Anti-methicillin Resistant *Staphylococcus aureus* Compound Isolation from Halophilic *Bacillus amyloliquefaciens* MHB1 and Determination of Its Mode of Action Using Electron Microscope and Flow Cytometry Analysis

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Abstract The aim of this study was to purify, characterize and evaluate the antibacterial activity of bioactive compound against methicillin-resistant *Staphylococcus aureus* (MRSA). The anti-MRSA compound was produced by a halophilic bacterial strain designated as MHB1. The MHB1 strain exhibited 99 % similarity to *Bacillus amyloliquefaciens* based on 16S rRNA gene analysis. The culture conditions of *Bacillus amyloliquefaciens* MHB1 were optimized using nutritional and environmental parameters for enhanced anti-MRSA compound production. The pure bioactive compound was isolated using silica gel column chromatography and Semi-preparative High-performance liquid chromatography (Semi-preparative HPLC). The Thin layer chromatography, Fourier transform infrared spectroscopy and proton NMR (¹H NMR) analysis indicated the phenolic nature of the compound. The molecular mass of the purified compound was 507 Da as revealed by Liquid chromatography-mass spectrometry (LC–MS) analysis. The compound inhibited the growth of MRSA with minimum inhibitory concentration (MIC) of 62.5 µg mL⁻¹. MRSA bacteria exposed to 4× MIC of the compound and the cell viability was determined using flow cytometric analysis. Scanning electron microscope and Transmission electron microscope analysis was used to determine the ultrastructural changes in bacteria. This is the first report on isolation of anti-MRSA compound from halophilic *B. amyloliquefaciens* MHB1 and could act as a promising biocontrol agent.

Keywords Saltpan · Halophilic *Bacillus amyloliquefaciens* · Phenolic compound · Anti-MRSA activity · Flow cytometry · Electron microscope

Introduction

Over the last few decades, the emergence of multidrug resistant pathogens imposes many health related problems in humans worldwide [1]. Among them, methicillin-resistant *Staphylococcus aureus* (MRSA) is an important nosocomial pathogen and has been associated with the major cause of skin and soft tissue infections [2]. MRSA was first reported in England [3]. Approximately 11,000 deaths were reported annually due to MRSA related diseases, including necrotizing pneumonia, osteomyelitis and sepsis [4]. There are two types of MRSA infection, hospital associated (HA) and community associated (CA) both differing in their epidemiology, DNA sequence, populations infected and sites of infection [5]. MRSA confers resistance to almost all commercially available antibiotics except vancomycin and teicoplanin [6, 7]. For many years, vancomycin has been used as the effective drug for MRSA infections. However, due to the emergence of vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA), the novel and potent anti-MRSA compounds are in urgent need to combat the pathogen.

Nowadays, the discovery of novel compounds from terrestrial sources have been decreased due to the re-isolation of known products. Hence, many investigators have focused on marine sources for the isolation of unusual natural products with a unique chemical structure [8, 9]. Moreover, from marine sources, many numbers of anti-MRSA metabolites have been isolated from different types

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of bacteria such as *Pseudomonas fluorescens*, *Actinomyces*, *Streptomyces*, *Marinispora* and *Bacillus* species. [10]. *Bacillus* sp. are ubiquitous in the marine environment and can exist under extreme conditions such as temperature, pressure, salinity and pH [11, 12]. Some anti-MRSA compounds were isolated from marine *Bacillus* sp. including Bogorol A, a cationic peptide antibiotic from *Bacillus laterosporus* PNG276 [13] and Loloatins A, B, C and D from *Bacillus* sp. MK-PNG-276A [14]. Phenolic compounds are a group of secondary metabolites that contain one or more hydroxy derivatives of benzene rings. These compounds exhibit diverse biological activities and significantly reduce the risk of some health related problems due to their antioxidant, antimutagenic, anti-inflammatory, antibacterial and antifungal properties [5]. Halophiles are salt-loving organisms that are able to survive in highly saline conditions and can be classified as slightly, moderately or extremely halophilic depending on their requirement for sodium chloride. Only a few reports are available on the isolation of anti-MRSA compounds from saltpan *Bacillus* species and it is yet to be thoroughly investigated for the discovery of novel compounds. Moreover, isolation of anti-MRSA compounds from halophiles of Indian saltpan have not been reported so far. With this background, we aimed to isolate anti-MRSA compound from bacteria at the Indian salt pan and to determine its mode of action.

Materials and Methods

Test Strain

MRSA strain was procured from the SRM Medical Research Centre (SRM MRC), SRM University, Chennai, India. Safety precautions were undertaken before handling the pathogen. The pathogenic strain was carefully transferred to the laboratory and Biosafety level 2 (BSL 2) cabinet was used throughout the experiment. The stock culture was maintained in Trypticase Soy Broth (TSB) medium (Hi-Media Laboratories, Mumbai, India) containing 20 % (v/v) glycerol at -70°C .

Preliminary Screening of Potent Isolate Against MRSA

The salt pan soils were collected near the Tuticorin coastal region (lat $8^{\circ} 76' \text{N}$ and long $78^{\circ} 15' \text{E}$), south east coast of India. A total of 324 colonies was isolated using Zobell Marine Agar 2216 medium (Hi-Media Laboratories, Mumbai, India) [15]. In the dual culture assay [16], the pure colony of all isolates were streaked perpendicularly on

the Mueller–Hinton Agar (MHA, Hi-Media Laboratories, Mumbai, India) plates swabbed with the MRSA bacterial suspension (5×10^5 CFU/mL). The plates were incubated at 37°C for 24 h and the zone of inhibition was measured. Among these, MHB1 isolate showed efficient anti-MRSA activity and determined for its tolerance to salinity with different sodium chloride (NaCl) concentrations (0, 5, 10, 15, 20, 25 and 30 %). The morphological and biochemical characteristics of MHB1 were determined according to the Bergey's manual of systematic bacteriology. The 16S rRNA gene sequence of MHB1 isolate was submitted to NCBI GenBank under the accession number KC952012 [gi 507718894].

Optimization, Extraction and Purification

The MHB1 isolate was optimized on Zobell Marine Broth (ZMB) 2216 medium (Hi-Media Laboratories, Mumbai, India) using various parameters like temperature (25 to 40°C), pH (5 to 12), NaCl (0 to 30 %), incubation period (1 to 5 days), 1 % (w/v) carbon (glucose, starch, fructose, xylose, maltose and sucrose) and 0.1 % (w/v) nitrogen (Beef, yeast, peptone, ammonium chloride, sodium nitrate and malt extract) sources [17]. The Carbon/Nitrogen (C/N) ratio was optimized for the production of maximum anti-MRSA compound. The optimized culture was then centrifuged at 8000 rpm for 20 min at 4°C . The cell-free supernatant (CFS) of 100 mL was extracted with an equal volume of solvent gradients, hexane, ethyl acetate, dichloromethane, chloroform and methanol. The solvent layers were concentrated using a rotary evaporator. The ethyl acetate extract showed the highest anti-MRSA activity as compared to other solvent extracts. Further, shake-flask method was used for the mass cultivation of MHB1 in optimized ZMB medium (8 L) and the cell-free supernatant (CFS) was extracted with an equal volume of ethyl acetate (8 L). The solvent layer was again concentrated using a rotary evaporator and the crude extract of about 1.2 g was obtained. It was then subjected to silica gel column (32.5 cm $\times$ 2.2 cm, 60–120 mesh, Hi-Media Laboratories, Mumbai, India) chromatography using the stepwise gradient of hexane and ethyl acetate (100:0, 80:20, 60:40, 40:60, 20:80, 0:100) as eluents. The fraction that showed activity against MRSA was further purified using Semi-preparative High-performance liquid chromatography (Semi-preparative HPLC) using ZORBAX Eclipse XDB-C18 column (9.4 $\times$ 250 mm, Agilent Technologies, USA) at a flow rate of 3 mL/min [18] with a solvent gradient of 50 % methanol, 50 % water to 100 % methanol over 30 min to obtain four fractions. The collected fractions were then concentrated using a freeze dryer and checked for anti-MRSA activity.

Chemical Characterization of Anti-MRSA Compound

The Thin layer chromatography (TLC Silica gel 60 F254, Merck Millipore manufacturers, Germany) was carried out using Benzene: methanol (2:1) and sprayed with the ferric chloride reagent. The infrared (IR) spectrum was measured on a Cary 600 series Fourier transform infrared spectroscopy (FTIR) spectrophotometer (Agilent Technologies, USA). High-performance liquid chromatography (HPLC) analysis was performed on ZORBAX Eclipse plus C18 column (4.6 × 250 mm, Agilent Technologies, USA) with a photodiode array detector. Accurate mass measurements were performed on the Shimadzu LCMS-2020 mass spectrometer equipped with an electrospray ionization (ESI) source (Shimadzu Scientific Instruments, Columbia, Maryland, USA). Lab solution software was used for data acquisition and processing. The proton nuclear magnetic resonance (^1H NMR) spectra was recorded at 500 MHz using a Bruker AC-300 spectrometer (Agilent Technologies, USA) with tetramethylsilane (TMS) as an internal reference and dimethyl sulfoxide (DMSO) as solvent.

In Vitro Susceptibility Testing

According to the Kirby-Bauer disk diffusion method [19], the discs were impregnated with the 15 μg of crude and purified compound and was placed onto MHA plates swabbed with the bacterial suspension (5×10^5 CFU/mL). The zone of inhibition was measured after incubation at 37 °C for 24 h. The Minimum inhibitory concentration (MIC) of bioactive compound was investigated as per the National Committee for Clinical Laboratory Standards (NCCLS) [20]. For determining the MBC, the MIC cultures were plated on Nutrient Agar (Hi-Media Laboratories, Mumbai, India) and incubated at 37 °C for 24 h.

Time Kill Studies

Time-kill studies were done as described previously [21, 22]. The 2× and 4× MIC of purified compound was added separately to the MRSA culture density of 10^7 CFU/mL and incubated at 37 °C. MRSA without compound was used as the growth control. At 2 h time intervals for a period of 24 h, the samples were withdrawn, serially diluted, plated on MHA and incubated at 37 °C for 24 h. Rates of killing were determined by measuring the reduction in viable bacteria ($\log_{10}$ CFU/mL). The killing curve was constructed by plotting numbers of viable cells against time. All experiments were performed in triplicates.

Electron Microscopic Analysis

The ultrastructural changes in MRSA induced by purified compound were determined by Scanning electron microscope (SEM) and Transmission electron microscope (TEM) analysis [23]. The MRSA strain containing 10^7 CFU/mL was treated with 4× MIC of compound for 2 h at 37 °C. The untreated bacteria was used as the negative control. The bacterial suspensions were placed onto the glass coverslip fixed with 2 % glutaraldehyde for 1 h. It was then washed thrice with distilled water and post fixed with 0.2 % osmium tetroxide for 1 h. Then the samples were dehydrated with ethanol series (30, 50, 70, 85 and 100 %) and air dried. Finally, the ultrastructural changes in MRSA cells were examined under SEM using Quanta 200 FEG (FEI company, USA) at an accelerating voltage between 2 kV and 19 kV under standard operating conditions. For TEM analysis, the sample preparation was described as above for SEM and the cells were examined by using FEI-Philips TECHNAI 10 TEM (FEI company, USA) under standard operating conditions.

Flow Cytometry Analysis

The cell viability of MRSA treated purified compound was determined by using BD FACScalibur flow cytometry system (BD Biosciences, USA) [24]. The bacterial suspension of 10^7 CFU/mL was treated with 4× MIC of compound for 30 min, 1 h and 2 h. For negative control, the untreated (live) bacterial suspension was used. The bacterial cells were then harvested, washed and resuspended in phosphate buffer saline (PBS). The propidium iodide (PI, Sigma-Aldrich, USA) was added to the cell suspension (final concentration 2 $\mu\text{g/mL}$) and incubated at room temperature for 15 min to allow dye uptake. The dead bacteria are stained fluorescent red by PI and was collected using a 617 nm band-pass filter. The BD CellQuest pro software was used for data analysis.

Results

Identification of MHB1 Isolate with Anti-MRSA Activity

Among the 324 isolates from saltpan soils, MHB1 isolate showed potent anti-MRSA activity with a zone of inhibition of 14 ± 1 mm using the dual culture assay (Fig. 1). The MHB1 was identified as *Bacillus amyloliquefaciens* based on its morphological, biochemical characteristics and 16S rRNA sequencing analysis. To check the tolerance to salinity, *B. amyloliquefaciens* MHB 1 was grown in ZMB media with different salt (NaCl) concentrations ranging

Fig. 1 Antagonistic activity of potent isolate MHB1 using the dual culture assay

from 0 to 30 %. The bacteria tolerated the NaCl concentration at 8 % and hence it was determined to be extreme halophile. The maximum anti-MRSA activity of this halophilic *B. amyloliquefaciens* was attained when ZMB was optimized with 1 % (w/v) fructose, 0.1 % (w/v) yeast, which corresponded to a C/N ratio of 30.76, temperature at 32 °C, pH 8.0 and 12 % NaCl. After 48 h of incubation, the activity was increased and remained stable between 48 and 72 h and then remarkably decreased after 72 h.

Purification of Anti-MRSA Compound

The crude extract (~1.2 g) obtained from *B. amyloliquefaciens* was loaded onto silica gel column with hexane and ethyl acetate as eluting solvents. Totally, 54 fractions were collected. The collected fractions were pooled together

based on Retention factor (R_f) value of TLC and the pooled fractions were labeled as fraction A, B, C, D, E, F, G. The TLC analysis of all pooled fractions showed the presence of compound mixtures when observed under UV light at 254 nm (Fig. 2a). The R_f values for each fraction obtained as follows: Fraction A ($R_f = 0.975$), Fraction B ($R_f = 0.95, 0.825, 0.725$), Fraction C ($R_f = 0.825, 0.725$), Fraction D and E ($R_f = 0.725, 0.625$), Fraction F ($R_f = 0.725, 0.625, 0.55$) and Fraction G ($R_f = 0.55$). Among all fractions, the potent anti-MRSA activity was observed in fraction D. For the second step purification of active fraction D, Semi-preparative column was used and four fractions labeled as FD1, FD2, FD3 and FD4 were collected. Among these, the highest activity was observed in FD2.

Compound Characterization with Spectroscopic Analysis

The TLC analysis of fraction FD2 ($R_f = 0.725$) showed the presence of single band (Fig. 2 (ii)) with yellow colour spot when sprayed with ferric chloride reagent and indicated the presence of phenolic derivative compound. The UV spectrum showed absorbance at 314 nm. Figure 3 represents the HPLC chromatogram of purified compound and the single peak was observed with the retention time of 9.77 min. Based on LC–MS analysis, the molecular weight of the phenolic compound was identified as 507 Da (Fig. 4). The small additional peak with 413 Da was obtained from parent molecular ion. Owing to the electron impact on parent compound, it undergoes fragmentation reaction and produces the stable fragment of 413 Da with low intensity. The possible explanation of low intensity is that the low relative abundance and stability of the fragment. The FTIR spectrum (Fig. 5) showed a broad stretching peak at 3379.4 cm^{-1} which clearly indicated the

Fig. 2 TLC analysis of (a) pooled fractions A, B, C, D, E, F, G and (b) pure compound in fraction D2 (FD2). The band with R_f value of 0.725 showed the efficient anti-MRSA activity as compared with other bands

Fig. 3 HPLC profile of the purified bioactive compound

Fig. 4 LC–MS analysis of bioactive compound with molecular weight of 507 Da. The small peak with 413 Da was attained from parent compound with low intensity

Fig. 5 FTIR spectrum of the anti-MRSA compound from MHB1 isolate

presence of the hydroxyl (–OH) group. In ^1H NMR spectrum (Fig. 6), the chemical shift was appeared in the range of $\delta = 8\text{--}6$ ppm, which confirmed the presence of –OH group on the chemical structure of compound and it is aromatic in nature. Hence, overall spectral analysis revealed the presence of phenolic nature of the compound.

Anti-MRSA Activity of Phenolic Compound

The phenolic compound was tested against MRSA and the zone of inhibition measurement was used to evaluate its antagonistic activity. Figure 7 shows the zone of inhibition of 17.66 ± 0.57 mm for pure compound as compared to

Fig. 6 ^1H NMR spectrum of the antibacterial compound in DMSO

that of the crude which was, 8.33 ± 1.15 mm. The MIC of the purified compound with $62.5 \mu\text{g/mL}$ showed efficient activity and was found to be bactericidal as $\text{MBC}/\text{MIC} \leq 2$. Figure 8 represents the time-kill curves for phenolic compound against MRSA. Maximum killing was observed in 4 h, at concentrations of $4 \times \text{MIC}$ or higher. It caused a 2-log reduction in the number of CFU/mL after compound addition. Killing was less rapid with $2 \times \text{MIC}$ of the same compound for which more than 6 h was required to cause a 2-log drop in the numbers of CFU/mL.

Mode of Action

The SEM and TEM analysis of MRSA cells treated and untreated with phenolic compound were shown in Fig. 9. SEM Micrographs (Fig. 9A) showed the surface of the untreated MRSA cell was smooth and showed the typical cocci shape. After treatment with $4 \times \text{MIC}$ of compound for 2 h, cells showed the blebs formation on its surfaces (Fig. 9B). TEM Micrographs (Fig. 9c) showed the normal cell shape with an undamaged structure and the inner and outer membranes remained intact. In treated cells, the membranes were damaged severely (Fig. 9d) and the cell contents were expelled into the extracellular space.

Flow cytometry analysis was performed to determine the anti-MRSA effect of $4 \times \text{MIC}$ of compound for 30 min, 1 h, 2 h (Fig. 10). The bacterial cell membrane was disrupted and the nucleic acid dye PI penetrated the membrane and was taken up by the damaged cells. Hence, these cells emitted red fluorescence and the live cells emitted green fluorescence. Based on the side light scatter and PI, the R2

and R3 gates were used to identify live and damaged or dead cells, respectively. Initially, the percentage of live cells was about 99.76 %. But after treatment it was reduced to 87.72 % in 30 min, 53.37 % in 1 h and 42.89 % in 2 h.

Discussion

Nowadays, the emergence of drug resistant pathogens has become a worldwide health problem. Among them, MRSA has been considered as an important nosocomial pathogen among the human population [1, 2]. The thick cell wall of *S. aureus* confers resistance towards all β -lactam antibiotics, including methicillin, oxacillin, penicillin and amoxicillin [25]. The increase in resistance of MRSA to 'last resort' antibiotics (vancomycin and teicoplanin) has created a pressing need in the development of novel antibiotics [7]. Previous studies reported the isolation of anti-MRSA substances, especially from marine *Pseudomonas* species. Darabpour et al. [26] collected the sediment samples from Persian Gulf and isolated the *Pseudoalteromonas piscicida* PG-01 showing potent anti-MRSA activity. Similarly, *Pseudoalteromonas phenolica* O-BC30 [7] and *Pseudomonas* sp. UJ-6 [27] were isolated from sea water and their inhibitory activity was evaluated towards MRSA. Surprisingly, for the first time, the extreme halophile *B. amyloliquefaciens* MHB1 was isolated from marine saltpan and was found to inhibit the MRSA pathogen. Saltpan microbes remains unexplored in the isolation of novel secondary metabolites against drug resistant pathogens.

Fig. 7 In vitro anti-MRSA activity of crude and purified compound by disk diffusion method

Fig. 8 Time kill curves with MRSA strain. Bactericidal activity of phenolic compound at multiples of the MIC. Symbols: ▲ growth control; ■ compound 2× MIC; ● compound 4× MIC

It was previously reported the structurally diverse anti-MRSA secondary metabolites such as Abyssomicin C, Bogorol A, 2,4-Diacetylphloroglucinol, Fijimycin A, Lipoxazolidinone A–C, Loloatins A–D, MC21-A, Marinopyrroles A, Marinopyrroles B and Moiramides B have been isolated from different marine bacteria [10]. Herein, the purified metabolite was isolated from saltpan bacteria and its maximum anti-MRSA activity was attained by optimizing the culture condition in ZMB broth. It has been reported that the culture optimization in media using different parameters including temperature, pH, incubation period, carbon and nitrogen sources influenced the

production of the antimicrobial compound [28]. In the current study, the nutritional and environmental parameters were optimized in ZMB medium for the efficient production of anti-MRSA compound. The optimized C/N ratio of 30.76, temperature at 32 °C, pH at 8.0 and NaCl at 12 % in the ZMB medium enhanced the anti-MRSA activity. Further, extraction and chromatographic purification steps resulted in the isolation of purified compound which belonged to the phenolic group based on TLC, LC–MS, ¹H NMR and FTIR analysis. Earlier studies revealed that this phenolic group of compounds exhibited antagonistic activity against MRSA. Mohammad et al. [4] synthesized the different phenylthiazole compounds and tested for MRSA with MIC determination. Patel et al. [5] synthesized the phenolic group of compounds with a unique chemical structure and checked against a panel of clinically relevant MRSA strains obtained from American Type Culture Collection (ATCC). Most of the synthesized compounds were found to have bactericidal activity at concentrations of two to fourfold higher than their MIC. In general, the chemically synthesized compounds cause hazards to humans and to the environment and hence naturally obtained compounds would be more beneficial. To our knowledge, this is the first report on purification of anti-MRSA compound from naturally derived saltpan source, *B. amyloliquefaciens*.

The bacterial cell membrane is responsible for many essential functions such as transport, osmoregulation, respiration processes, biosynthesis and cross-linking of peptidoglycan and synthesis of lipids. The membrane integrity is important for all these functions and its disruption causes metabolic dysfunction, which lead to the cell death [23]. In the present study, the ultrastructural changes in MRSA pathogen after treatment with 4× MIC of phenolic compound was analyzed by Electron microscope. Both SEM and TEM analysis revealed the efficient activity of the purified compound on MRSA, especially in TEM, the cell membrane damage was observed in 2 h. It was found that such membrane damage would promote the penetration of compound leading to the disruption of inner cell cytoplasmic contents. Some researchers have already reported the SEM and TEM analysis of bacterial cell damage by antimicrobial peptides gramicidin S and the peptidyl-glycylleucine-carboxamide (PGLa). SEM analysis showed the formation of blisters on the surface of *Escherichia coli* and deep craters in *S. aureus* whereas in TEM analysis, numerous bubbles protruded from the cell surface of *E. coli* and the non-membrane-enclosed bodies were observed in *S. aureus* [23]. Several groups of natural or semi-synthetic anti-MRSA agents including ceragenins, lipopeptides, lipoglycopeptides and glycopeptides that target the bacterial membrane with different mechanisms of action were identified [29]. Flow cytometry is a sensitive

Fig. 9 SEM and TEM images of phenolic compound treated and untreated MRSA pathogen. Untreated MRSA (a) and (c) cells are compact and evenly shaped. After treatment with $4\times$ MIC of compound for 2 h, cell surfaces showed the blebs (b) formation and cell membrane was damaged (d) leading to the release of cytoplasmic contents to the outer space which were indicated by arrows. The width of the images corresponds to a distance of $3\text{ }\mu\text{m}$ (a), $1\text{ }\mu\text{m}$ (b), $0.5\text{ }\mu\text{m}$ (c), $2\text{ }\mu\text{m}$ (d)

Fig. 10 Flow cytometric analysis represents the dot plot profiles of MRSA cells treated with $4\times$ MIC of phenolic compound for 30 min, 1 h and 2 h. For negative controls, suspension of fresh live (untreated) cells were analyzed. The percentages of live (green fluorescence) and dead cells (red fluorescence) are mentioned. All of the profiles were analyzed with gates placed on R2 and R3. SSC-H, side-scatter height (color figure online)

technique that allows us to determine the viability of bacteria on a single cell level using fluorescent probes [30]. Many investigators have used this method to study the interaction between antibacterial agents and bacteria. The cell viability of antibiotics (amoxicillin, tetracycline and erythromycin) treated *S. aureus* and *Micrococcus luteus* were studied by Novo et al. [24] using flow cytometric analysis. In the above analysis, the fluorescent probe TO-PRO[®]-3 was used for binding to the damaged cells. Higgins et al. [31] studied the disruption of cell membrane integrity induced by Telavancin (lipoglycopeptide) in MRSA by using two nucleic acid stains SYTO9 and PI. SYTO9 is a membrane-permeable dye that causes the emission of green fluorescence in viable cells with intact membrane. In contrast, PI is a membrane-impermeable dye that binds to the dead cells with compromised membranes and causes emission of red fluorescence. In our study, the cell viability was determined using nucleic acid probe, PI by flow cytometry based on different time intervals. After treatment with phenolic compound, the cell membrane got depolarized, triggered the release of potassium (K⁺) ions and increased the uptake of PI. The damaged MRSA cells emitted red fluorescence as a result of PI intercalation with the nucleic acids. The results obtained from this study are promising, however; further studies are required to elucidate the structure of this bioactive compound.

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