Original Article

DOI: 10.5582/ddt.2018.01042

Fungal-derived xenobiotic exhibits antibacterial and antibiofilm activity against *Staphylococcus aureus*

Siddhartha Kumar[1], Arvind Kumar[1], Manisha Kaushal[2], Prince Kumar[3], Kasturi Mukhopadhyay[3], Antresh Kumar[1,*]

[1] *Department of Biotechnology, Central University of South Bihar, Gaya, India;*
[2] *Department of Botany, Gurunakak Degree College Umra kala Siohara, Bijnor, U.P. India;*
[3] *School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India.*

Summary ***Staphylococcus aureus*** **is an opportunistic pathogen, responsible for superficial and invasive infections both in nosocomial and community-acquired settings. The incidences of infection have become more problematic attributable to emerging drug resistance and biofilm formation. These challenges suggest the need for new antimicrobial agents against *S. aureus*. In present work, we purified a fungal xenobiotic (FI3) which elicits a potent antimicrobial activity against a list of tested microbes including methicillin sensitive (MSSA) and methicillin resistance (MRSA) *S. aureus*. The cell growth of MSSA and MRSA were completely ceased with the 1× minimum inhibitory concentration (MIC); 32 µg/mL and 128 µg/mL, respectively. The cell viability severely decreased within 90 min, due to disturbance of membrane homeostasis. This bactericidal effect was enhanced at lower pH (pH 4) with a speculation to retain positive charge. The FI3 potently disrupts biofilm adherence at 64 µg/mL and found to be a safe with no toxic effect on mammalian tissue. FI3 also leads to increase the potency of tested antibiotics. Taken together, we established that FI3 has a potent antimicrobial activity against tested microbes and safer to human tissue. It may be proven a leading molecule for the treatment of bacterial infections.**

Keywords: Staphylococcal infections, antibacterial, antibiofilm, fungal xenobiotic, Staphylococcus aureus

1. Introduction

Staphylococcus aureus is a dangerous human pathogen which causes a diverse range of superficial and invasive diseases such as acute skin and tissue lesions to severe necrotizing pneumonia, osteomyelitis endocarditis, septicemia, and catheter-associated bacteremia (*1-4*). This notorious bacterium continues to parade higher morbidity, mortality, and a significant financial burden to the public. Individuals at high risk for *S. aureus* infection include patients with surgical, organ transplantation, indwelling catheters, ventilator-assisted respiration, diabetics, tracheal intubation, late-stage renal disease, immunosuppressive or cancer therapy and individuals with low-birth weight neonates, and hospital residents (*5-8*). In spite of high rate of infections, continuous and rapidly emerging drug resistance against available different antibiotics has made it difficult to treat (*9*). Secretion of virulence factors and formation of biofilm in *S. aureus* are the two hallmark determinants that majorly contribute to therapeutic failure. A multitude of virulence factors including enterotoxins, pore-forming toxins (α-hemolysin, Panton-Valentine leukocidin) is considered to be the key factors essential for the establishment of infection (*10*). In contrast, formation of multicellular biofilm over on cell boundary acts as a major obstacle for drugs to enter inside and that makes them 100 folds more resistant to antibiotics, antimicrobials, and immune defense (*11,12*). Many *Staphylococcus* species showed an increased resistance to available drugs (*13,14*). Consequently, the prognosis of such infections is problematic and has now

**Address correspondence to:*
Dr. Antresh Kumar, Centre for Biological Science (Biotechnology), Central University of South Bihar, Patna 800014, Bihar, India.
E-mail: antreshkumar@cub.ac.in

become an alarming issue both in developed as well as developing countries.

The drug resistance condition is developed by frequent and indiscriminate uses of antibiotics. More than 11,000 people died from drug resistance *S. aureus* related infections in the U.S. (CDC report 2013) and now the situations have reached to an epidemic proportion with a serious health concern (*15*). In India, the incidence of drug resistant *S. aureus* infections has endemic nature and it increased steadily up to 54% both in hospital-acquired (HA) and community-acquired (CA) infections (*16-18*). There is a major deficit of effective and sustainable treatment against *S. aureus*. Hence, there is an urgent need for finding new antibacterial and novel approaches against both planktonic and sessile bacteria.

Since a long time, the discovery of new active compounds from both natural and synthetic sources has been explored and still gaining much attention (*19,20*). Fungi have been used as a natural source of traditional medicine and variety of drugs has been derived and still, the research on fungi is of a great interest. Antibiotics penicillin, streptomycin, immunosuppressant cyclosporine, anti-hypercholesterolemic compactin, and lovastatin are such examples. In fact, fungal secondary metabolites have a vital ecological role; they act as a weapon of defense and competitor to protect themselves against parasites and predators. The endophytic fungi, habitat to colonize on plants, represent a storehouse of the diverse array of bioactive secondary metabolites which can prove to be a miracle and combat plethora of pathogens including resistant microorganisms. With this background, the objective of this study is to evaluate the antimicrobial and anti-biofilm activity of the endophytic fungal xenobiotic (FI3) against methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). We showed that FI3 leads to eradicate *S. aureus* infection load speedily by targeting cell membrane and biofilm adherence of Staphylococcal strain without showing any detrimental effect to *E.coli* and human tissue.

2. Materials and Methods

2.1. *Bacterial and fungal strains and culture media*

The methicillin-sensitive *S. aureus* (MSSA); ATCC29213 and methicillin-resistant *S. aureus* (MRSA); ATCC33591, *Pseudomonas aeruginosa* MTCC1034, *Bacillus subtilis* MTCC441 and, *Escherichia coli* strains were used in this study. MSSA was received as a gift by Prof. Kasturi Mukhopadhyay, JNU, New Delhi, India and *P. aeruginosa* MTCC1034, *B. subtilis* MTCC441, and *E. coli* were purchased from MTCC, Chandigarh, India. These strains were used for analysis of drug susceptibility assay. Drug susceptibility test was also performed using MRSA. *S. aureus* strains were cultured in Muller-Hinton Broth/Agar (MHB/MHA). Tryptic Soya Agar/Broth (TSA/TSB) was used for biofilm study. *P. aeruginosa* MTCC1034, *B. subtilis* MTCC441 and *E. coli* were cultured in LB (Luria-Bertani) broth. The fungal isolates (FI) were isolated from the bark and dried tissue of the plant from the Sanjay Gandhi Zoological Park (SGZP), Patna, Bihar and also received from the National Culture Collection of Pathogenic Fungi (NCCPF), PGIMER, Chandigarh, India. All media were purchased from the Hi-media laboratory.

2.2. *Purification of fungal xenobiotic (FI3)*

The fungal isolates maintained on YEPD agar plate (seed culture) was inoculated in YEPD medium and allowed to incubate on shaking condition for six days at 30ºC. Cells were pelleted by centrifugation at 12,000× g for 15 min and the culture supernatant was filtered through Whatman filter No 1. The cell-free supernatant was extracted as described by Samuel *et al*. (*21*). The supernatant was gently agitated for 30 min after addition of equal volume of chloroform. This step was repeated thrice by adding fresh Chloroform. Fractions recovered from chloroform extraction were evaporated in the rotatory evaporator, dried under vacuum, weighed and dissolved in phosphate buffer saline (PBS) for purification. The sample was loaded on C-18 column using a gradient flow rate with 1 mL/min of acetonitrile (A) and water (B) mobile solvents. The chromatogram was run as A5% + B95% (0 min); A15% + B85% (0-5 min); A95% + B5% (5-25 min); A15% + B85% (25-40 min). The prominent peak with high intensities were collected in a sterile glass vial for further processing and antimicrobial analysis. Peak No. 6 showed postive results was collected and termed this xenobiotic as FI3.

2.3. *Susceptibility assay*

The antimicrobial susceptibility of FI3 was determined by two different methods mentioned here under.

2.3.1. *Spot assay*

FI3 was initially screened for antimicrobial activity against *S. aureus* by spot assay described elsewhere (*22,23*) with minor modifications. For this, both MSSA and MRSA cells were cultured in Muller-Hinton broth (MHB) till the cell density (Abs 600 nm) reaches 0.5. It was achieved at the mid-exponential phase of the cell growth (data not shown). One OD_{600nm} of *S. aureus* cell density was calculated which equals to 2×10^8 cells/mL (*24*). The cell pellet was resuspended in PBS to maintain 0.1 OD for spotting. 2×10^6 (0.01 OD_{600nm}) MSSA or MRSA cells were uniformly spread over the surface of the Muller-Hinton agar (MHA) plate and FI3 was put on plate for antimicrobial screening for

calculation of zone of inhibition. 5 µL of ten folds serial dilutions were spotted on MHA plate in the absence and presence of FI3 and other tested drugs. The difference in *S. aureus* cell growth was observed after incubation for 24 h at 37°C.

2.3.2. *Minimum inhibitory concentration (MIC)*

MIC was determined by broth microdilution method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (*25*). The assay was done in three independent experiments. Briefly, different dilutions of FI3 and other tested drugs *viz*. Tet (tetracycline), Genta (gentamycin), and Van (vancomycin) were prepared in 96 wells plate by addition of final volume 100 µL of MHB medium in each well of the plate. Subsequently, it was serially diluted in 1:2 ratios. After successful dilutions of tested compounds and drugs, 2×10^6 cells of mid-exponential phase *S. aureus* (MSSA and MRSA), *P. aeruginosa*, *B. subtilis* and *E. coli* were added in each well. MIC value was calculated by observing the optical density (OD_{600nm}) after 24 h of plate incubation at 37°C. 1× MIC was defined as the concentration of tested compound (FI3) that enables to restrict cell growth 100% as compared to the cell growth control. The FI3 showing 100% cell growth inhibition was considered as the 1× MIC.

2.4. *Cell viability assay*

To determine the antimicrobial activity of FI3, 2×10^6 MSSA cells of mid exponential phase were taken as described elsewhere (*26*). The cells were incubated with varying concentration of FI3 *i.e*. 16 µg/mL, 32 µg/mL, 64 µg/mL and 128 µg/mL, those correspond to 0.5× MIC, 1× MIC, 2× MIC and 4× MIC, respectively. The mixture of tube content was incubated at 37°C at 120 rpm. After each 30 min of interval up to 90 min, 10 µL of tube content was taken and serially diluted up to 100 times in PBS to reduce FI3 and then spread out on MHA plate at 37°C. Viable colonies were calculated after overnight incubation.

2.5. *Biofilm attachment assay and inhibition of biofilm formation*

The effect of FI3 on biofilm inhibition of MSSA was tested as described elsewhere (*27-29*). Briefly, the overnight culture of *S. aureus* was diluted up to 2×10^5 cells/mL in TSB. This diluted cell suspension with 5 mM glucose was seeded in to 24-well polystyrene plate in addition to introducing different concentrations of FI3. The assay was done in three independent experiments. In biofilm attachment assay, sterile glass slide (10 × 10 mm) was put in the wells. The plates were incubated at 37°C for 48 h and 3 h for inhibition of biofilm formation and biofilm attachment assay, respectively. After incubation, both slides and wells were washed with 250 µL of PBS to remove planktonic bacteria and air dried. The cells were then fixed with 200 µL of methanol for 15 min and plates were allowed to dry. The plate wells or glass slides were stained with 200 µL of 0.1% (v/v) crystal violet for 5 min. Excess stain was gently rinsed off and plates were air-dried. After staining, the attachment of biofilm was observed under the inverted microscope. The stained wells were re-solubilized in 200 µL of 95% (v/v) ethanol and cell concentration was measured at OD_{595nm} for biofilm inhibition analysis respectively (*30*). The FI3 untreated *S. aureus* cells were considered as a positive control in both experiments.

2.6. *Haemolysis assay*

To test the hemolytic activity FI3, chicken blood and human red blood cells (RBCs) were taken as reported elsewhere (*31,32*). Briefly, fresh human blood was collected and layered on histopaque-1077. The RBCs cells were recovered from the pellet by centrifugation at 400× g for 30 min. The pellet was washed with 0.9% (w/v) NaCl for three times and finally resuspended in 0.9% NaCl. Then, various concentrations (16-256 µg/mL) of FI3 were added in 95 µL of the RBCs cells suspension and it was then incubated at 37°C with gentle mixing for 3 h. After incubation, the supernatant was recovered by centrifugation as above. The cell lysis was quantified at 415 nm. As a positive control, 1% triton X-100 was added in RBCs suspension for measuring the complete lysis and in the negative control, RBCs suspension was incubated only in the presence of PBS. Haemolysis was calculated as: Percentage haemolysis = $[(A - A0)/(A100 - A0)] \times 100$. Where: A, represents absorbance of the FI3 treated sample at 415 nm and A0 and A100 represent zero percent and 100% lysis determined in PBS and 1% Triton X-100, respectively.

2.7. *Cytoplasmic membrane permeability assay*

To investigate the membrane permeabilizing property of the FI3 against *S. aureus* cell membrane, calcein release assay was performed using calcein acetoxymethyl ester (calcein-AM) as described elsewhere with minor modifications (*33*). Calcein-AM is a non-fluorescent, lipid-soluble, cell membrane permeable dye. Upon hydrolysis by cytoplasmic esterase, it breaks into non-permeable, fluorescent calcein with an excitation (Ext) and emission (Em) 494 nm and 527 nm, respectively. Briefly, *S. aureus* cells were harvested to mid-exponential phase and diluted to maintain 2×10^4 cells/mL in PBS, supplemented with 5 mM glucose. Cells were then incubated with 2 µM calcein-AM for 1h for maximal uptake of dye followed by addition of 1× MIC concentration (32 µg/mL) of FI3 and then

fluorescence was monitored at different time intervals (up to 90 min). Gramicidin B (35 µg/mL) was used as a positive control. During analysis, 1 mL of the sample was withdrawn at 15 min intervals and supernatants were recovered by centrifugation for fluorescence measurement using Spectrofluorometer (Perkin Elmer, LS 55).

2.8. *Propidium iodide (PI) uptake assay*

PI is a non-permeable, cell membrane, fluorescent dye that having excitation (Ext) and emission (Em) wavelength 544 nm and 620 nm, respectively. When the bacterial cell membrane is rendered permeable, PI enters and interacts with cellular DNA and fluorescence can be detected by spectrofluorometer. This assay was done according to the method described by Madhuri *et al*. (*33*). For this, *S. aureus* cells were harvested to mid-exponential phase and cells turbidity were diluted to maintain 2×10^4 cells/mL in PBS buffer. 2 mM PI was supplemented in the cell culture and incubated at 37°C for 10 min for dye equilibration. Cells were then exposed to 1× MIC of FI3 (32 µg/mL) at 37°C for different time periods up to 120 min. Gramicidin B (35 µg/mL) was used as a positive control since it kills the bacteria through membrane disruption (*34*). Fluorescence was measured at every 30 min intervals for 3 h using Spectrofluorometer (Perkin Elmer, LS 55).

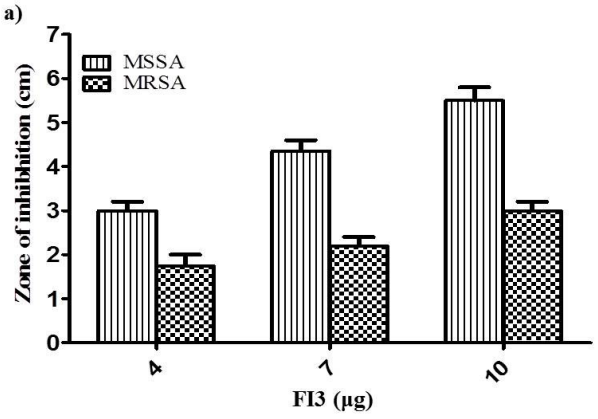
2.9. *Statistical analyses*

Statistical analyses were assessed using GraphPad prism 6.0 (Graph Pad Software, La Jolla, CA). *p* values were calculated *via* Student *t*-test. $p < 0.05$ was deemed significant.

3. Results

3.1. *Susceptibility profile of FI3 against both methicillin sensitive and resistant S. aureus*

The antimicrobial activity of fungal isolates was tested by spot assay for initial screenings, one such fungal isolate (FI) was identified as *Aspergillus nidulans* based on 18S rDNA sequence analysis (data not shown). The antimicrobial activity of different solvent extracts of *A. nidulans* was tested. The chloroform extract showed the highest antimicrobial activity among the all tested solvents. The solvent extract was then powdered and dissolved in PBS, filter through the 0.22 µm filter. The antimicrobial xenobiotic (FI3) was purified by reverse phase chromatography using C-18 column (Figure 1S, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=28*). Figure 1a



b)

Strains	1X MIC
MSSA (ATCC29213)	32µg/mL
MRSA (ATCC33591)	128µg/mL
P. aeruginosa MTCC1034	128µg/mL
B. subtilis MTCC441	256µg/mL
E.coli	ND*

*ND= not detectable

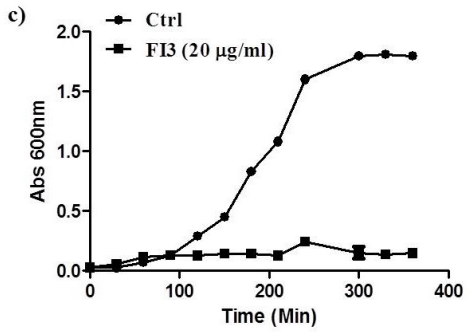


Figure 1. Susceptible profile of FI3 against *S. aureus*. Six days aged fungal culture broth extracted with equal volume Chloroform. Chloroform extract (FI3) was then evaporated and it was dissolved in PBS **(a)**. FI3 was spotted at the indicated amounts (4 µg, 7 µg, and 10 µg) on MSSA and MRSA suspended in MHA plate. Plate incubated at 37°C for 24 h and zone of inhibition was calculated. **(b)** Determination of MIC of FI3 against MSSA, MRSA, ***P. aeruginosa B. subtilis*** and ***E. coli*** bacterial strains. The 1× MIC) was measured that can be defined as the concentration of FI3 at which 100% of cell growth was inhibited. **(c)** Effect of FI3 (20 µg/mL) on MSSA growth.

illustrates that cell growth of both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistance *S. aureus* (MRSA) was inhibited in the presence of varying concentration of FI3. These results clearly demonstrate that FI3 is effective against both MSSA and MRSA. The antimicrobial activity of FI3 was further assessed against a panel of Gram+ve and Gram−ve strains using microdilution assay. Figure 1b demonstrates that FI3 completely inhibits the cell growth of the tested bacteria strains including resistant MRSA. The growth of MSSA and MRSA were completely arrested (1× MIC) at 32 µg/mL and 128 µg/mL, FI3 respectively. FI3 also retains its MIC values for *P. aeruginosa* MTCC1034 and *B. subtilis* MTCC441, 128 µg/mL and 256 µg/mL, respectively. On the contrary, FI3 has no appreciable inhibition of *E. coli* growth and was found to be insensitive even at higher concentration. Strikingly, MSSA growth was not recovered and remain suppressed when growth curve was performed in the presence of more than 0.5× MIC (16 µg/mL) concentration of FI3 (Figure 1c). These findings revealed that the FI3 effectively inhibits the growth of *S. aureus* (MSSA and MRSA), *P. aeruginosa* and *B. subtilis* without affecting the growth of *E. coli.*

3.2. *Combined effect of FI3 with topical antimicrobials*

Combination of two or more antimicrobials together has been explored as an alternative strategy for the treatment of systemic *S. aureus* infections. In order to test the combinatorial effects of FI3 together with tested topical antibiotics on *S. aureus* susceptibilities, broth microdilution and spot assays were performed. Figure 2a illustrates the drug susceptibility profile of three different antibiotics named as Tet, Genta, and Van in combination with FI3. The MIC of these individual antibiotics was measured as 1 µg/mL, 1 µg/mL, and 2 µg/mL, respectively (Figure 2a). The drug susceptibility of Tet, Genta, and Van was further enhanced when cells were grown with 8 µg/mL FI3 along with tested antibiotics. The MIC values of tested antibiotics; Tet, Genta, and Van in combination with FI3 were now calculated as 62.5 ng/mL, 125 ng/mL, and 250 ng/mL, respectively. Spot assay further confirmed that the supplementation of FI3 together with tested antibiotics were noticeably more susceptible to Tet, Genta, and Van than the individual effects of the tested antibiotics (Figure 2b).

3.3. *Bactericidal activity of FI3*

To assess the bactericidal activity of FI3, mid-exponential phase cells were treated with different concentration of FI3 *i.e.* 0.5× MIC (16 µg/mL), 1× MIC (32 µg/mL), 2× MIC (64 µg/mL) and 4× MIC (128 µg/mL) for 30 min, 60 min, and 90 min of action time. Figure 3a illustrates that the FI3 treatment shows time and concentration-dependent of cell killing. For example, at 0.5× MIC concentration, 19%, 24%, and 53% cells were killed within 30 min, 60 min, 90 min, respectively whereas at 1× MIC, 38%, 67% and 96% cells were killed in 30 min, 60 min and 90 min, respectively. The cells were found to be completely eliminated with no significant viable cells (± 2.5%)

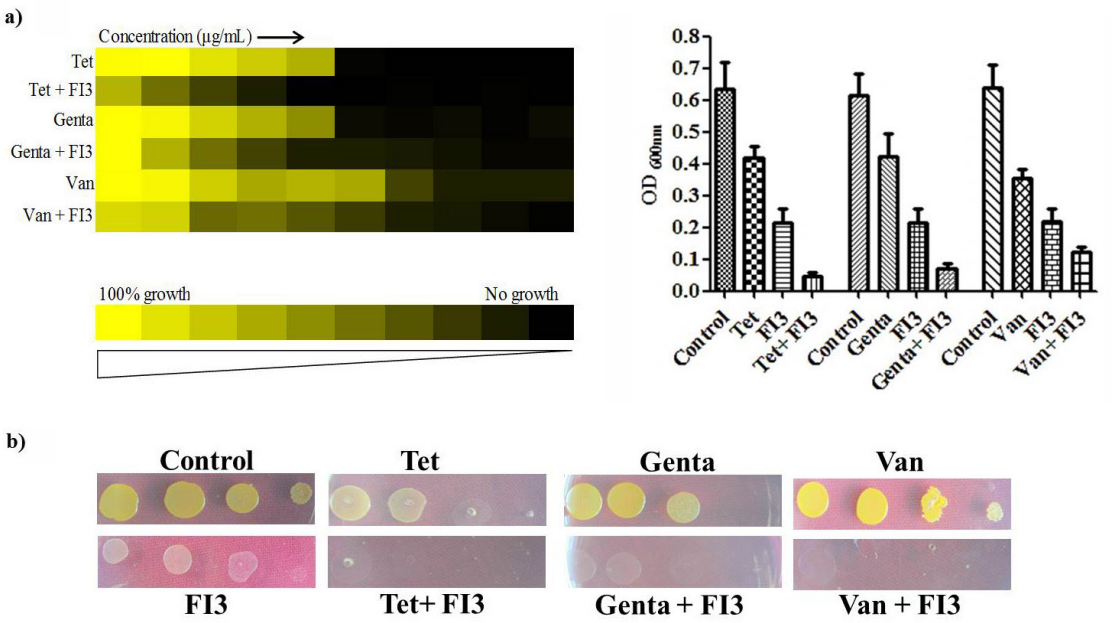


Figure 2. Combinatorial effect of FI3 with tropical antimicrobials. (a) Broth dilution assay to determine MIC value of tested antimicrobial drugs Tet (tetracycline), Genta (gentamycin), and Van (vancomycin) in absence and presence of FI3. Data was quantitatively showed with color (see the color bar in lower panel), where each shade of color represents relative optical densities of the cell and as bar graphs (see right panel). **(b)** Spot assays of *S. aureus* in presence of Tet, Genta, and Van (62.5 ng/mL, 62.5 ng/mL, and 125 ng/mL) alone and with FI3 (8 µg/mL).

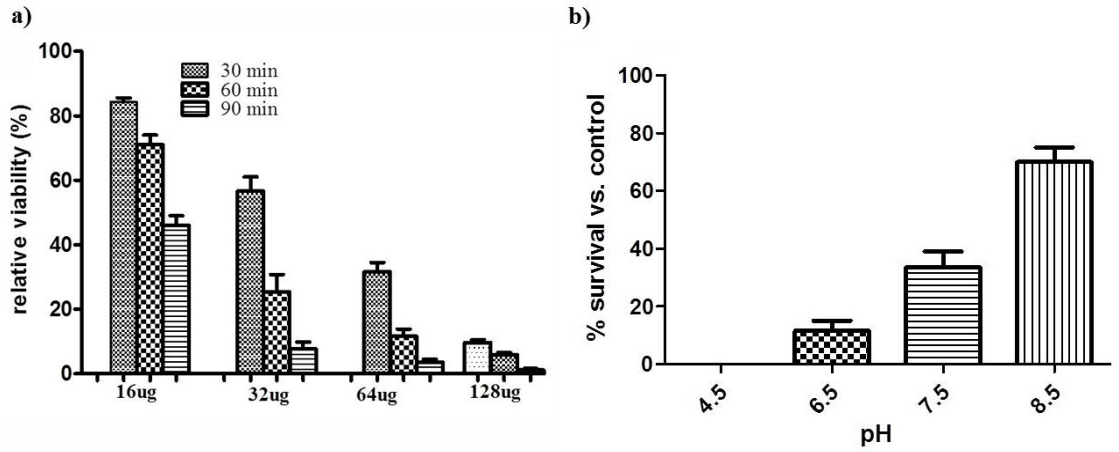


Figure 3. Antimicrobial activity of FI3. (a) Cell killing assay to determine the antimicrobial activity of FI3. Mid-exponential phase *S. aureus* cells were treated with 0.5×, 1×, 2× and 4× MIC *i.e.* 16 µg/mL, 32 µg/mL, 64 µg/mL, and 128 µg/mL, respectively. Cells were incubated up to 90 min at 30 min intervals. **(b)** *S. aureus* cells were grown till mid-log phase and cells were exposed to 16 µg/mL (0.5× MIC), a sub-inhibitory concentration of FI3 for 2 h at different pH.

within 90 min exposure of 1× MIC or at higher concentrations. These results ensure that the FI3 has a potent antimicrobial activity.

To access the effect of pH on cell susceptibility of *S. aureus* in response to FI3 treatment (0.5× MIC), cell viability assay was performed at varying pH ranging from 4.5 to 8.5. Figure 3b illustrates the impact of pH on the antimicrobial activity of FI3. Cells viability at individual pH was measured in presence or absence of FI3. Without FI3 treatment at different pH was considered as control (Ctrl). The relative cell viability (FI3/Ctrl) was analyzed at different tested pH for monitoring pH dependent effect of FI3. The data suggested that antimicrobial activity of FI3 is pH dependent and more susceptible at lower pH. The increased susceptibility at lower pH is expected to be increased a net positive charge on the molecule. This gain in the positive charge on FI3 molecule tends to interact to the negative charged cell membrane more firmly.

3.4. *Membrane permeability effect of FI3*

The staphylococcal cell permeabilization effect of FI3 was tested by quantification of preloaded calcein leakage *via* spectrofluorimetric analysis. Gramicidin B; a known pore-forming peptide was used as a positive control. For this, we performed cell permeability analysis by measuring calcein258 AM hydrolysis. Calcein AM is a cell-permeable, non-fluorescent dye which converts into a green fluorescent calcein after acetoxymethyl (AM) ester hydrolysis by intracellular esterase. When cells membrane becomes more permeable, more amount of calcein AM dye enters the cell and get hydrolyzed into membrane-impermeable green fluorescent calcein dye. Dead cells with compromised cell membrane do not retain calcein fluorescence inside the cell and release outside the cell membrane that can be measured at 515nm. Similar to the positive control, cells treated with 0.5× MIC FI3 (16 µg/mL) increased calcein fluorescence that indicates enhanced cell permeability. The cell permeability was further corroborated when cells were spotted with cell-disrupting chaotropic agent SDS and by PI uptake assay. Figure 4B illustrates that cells treated with SDS became hyper-sensitive in presence of FI3 and PI was 45% up taken upon treatment of 0.5× MIC FI3 as compared to positive control (Figure 4c).

3.5. *FI3 potentially clears up matured biofilm*

To consider the potential staphylocidal activity of FI3, we also considered the possibility that FI3 would also be effective against matured biofilm. *S. aureus* cells were grown in biofilm forming medium with the subsequent increasing order of FI3 concentration and biofilm mass was measured after 2 h of treatment. More than 90% biofilm formation was inhibited at 64 µg/mL (2× MIC) concentration (Figure 5a). FI3 showed a significant inhibitory activity against *S. aureus* biofilm formation at 48 h (data not shown). The biofilm inhibitory effect of FI3 was further confirmed by microscopic analysis. Cells treated with 1× MIC (32 µg/mL) and 2× MIC (64 µg/mL) concentration of FI3 reduce the biofilm cell aggregation (cleared up) approximately 40% and 89%, respectively as compared to untreated (control) (Figure 5b).

3.6. *Haemolytic effect of FI3*

The antimicrobial and antibiofilm activity of FI3 against both planktonic as well as surface-attached cells prompted us to further find out any toxic effect of FI3 on human tissue. This was achieved by using human as well as chicken blood tissues. For this, RBC cells were treated with subsequently increasing concentration of FI3. 0.9% NaCl and 1% Triton X-100 were taken as a negative and positive control respectively. The FI3 did not show any haemolytic effect on both human as well as chicken blood tissues as compared to tested control. This study suggests that FI3 is safer with low hemolytic

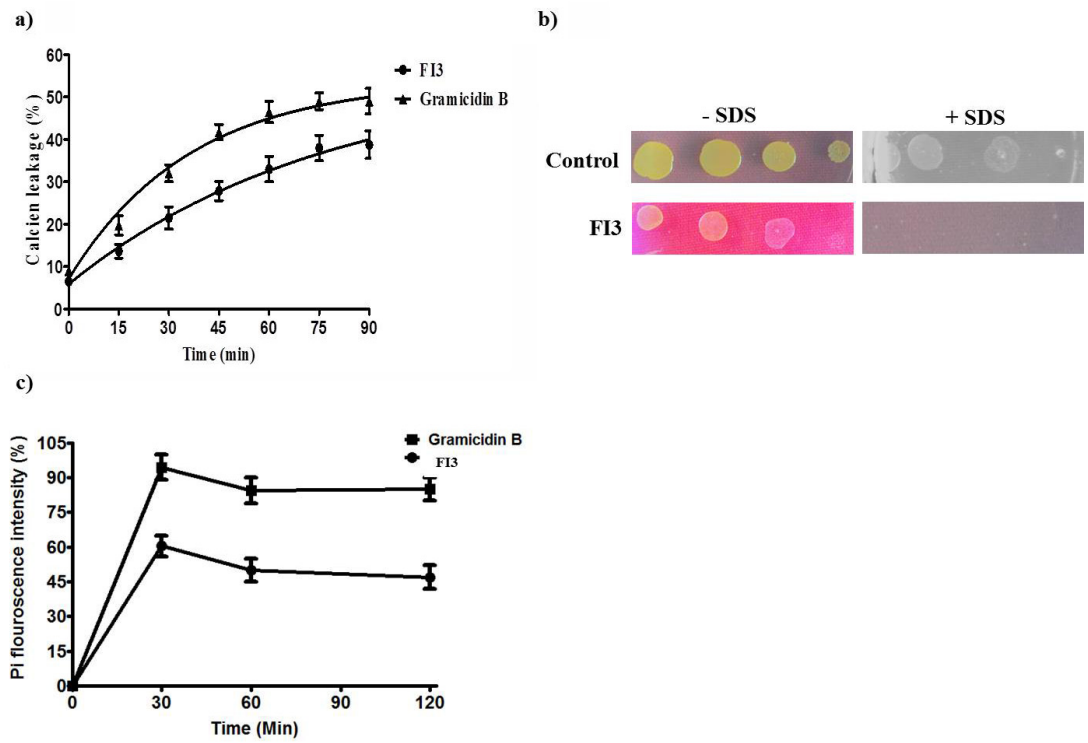


Figure 4. Cell membrane permeabilization activity of FI3 against *S. aureus*. (a) To assess the FI3 effect on membrane permeabilization, mid-log phase cells treated with 32 µg/mL (1× MIC) FI3 up to 90 min at 15 min intervals. 35 µg/mL gramicidin was used as a positive control. **(b)** Spot assay of *S. aureus* in the absence (negative control) and presence of 16 µg/mL (0.5× MIC), a sub-inhibitory concentration of FI3 and cell perturbation agent SDS at 0.03%. **(c)** PI uptake assay performs to determine cell deprivation in presence of 16 µg/mL, FI3 and 35 µg/mL, gramicidin B (positive control).

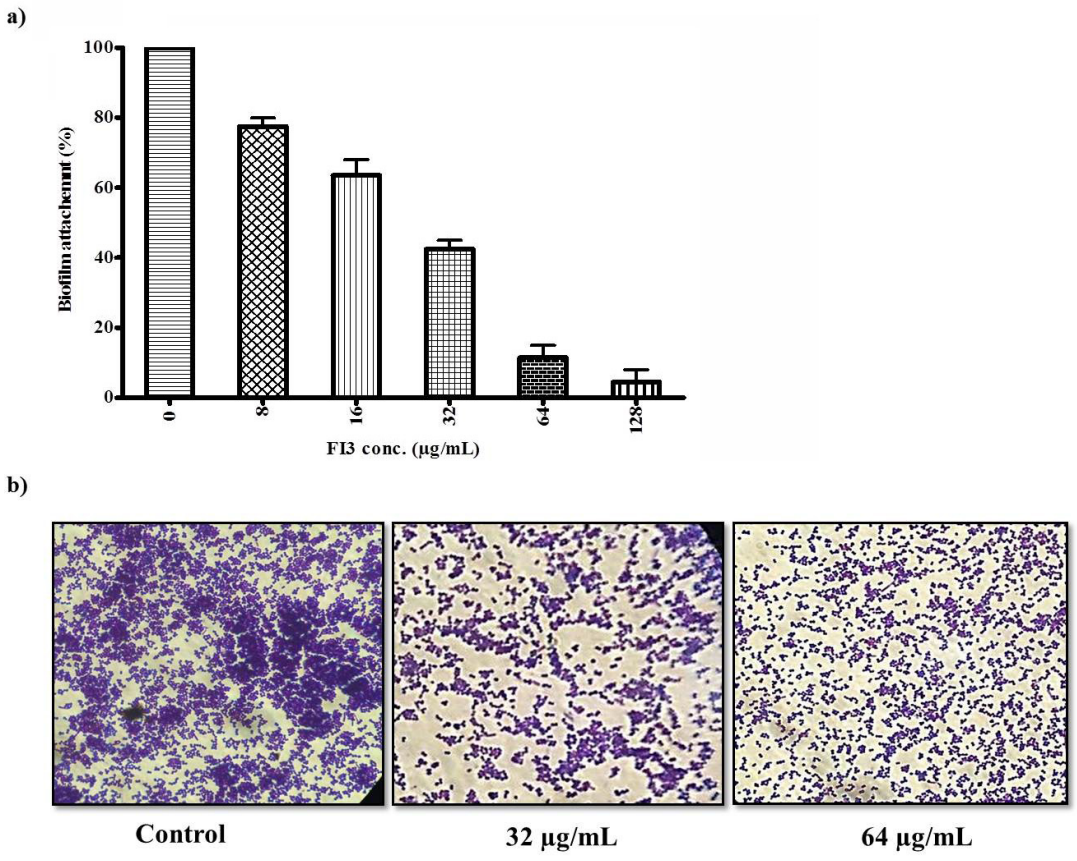


Figure 5. Anti-biofilm activity of FI3. The 2×10^5 cells/mL *S. aureus* cells suspended in the TSB-glucose medium, were seeded in pre- kept glass slide, in 24 well polystyrene plate and added different concentrations of FI3 (in triplicate) as indicated. The plate was incubated at 37°C for **(a)** 4 h for biofilm attachment analysis and **(b)** 48h for inhibition of biofilm formation.

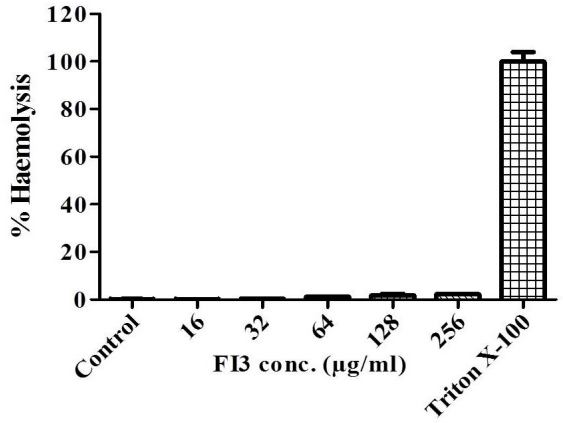


Figure 6. Hemolytic effect of FI3 on mammalian cells. FI3 toxicity was measured against human blood RBCs by measuring hemolytic activity as described elsewhere (Chopra *et al*., 2015). Triton X-100 as a positive control and NaCl (0.9%) as a negative control.

activity to the human as well chicken tissue as depicted in Figure 6.

4. Discussion

The prevalence of methicillin resistance *S. aureus* (MRSA) globally cause a major risk in the treatment of hospital-acquired (HA) and community-acquired (CA) infections (*35*) and become endemic in India (*15*). Rapidly emerging such threats and clinical complications in Staphylococcal infections demand the discovery of novel antibacterial candidates against both planktonic and drug resistant sessile bacteria. Since a long time, the discovery of new active compounds from natural sources has been explored and still gaining much attention. Natural products isolated from fungi have been represented one of the most successful source for the treatment of such infectious diseases. Indian geographical diversity is favorable to fungi which covers one-third of fungal diversity of the World. More than 75% of the total fungal species remain undiscovered and need to be explored against a plethora of pathogens especially multidrug-resistant microorganisms. The objective of the present study was to examine antibacterial properties of *A. nidulans* fungal xenobiotic in different micro-environmental conditions. The purified fungal xenobiotic (FI3) showed a strong antibacterial activity against different bacteria such as *P. aeruginosa* MTCC1034, *B. subtilis* MTCC441, MSSA and MRSA strains with a MIC 128 µg/mL, 256 µg/mL, 32 µg/mL and 128 µg/mL, respectively (Figure 1c). Similar antibacterial effect of natural products extract was earlier reported (*19,36,37*). Exploration of combined effects of two or more antimicrobials together can be an alternative strategy for the treatment of life-threatening multi-drug resistant *S. aureus* infections. The clinical impacts of such strategy are not only to synergize the bacteriostatic or bactericidal activities but also suppress the multi-drug resistance (MDR) problem in systemic *S. aureus* infections. Interestingly, FI3 was capable to synergize the antimicrobial effect up to 15 folds when it used in combination with Tet, Genta, and Van as compared to individual effects of the tested antibiotics (Figure 3). In addition, FI3 also has a strong antimicrobial activity that effect was observed dose-dependent. When *S. aureus* cells exposed with 16 µg/mL (0.5× MIC) FI3, 19%, 24%, and 53% cells were killed within 30 min, 60 min, 90 min, respectively whereas at 1× MIC (32 µg/mL), 38%, 67%, and 96% cells were killed in 30 min, 60 min and 90 min, respectively. FI3 completely eliminated with no significant viable *S. aureus* cells within 90 min exposure. The antimicrobial activity of FI3 was maximum at pH 4 compared to other tested pH (Figure 3b). The increased susceptibility at lower pH is expected to be increased a net positive charge on the molecule which promotes interaction with negatively charged cell surface thereby showed higher bactericidal activity. Similar results were observed when α-MSH (α-melanocyte stimulating hormone) peptide was tested against *S. aureus* at varying pH (*33*). The antimicrobial effect of FI3 was due to cell membrane disruptions which correlated by enhancement of cell permeability. The cell membrane permeability increased by 33% when cells were incubated for 90 min exposed with 0.5× MIC FI3. Such membrane disruption effect was further corroborated by cells spotted with cell-disrupting chaotropic agent SDS and PI uptake assay. In presence of SDS, FI3 made cells hyper-sensitive similar to the study reported by Pal *et al*. (*38*) and 45% higher PI was taken up upon treatment of 0.5× MIC FI3 as compared to positive control.

Formation of biofilm over the cell surface of *S. aureus* makes it difficult for antibiotics and immune cells to attack. Biofilm forming cells elicit several folds higher drug-resistant and adhered to implanted medical devices and damaged tissue and are one of the major attributes of acute and chronic infections (*39,40*). In this context, it is interesting to note that FI3 effectively inhibited biofilm formation of *S. aureus* and almost 89% biofilm was cleared up by the treatment of 64 µg/mL (2× MIC) FI3 (Figure 5a). A similar antibiofilm effect was also reported by Bakkiyaraj *et al*. (*19*). In contrary, ebselen that strongly shows antibacterial activity against *S. aureus* is strongly inhibited biofilm formation at 16× MIC concentration (*41*). It has also been remarkably noted further that FI3 showed low hemolytic effect on human blood tissues and safe to mammalian cells (Figure 6). Moreover, FI3 has a good bactericidal activity against both *S. aureus* strains with little difference in susceptibility to MSSA and MRSA strains. This high bactericidal activity of FI3 against both MSSA and MRSA strains may have tremendous therapeutic consequence.

Taken together, our results demonstrate that the purified FI3 of fungal *A. nidulans* shows a potent

bactericidal and anti-biofilm activity against tested microbes including *S. aureus*. FI3 rapidly ceases the growth of both the sensitive (MSSA) and drug-resistant (MRSA) *S. aureus*, without affecting *E. coli* growth. The growth of MSSA was rapidly ceased at 32 µg/mL (1× MIC) within 90 min. In spite of antimicrobial activity, biofilm formation of *S. aureus* was also remarkably inhibited at 64 µg/mL, without showing any hemolytic effect on human blood cells. Both bactericidal, antibiofilm activity of FI3 can be used for the treatment of bacterial infections and may be proven a leading molecule.

Acknowledgements

We thank to Prof. Rajendra Prasad, Amity University, Gurgaon, and Dr. Ajay Kumar Singh, Associate Professor, Department of Bioinformatics, for motivating us to initiate this study. The work presented in this paper has been supported in part by grants to A.K. from Science & Engineering Research Board (SERB), Department of Science & Technology (DST), India, [SB/EMEQ-278/2013, 29-10-2013].

References

1. Lowy FD. *Staphylococcus aureus* infections. N Engl J Med. 1998; 339:520-532.
2. Odell CA. Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) skin infections. Curr Opin Pediatr. 2010; 22:273-277.
3. Skiest DJ, Cooper TW. High recurrence rate of CA-MRSA skin and soft tissue infections. Arch Intern Med. 2007; 167:2527-2527.
4. Mohammad H, Thangamani S, Seleem MN. Antimicrobial peptides and peptidomimetics-potent therapeutic allies for staphylococcal infections. Curr Pharm Design. 2015; 21:2073-2088.
5. Peters PJ, Brooks JT, McAllister SK, Limbago B. Methicillin resistant *Staphylococcus aureus* colonization of the groin and risk for clinical infection among HIV-infected adults. Emerg Infect Dis. 2013; 19:623-629.
6. Chambers HF, DeLeo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol. 2009; 7:629-641.
7. Missiakas D, Schneewind O. *Staphylococcus aureus* vaccines: Deviating from the carol. J Exp Med. 2016; 213:1645-1653.
8. Spellberg B, Daum R. Development of a vaccine against *Staphylococcus aureus*. Semin Immunopathol. 2012; 34:335-348.
9. Koziel J, Maciag-Gudowska A, Mikolajczyk T, Bzowska M. Phagocytosis of *Staphylococcus aureus* by macrophages exerts cytoprotective effects manifested by the upregulation of antiapoptotic factors. PLoS One. 2009; 4:e5210.
10. Ohlsen K, Koller KP, Hacker J. Analysis of expression of the alpha-toxin gene (*hla*) of *Staphylococcus aureus* by using a chromosomally encoded *hla*::*lacZ* gene fusion. Infect Immun. 1997; 65:3606-3614.
11. Gilbert P, Allison DG, McBain AJ. Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? J Appl Microbiol. 2002; 92:98S-110S.
12. Kiedrowski MR, Horswill AR. New approaches for treating staphylococcal biofilm infections. Ann N Y Acad Sci. 2011; 1241:104-121.
13. Ceyssens PJ, Bambeke FV, Mattheus W, Bertrand S. Molecular analysis of rising fluoroquinolone resistance in Belgian non-invasive *Streptococcus pneumoniae* isolates (1995-2014). PLoS One. 2016; 26:e0154816.
14. McManus BA, Coleman DC, Deasy EC, Brennan GI. Comparative genotypes, Staphylococcal cassette chromosome mec (SCCmec) genes and antimicrobial resistance amongst *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* isolates from infections in humans and companion animals. PLoS One. 2015; 10:e0138079.
15. Klevens RM, Edwards JR, Richards CL, Horan TC. Estimating health care-associated infections and deaths in U.S. hospitals, 2002. Public Health Rep. 2007; 122:160-166.
16. Sunagar R, Hegde NR, Archana GJ, Sinha AY. Prevalence and genotype distribution of methicillin-resistant *Staphylococcus aureus* (MRSA) in India. J Glob Antimicrob Resist. 2016; 7:46-52.
17. Bouchiat C, El-Zeenni N, Chakrakodi B, Nagaraj S. Epidemiology of *Staphylococcus aureus* in Bangalore, India: emergence of the ST217 clone and high rate of resistance to erythromycin and ciprofloxacin in the community. New Microbes New Infect. 2015; 7:15-20.
18. Nagaraju U, Bhat G, Kuruvila M, Pai GS. Methicillin-resistant *Staphylococcus aureus* in community-acquired pyoderma. Int J Dermatol. 2004; 43:412-414.
19. Bakkiyaraj D, Pandiana STK. *In vitro* and *in vivo* antibiofilm activity of a coral associated actinomycete against drug resistant *Staphylococcus aureus* biofilms. Biofoul. 2010; 26:711-717.
20. Hertiani T, Edrada-Ebel R, Ortlepp S, Van Soest RW. From anti-fouling to biofilm inhibition: new cytotoxic secondary metabolites from two Indonesian *Agelas* sponges. Bioorg Med Chem. 2010; 18:1297-1311.
21. Samuel S, Mathew BS, Veeraraghavan B, Fleming DH. *In vitro* study of elution kinetics and bio-activity of meropenem-loaded acrylic bone cement. J Orthop Traumatol. 2011; 13:131-136.
22. Kumar A, Shukla S, Mandal A, Shukla S. Divergent signature motifs of nucleotide binding domains of ABC multidrug transporter, CaCdr1p of pathogenic *Candida albicans*, are functionally asymmetric and non-interchangeable. Biochim Biophys Acta. 2010; 1798:1757-1766.
23. Mandal A, Kumar A, Singh A, Lynn AM. A key structural domain of the *Candida albicans* Mdr1 protein. Biochem J. 2012; 445:313-322.
24. Singh M, Mukhopadhyay K. C-terminal amino acids of alpha-melanocyte-stimulating hormone are requisite for its antibacterial activity against *Staphylococcus aureus*. Antimicrob Agents Chemother. 2011; 55:1920-1929.
25. Clinical and Laboratory Standards Institute Performance standards for antimicrobial susceptibility testing. Nineteenth information supplements. CLSI documents M100-S19. 2009; CLSI, Wayne, Pa, USA.
26. Singh M, Gadepalli R, Dhawan B, Mukhopadhyay M. Combination of alpha-melanocyte stimulating hormone with conventional antibiotics against methicillin resistant *Staphylococcus aureus*. PLoS One. 2013; 8:e73815.

27. Chopra L, Singh G, Jena KK, Sahoo DK. Sonorensin: a new bacteriocin with potential of an anti-biofilm agent and a food biopreservative. Sci Rep. 2015; 5:13412.
28. Overhage J, Campisano A, Bains M, Torfs EC. Human host defense peptide LL-37 prevents bacterial biofilm formation. Infect Immun. 2008; 76:4176-4182.
29. Mataraci E, Dosler S. *In vitro* activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin resistance *Staphylococcus aureus* biofilms. Antimicrob Agents Chemother. 2012; 56:6366-6371.
30. Durham-Colleran MW, Verhoeven AB, Van-Hoek ML. *Francisella novicida* forms *in vitro* biofilms mediated by an orphan response regulator. Microb Ecol. 2010; 59:457-465.
31. Joshi S, Bisht GS, Rawat DS, Kumar A. Interaction studies of novel cell selective antimicrobial peptides with model membranes and *E. coli* ATCC 11775. Biochim Biophys Acta. 2010; 1798:1864-1875.
32. Mangoni ML, Carotenuto A, Auriemma L, Saviello MR. Structure-activity relationship, conformational and biological studies of temporin L analogues. J Med Chem. 2011; 54:1298-1307.
33. Madhuri, Shireen T, Venugopal SK, Ghosh D. *In vitro* antimicrobial activity of alpha-melanocyte stimulating hormone against major human pathogen *Staphylococcus aureus*. Peptides. 2009; 30:1627-1635.
34. Koo SP, Bayer AS, Yeaman MR. Diversity in antistaphylococcal mechanisms among membrane-targeting antimicrobial peptides. Infect Immun. 2001; 69:4916-4922.
35. Michael ZD, Robert SD. Community-associated methicillin-resistant *Staphylococcus aureus*: Epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev. 2010; 23:616-687.
36. Pannapa P, Bajaree J, Acharawan T, Pattra S. Evaluation of endophytic fungi extract for their antimicrobial activity from *Sesbania grandiflora* (L.) Pers. Int J Pharm Biomed Res. 2012; 3:132-136.
37. Liang H, Xing Y, Chen J, Zhang D, Guo S, Wang C. Antimicrobial activities of endophytic fungi isolated from *Ophiopogon japonicus* (Liliaceae). BMC Complement Altern Med. 2012; 12:238.
38. Pal R, Hameed S, Fatima Z. Iron deprivation affects drug susceptibilities of mycobacteria targeting membrane integrity. J Pathol. 2015; 2015:938523.
39. Tote K, Berghe DV, Deschacht M, de Wit K. Inhibitory efficacy of various antibiotics on matrix and viable mass of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. Int J Antimicrob Agents. 2009; 33:525-531.
40. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001; 358:135-138.
41. Thangamani S, Younis W, Seleem MN. Repurposing ebselen for treatment of multidrug- resistant staphylococcal infections. Sci Rep. 2015; 5:11596.

(Received May 30, 2018; Revised July 28, 2018; Accepted August 20, 2018)