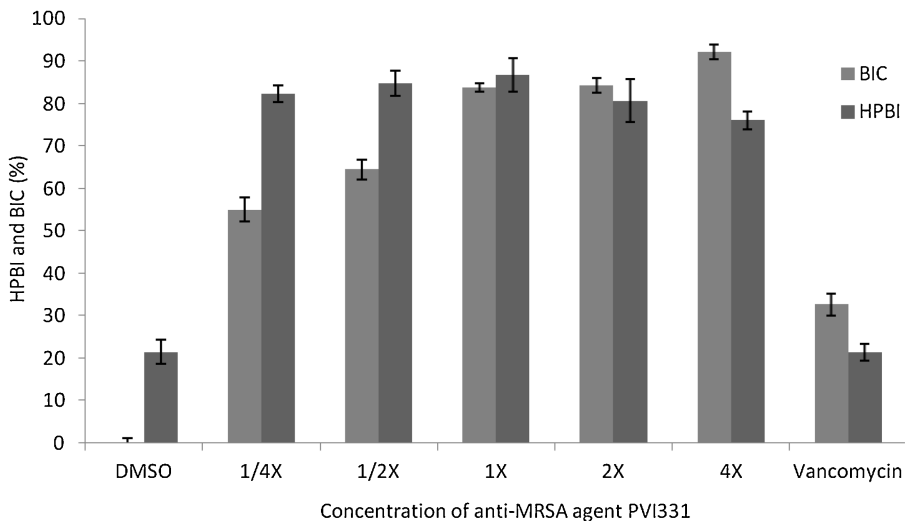

Fig. 6 – Antibiofilm activity of the extract from *Streptomyces albus* ICN33 against *S. aureus* ATCC25923.

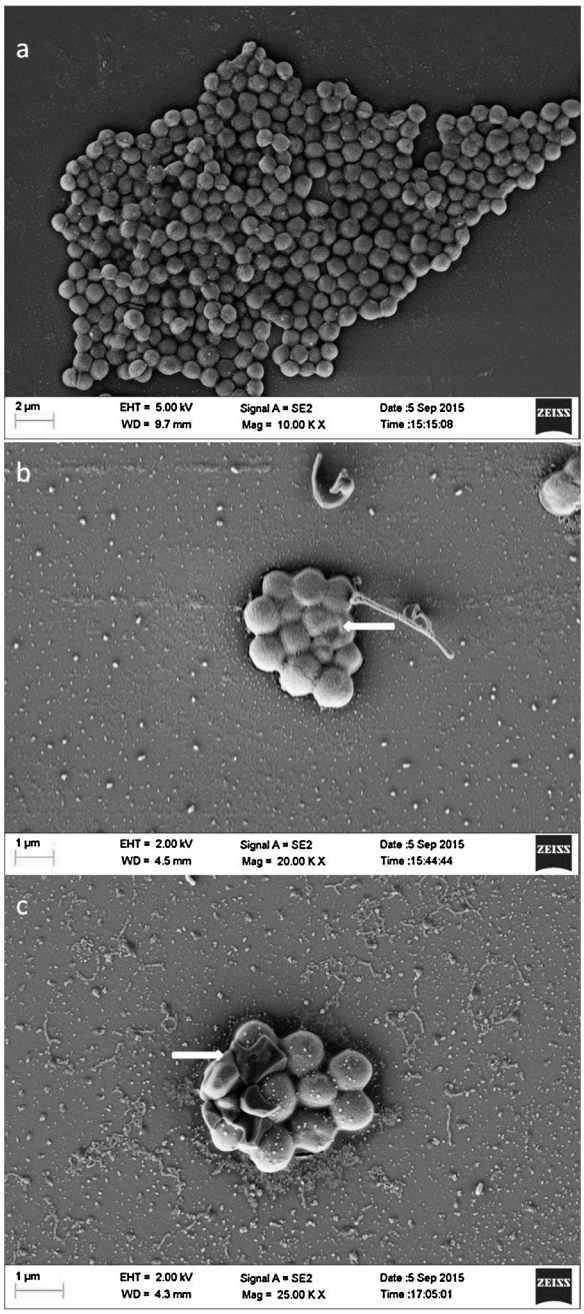

Fig. 7 – Micrographs of scanning electron microscopy of *S. aureus* ATCC 25923. (a) Control image show normal surface morphology. (b) SubMIC of PVI331 at 0.25 μg/ml treated cells show disintegration of cell wall (indicated by white arrow) and slight cellular damage. (c) SubMIC of vancomycin at 0.5 μg/ml treatment shows disruption and release of cell contents.

the same pattern of electrical excitation as the human heart (Staudt and Stainier, 2012). Pharmacological studies in zebrafish embryo revealed the compound PVI331 had found to be safe up to 30 μg/ml which is 30 times higher concentration than its MIC value. The compound revealed 48 ± 2 μg/ml of LC_{50}. 30 μg/ml was found to be maximal non-lethal concentration. The embryos treated with higher doses at 60 μg/ml exhibited mild cardiac edema and pericardial bulging. There has no significant changes in HBR was found up to 30 μg/ml and the higher assay concentration at 70 μg/ml resulted in reduced HBR as 141.6 ± 3.37 beats/min whereas the HBR of the control embryos were found to be 168 ± 2.67 beats/min.

Concluding remarks

Taken together, our data shows the anti-MRSA compound PVI331 from *Streptomyces albus* ICN33 may possess a chemical novelty and need to be further structurally characterized and identified for its biosynthetic pathway. The potential of anti-MRSA compound in sponge associated *Streptomyces* is hopeful since it opens new opportunity for bioprospecting these kinds of bacteria for novel compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jab.2016.04.003.

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