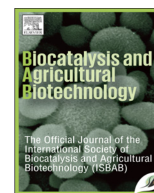
Biocatalysis and Agricultural Biotechnology 17 (2019) 750–757

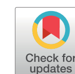
Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab



Extraction and partial purification of secondary metabolites from endophytic actinomycetes of marine green algae *Caulerpa racemosa* against multi drug resistant uropathogens



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ARTICLE INFO

Keywords:
Multi drug resistant bacteria
Actinomycetes
Thin layar chamatogaraphy
Minimum inhibition concentration
Scanning electron microscope

ABSTRACT

The aim of this study is to identify potential antibacterial compounds of endophytic actinomycetes (EA) from marine algae *Caulerpa racemosa* for inhibit the multi drug resistant (MDR) Gram negative bacteria (GNB) including *Eschericia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Proteus mirabilis* (*P. mirabilis*), *Klebsiella pnumonia* (*K. pnumoniae*) and *Enterobacter* sp., which were confirmed by UTI panel antibiotic discs. Among the 100 EA, five strains were showed better antogonistic activity against all the selected uropathogens. In the ethyl acetate extract, the NMS 5 strains of EA showed excellent anti-bacterial activity against all the MDR uropathogen. Further, the NMS 5 extract was partially purified from thin layar chramatogaraphy (TLC) and spot 3 of TLC crude compound showed 14, 12 mm zone of inhibition against both the uropathogens. In addition, the minimum inhibition concentration (MIC) of crude exhibited the inhibition percentage was 87 and 84 at 120 µg/mL concentration. The MIC was confirmed by minimum bactericidal concentration (MBC) and it was indicated same as MIC. The treated NMS 5 crude compound of *P. aeruginosa* and *K. pneumonia* cells were showed more dead cells, intracellular damage and morphological damage with disaggregated cells by confocal laser scanning electron microscope (CLSM) and scanning electron microscope (SEM). Hence, the present study was revealed that the algae derived EA have the potential anti-bacterial metabolites for inhibition of MDR bacteria and it can be applied for various other infections after further purification.

1. Introduction

Increasing antibiotic resistance bacteria in urinary tract infections (UTIs) are serious problem and greatest challenge in public health care (Khawcharoenporn et al., 2013). It refereed as the evolution of microorganisms such as bacteria, fungi, viruses and parasites that developed resistant nature to fight and neutralize an antimicrobial agent (Tenney et al., 2018; Mihankhah et al., 2017). In 2014, World health organization (WHO) was organized a global antimicrobial resistance (AMR) surveillance (Exner et al., 2017). The result of AMR surveillance was documented that the antibiotic resistance is a global threat to human health due to the development of resistance to one or more antibiotics like MDR (Exner et al., 2017; Gupta et al., 2017). For instance, in Gram positive bacteria (GPB), *Staphylococcus aureus* (*S. aureus*) are frequently developed resistant to methicillin and referred as methicillin-resistant *S. aureus* (MRSA) (Nepal et al., 2017). In case of (GNB), 60% of the bacteria are developed more resistant to all the current antibiotics and all classes of antibiotics including carbapenems fluoroquinolones cephalosporin (Hawkey et al., 2018; Rajivgandhi et al., 2019). All the GNB are communicated each other by quorum sensing, a signaling factors helps to die the infected materials in one bacteria to other and enter inside the cell at the transcriptome level (Barros et al., 2018). According to the WHO report in 2017, these bacteria are categorized in three groups based on the antibiotic resistance and termed as critical, high and medium priority (WHO, 2017). In the critical category *Acinetobacter*, *Pseudomonas* and various Enterobacteriaceae (including *Klebsiella*, *E. coli*, *Serratia*, and *Proteus* (Kumar et al., 2015; Shaikh et al., 2015). The excessive, unprescribed format and inappropriate dosages of antibiotic have led to spread of the various types of resistant organisms (Eshetie et al., 2015). Sometimes, it is evolved due to the major virulence factors like efflux pump, biofilm formation, β-lactamases and ureases (Rajivgandhi et al., 2018a, 2018b, 2018c, 2018d, 2018e, 2018f). Recent prediction of AMR report, worldwide annually deaths will be reach 10 million in 2050 due to he caused by MDR infections

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https://doi.org/10.1016/j.bcab.2019.01.016
Received 13 August 2018; Received in revised form 6 January 2019; Accepted 8 January 2019
Available online 09 January 2019
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(Ramírez-Castillo et al., 2018). There have been significant attempt to enhance novel classes of anti-microbial agents by various natural sources that altering the existing antibiotics, and synthesizing anti-microbial peptides.

Recent advancement in marine pharmacology propose different sources to evolve new formulations are based on the various types of secondary metabolites and flexible antimicrobial properties (Yang and Song, 2018). Among the various marine environment, actinomycetes produced more than 10,000 of bioactive compounds, are mainly derived from the Genus of *Streptomyces* (Rodríguez et al., 2018). The balance 25% of the bioactive materials are derived from rare actinomycetes (Subramani and Aalbersberg, 2013). Likewise, EA is categorized in rare actinomycetes group, it grow from inside the plant and algae (Matsumoto and Takahashi, 2017; Shan et al., 2018). EA and their compounds led to the production of new classes of antibiotics are in drug discovery programs, importantly to compete infections caused by MDR bacteria (Wahaab and Subramaniam, 2018). EA has acquired additional nutrients, carbon and various nitrogen sources from plant and algae derived materials by basic mutualistic mechanism (Tanvir et al., 2013). However, very limited researches are going on the field of EA from marine algae and discovery of their respective compound for MDR bacteria. Due to this defect, we have desired to investigation of novel compounds from EA may overcome these resilient bacteria in UTIs. Previously, the compounds of alnumycin (Bieber et al., 1998), munumbicins A–D (Castillo et al., 2006), coronamycins (Ezra et al., 2004), anthraquinones (Li et al., 2015a, 2015b) were reported from various EA *Streptomyces* sp. Very recently, Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (Rajivgandhi et al., 2018a, 2018b, 2018c, 2018d, 2018e, 2018f), 1, 4-diaza-2, 5-dioxo-3-isobutyl bicyclo[4.3.0]nonane (Rajivgandhi et al., 2019) and Bis (2-Ethylhexyl) Phthalate (Rajivgandhi et al., 2018a, 2018b, 2018c, 2018d, 2018e, 2018f) were reported from EA of *Nocardiopsis* sp. From the previous reports, we have confirmed that the EA is very effective marine bacteria and it can able to inhibit all types of human infections.

Thus, the focus of this study is to isolate EA from marine algae *Caulerpa racemosa* and purify their bioactive compounds for inhibit the MDR bacteria related to antibiotic resistance nature in UTIs.

2. Materials methods

2.1. Collection of samples

The young healthy green algae *Caulerpa racemosa* was collected from Gulf of Mannar region (Latitude 915'41.8800N, Longitude 7904'05.8100E), Rameswaram, Southeast coast of Tamil Nadu, India. The collected algae sample was covered with sterile zip lock to avoid contamination. The sample was taken to the laboratory with ice box. The algae sample was thoroughly washed with tap water for detachment of the free floating organisms means of epiphytes. After, the sample was initially surface sterilized with double distilled water 2 times between the time interval of 5 min and stored in 4 °C for further use.

2.2. Isolation of endophytic actinomycetes

The EA were isolated from *Caulerpa racemosa* by using actinomycete isolating agar (AIA) with the following method of Bibi et al. (2017). The collected algae samples were cut aseptically with 10 mm pieces by using sterile scale pale and washed with 70% ethanol for 5 min followed by sodium hypochloride for 30 min. After chemical treatment, the surface sterilized samples were rinsed thoroughly with sterile double-distilled water for 5 min, to remove unwanted surface contaminants. After washing, all the samples were dried and inoculated on the AIA plates. All the plates were incubated at 28 °C for 5–10 days. After 10 days incubation, powdery white, grayish white, pale yellow white color colonies were screened and stored in starch casein agar plates at 4 °C for further use.

2.3. Validation of endophytic actinomycetes

The isolated actinomycetes colonies grown out from the inoculated algae sample pieces or not was validated by validation process using International Strptomyces Project Medium (ISP-2) agar plates as the following method of Zin et al. (2017). Briefly, the unsterilized algae samples was directly inoculated on ISP-2 medium and last wash water sample 0.1 mL was spread on the ISP 2 media separately. Both the samples were maintained at 28 °C for 24 h. After incubation, no any microbial growth of the last wash water inoculated ISP 2 plates were considered as endophytes. In parallel, to found the surface contaminating microbes (Various morphology was observed both in macroscopic and microscopic analysis) with fungal contamination of unsterilized algal samples were also confirmedas endophytes.

2.4. Collection and identification of multi drug resistant bacteria

100 strains of GNB including *E. coli*, *P. aeruginosa*, *P. mirabilis*, *K. pnumoniae* and *Enterobacter* sp. (each five) which cause UTIs and obtained from Department of Microbiology, K.A.P.V. Government Medical College, Tiruchirappalli, Tamil Nadu, India. The morphological identification and biochemical characterization of selected bacteria were confirmed in our laboratory. After confirmation, all the bacteria were detected for their antibiotic resistance pattern using specific UTI panel discs of HX12 & HX053 including imipenom (IPM-10 μg), aztreonam (AT-30 μg), cefoperazone/ sulbactum (CFS-75/10 μg), piperacill/tazobactam (PIT-100/10 μg), ceftazidime (CAZ-30 μg), netillin (NET-30 μg) and cefotaxime (CAZ-30 μg), ciproflaxacin (CIP-5 μg), co-trimoxazole (COT-25 μg), gentamycin (GEN-10 μg), imipenem (IPM-10 μg), ticarcillin/clavulanic acid (TCC 75/10 μg) by Kirby-Bauer disc diffusion method as followed by Maruthupandy et al. (2018).

2.5. Primary screening of eandophytic actinomycetes

The primary screening of isolated EA strains were evaluated using the conventional cross-streak method (Balachandar et al., 2018) for detection of their antagonistic effect. The isolated actinomycetes strains were individually streaked at the centre of a muller hinton agar plates and incubated for 7 days at 28 °C. After showed with ribbon like clear actinomycete growth, the 24 h cultures of selected uropathogens were streaked perpendicularly (90º angles) and the plates were incubated at 37 °C for 24 h. After incubation, the zone of inhibition was measured from the positive actinomycetes strains and recorded for further use. The result was followed by the evidence of bergey's manual and indicated as following manner, − = no activity; + = weak activity (< 25% inhibition); + + = moderate activity (25–50% inhibition); and + + + = good activity (> 50% inhibition). Based on the broad spectrum activity, potential anti-bacterial activity of isolated EA strains were selected for this study. After, the culture filtrate of active strain was obtained from 7 days incubated starch casein broth and diluted with equal volume (1:1 ratio) of various organic solvents including ethyl acetate, alcohol, dichloromethane, chloroform and methanol and shaken for 30 min. After 30 min, the active metabolites were extracted and performed against selected uropathogens (Rajivgandhi et al., 2016).

2.6. Fermentation and extraction of bioactive compounds

Based on the primary screening with antagonistic activity, the excellent activity of EA strain was chosen for detection of their antimicrobial metabolites with active solvent of ethyl acetate by liquid-liquid extraction method (Rajivgandhi et al., 2018a, 2018b, 2018c, 2018d, 2018e, 2018f). The active EA strain was fermented in starch casein broth (SCA) and incubated at 28 °C for 15–20 days. To construct

a library of the secondary metabolites,were centrifuged at 10,000 rpm for 15 min. The pellet and supernatant were collected separately in beaker. After, the supernatant was recovered by using filtrated by Whattman No.1 filter paper. After filtration, the equal volume of ethyl acetate 1:1 (w/v) was mixed with the filtrate and shaken vigorously at 2 h for complete liquid-liquid extraction. From the extraction, the organic layer of the extract was collected and separated from aqueous layer using separating funnel. The collected organic layer was incubated at 30–45 °C with evaporation for receiving active metabolites and dissolved in DMSO for further investigation.

2.7. Partial purification of anti-bacterial compound

The DMSO mixed dried crude extract of the active actinomycete strain was partially purified by thin layer chromatography (TLC) using silica gel for TLC plate (Kumar et al., 2018). Briefly, 0.1 mL of mixed crude extract sample was spotted in 10 different TLC plate. The spotted plate was optimized by ascending solvent method using toluene-chloroform-methanol (5:8:3 v/v), chloroform-methanol (24:1, v/v), chloroform-ethyl acetate (7.5:17.5, v/v), methanol-dichloromethane-water (1:1:1, v/v) and benzene-acetic acid-water (4:1:5, v/v). After 30 min, all the spots were scrapped separately and stored for further use.

2.8. Secondary screening of endophytic actinomycetes

All the partially purified TLC elutions were separately tested against MDRs uropathogens of *E. coli*, *P. aeruginosa*, *P. mirabilis*, *K. pnumoniae* and *Enterobacter* sp. by agar well diffusion method (Siddharth and Vittal, 2018). Briefly, 24 h culture of all the MDR strains were spread on MHA plates and wells were cut into the plate. After, various concentration of partially purified elutions were added into each well. Whereas, the solvent ethyl acetate acted as a primary control and ceftazidime used as a positive control. Then, all the plates were incubated at 37 °C for 24 h. After incubation, the plates were observed with inhibition zone against the uropathogens, and the elution spots with highest zone was considered as an active compound spot. Finally, the active TLC spots were merged and dried for further use.

2.9. Determination of MIC

The lowest concentration of the dried TLC compound that inhibit the maximum growth of selected MDRs was refereed as MIC and tested by micro broth dilution method using ELIZA reader with 96 well polystyrene plate (Rajivgandhi et al., 2018a, 2018b, 2018c, 2018d, 2018e, 2018f). Briefly, various concentration (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μg/mL) of crude TLC compound was added into sterile tryptic soy broth containing 96 well plates before filled 100 μg/mL of MDR uropathogens. The final concentration was made at 300 μL. Whereas, the bacterial culture of fresh TSB without addition of crude compound was acted as a control. All the plates were shaken well and then incubated at 37 °C for 24 h. After 24 h incubation, no any visible growth containing wells of the plate with very least concentration was recorded and indicated as MIC. After, all wells of the 96-well plates were read with UV spectrophotometer at 570 nm for detection of percentage of inhibition. The results were calculated based on the control or treated plates and the percentage of inhibition (PI) was also calculated by triplicate value with following equation

$$PI = (\text{Control OD } 570\,\text{nm} - \text{Test OD } 570\,\text{nm})/\text{Control OD } 570\,\text{nm} \times 100.$$

2.10. Determination of MBC

The detection of MBC value in the TLC crude compound was detected according to the standard method by Miyasaki et al. (2013). After 24 h incubation, aliquot the 10 μL of the all MIC concentration, which showed no visible bacterial growth were taken from treated bacteria and streaked on fresh MHA plate. All the plates were incubated at 37 °C for 24 h. After incubation, the results were identified based on the bacterial growth development on MHA plates. MBC was considered as, both MIC and MBC values are equal or higher than MIC.

2.11. Live/dead cells identification

Based on the antibacterial effect, the detection of live/dead cells differentiation in crude TLC compound treated MDRs strains of *K. pnumoniae* and *Enterobacter* sp were visualized by CLSM (Rajivgandhi et al., 2019). Briefly, the cover glass slide were kept in the MIC treated uropathogenic culture in 6 well plates and incubated at 37 °C for 24 h. After incubation, the cover glasses were washed with three times of sterile PBS. The dried cells were stained with 10 mg/mL of acridine orange/ethidium bromide (AO/EtBr) for 15 min under dark condition. The stained bacterial cells were analyzed under CLSM analysis (Carl Zeiss, Jena, Germany) at 488 nm and a HeNe laser at 543 nm.

2.12. Scanning electron microscope analysis

The morphological differentiation of crude TLC compound treated MDRs *K. pnumoniae* and *Enterobacter* sp were analyzed by SEM (Das et al., 2017**)**. Briefly, the potential MIC of active EA crude compound was exposed to selected MDRU in 6 well plates. All the plates were incubated at 37 °C for 24 h. After incubation, the samples were centrifuged at 10,000 rpm for 15 min and the pellets were collected after discarding of supernatant. The pellets were washed three times with 10 mM sodium phosphate buffer (pH 7.4) and centrifuged again at 5000 rpm for 30 min with PBS. After centrifugation, the PBS was discarded in both the samples and pellets were collected. After, pellets of the cells were fixed with 50 μL of 2.5% glutaraldehyde for 2 h in 4 °C and washed three times using phosphate buffer solution (PBS). Then, the cells were dehydrated using various ethanol graded series (0%, 5%, 10%, 15%, 25%, 35%, 54%, 55%, 65%, 75%, 85%, 95% and 100%) and seen under SEM using an accelerating voltage of 10 kV. The cells were dried in cover glasses and coated with gold-palladium metal (60:40 alloys). The samples were visualized on a Cambridge Stereo scan 200 scanning electron microscope (model: VEGA3 TESCAN, Brno, Czech Republic) using an accelerating voltage of 20 kv.

3. Result

3.1. Isolation and validation of endophytic actinomycetes

The EA strains were isolated from the green algae of *Caulerpa racemosa* (Fig. 1a) and the EA strains were confirmed by their morphological, sporulation and pigmentation characters. After 7 days incubation, 100 strains of clear white, dried with powdery colonies and pale yellow color actinomycete colonies were observed in the AIA plates (Fig. 1b, c). All the isolated strains were streaked separately on fresh AIA plates and then streaked on SCA slants for pure culture identification. In validation, the last wash of surface sterilized *Caulerpa racemosa* water sample streaked plate showed no any contamination were observed in the ISP-2 plates (Fig. 1d). It clearly noticed that the screened isolates were emerged from the internal tissue of the algae and proved as an endophyte. Whereas, unsterilized algae inoculated control plates were indicated that the ISP plates showed actinomycetes growth with fungal contamination (Fig. 1e). The algae based endophytic nature contribute in metabolic pathways and enhance the potential of metabolites production with some own genetic information (Gong et al., 2018; Rajivgandhi et al., 2018a, 2018b, 2018c, 2018d, 2018e, 2018f). Hence, the overall result was confirmed that the isolated actinomycete strains were endophytes. Recently Rajivgandhi et al. (2019) reported that the marine algae derived actinomycetes has more antibacterial and cytotoxicity effect and its produced potential secondary metabolites due

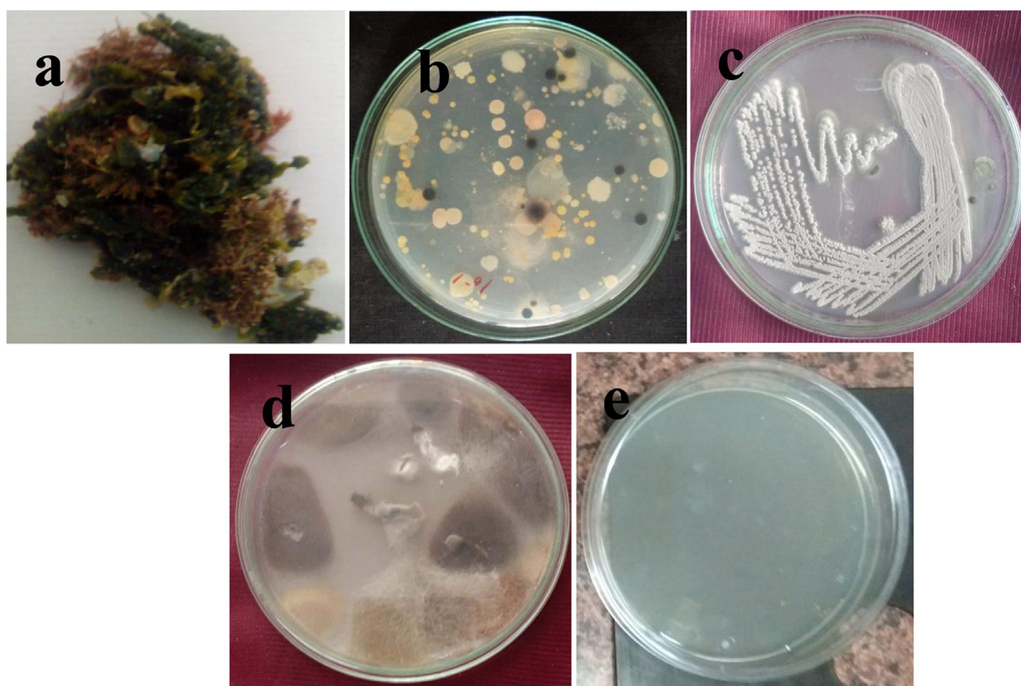

Fig. 1. Isolation and validation of endophytic actinomycetes from marine algae *Caulerpa racemosa* (a), isolation of endophytic actinomycetes in AIA plate (b), pure culture of actinomycetes (c), no microbial growth was present in the last wash sample streaking plate (d) and unsterilized algal sample of actinomycetes with fungal contamination (e).

to the received various nutrients from algae.

3.2. Collection and identification of multi drug resistant bacteria

Based on the Bergey's manual, the obtained bacterial samples were checked in our laboratory and confirmed as all are same and GNB. After confirmation, the selected bacteria and its multi drug resistance characteristic nature were confirmed by using specific HEXA UTI discs method. After 24 h incubation, 2, 3, 4, 2, 0 strains of *E. coli*, *P. aeruginosa*, *P. mirabilis*, *K. pnumoniae* and *Enterobacter* sp. were showed multi drug resistant activity, respectively. Based on the WHO report, we have chosen "Critical 1" category of the each one MDR *P. aeruginosa* and *K. pnumoniae* for this study and were showed highly resistant to all tested antibiotics. Both the bacteria were exhibited at 10, 6, 18, 24, 11, 7, 9, 4 mm inhibition zone against AT, CAZ, NET and IMP, respectively (Fig. 2a,c). Whereas, 2, 5, 2 mm zone of inhibitions were observed against CIP, GEN and TOC of *P. aeruginosa* (Fig. 2b) and 10, 2 mm zone of inhibition against COT and GEN of *K. pneumonia* (Fig. 2d). All other antibiotics did not produced any inhibition zone against both the uropathogens were also clearly observed. Based on the CLSI guidelines, partial zone of inhibition around the tested antibiotics were considered as sensitive against both the uropathogens (Rajivgandhi et al., 2018a, 2018b, 2018c, 2018d, 2018e, 2018f). The inhibition zones were not attained the inhibition of uropathogens due to the production of efflux pump, enzyme production, QS inhibition and biofilm formation (Fair and Tor, 2014). The other tested uropathogens *E. coli*, *P. mirabilis*, *Enterobacter* sp. were more sensitive to tested antibiotics were also observed. All the results were proved that the *P. aeruginosa* and *K. pneumonia* has developed more resistance against all current antibiotics than other uropathogens and it was correlated with WHO report (WHO, 2017; Barros et al., 2018). Hence, we have chosen these two MDR bacteria *P. aeruginosa* and *K. pneumonia* for further inhibition study.

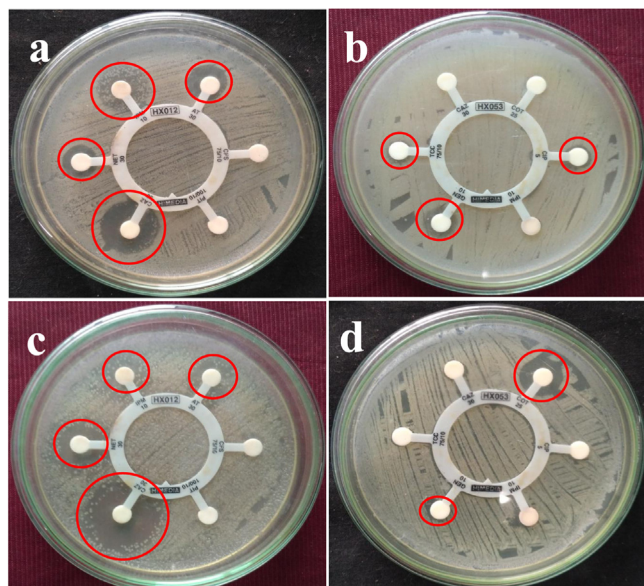

Fig. 2. Detection of multi drug resistant effect of *P. aeruginosa* (a, c) and *K. Pneumoniae* (b, d) against HX012 and HX053, respectively.

3.3. Antagonistic activity of isolated EA strains

Among the 100 isolated EA strains, 5 EA strains were only showed excellent antagonistic activity against selected MDRs pathogens of *P. aeruginosa* and *K. pnumoniae.* The other actinomycetes were not produced better antagonistic activity against tested uropathogens. Based on the zone variation, these five EA strains were chosen for further studies and these strains were named as NMS 1, NMS 2, NMS 3, NMS 4 and NMS 5 (Table 1). In primary screening, the culture filtrate of NMS 5 strains were showed excellent inhibitory activity against both the uropathogens (Fig. 3) in ethyl acetate than other EA strains. The other

Table 1
Identification of endophytic actinomycetes.

S. no.	Strains	Antagonistic activity	Category
1	NMS 1	Better Activity	+
2	NMS 2	Moderate activity	+ +
3	NMS 3	Moderate activity	+ +
4	NMS 4	Moderate activity	+ +
5	NMS 5	Excellent Activity	+ + +

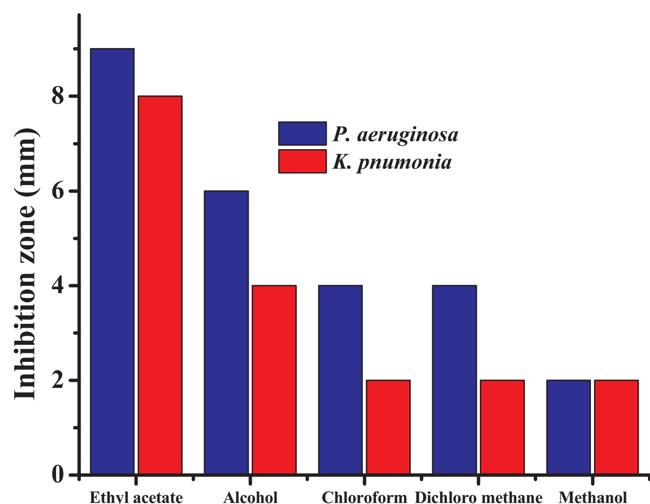

Fig. 3. Antibacterial activity of NMS 5 culture filtrate against selected MDR uropathogens using various solvents.

solvents filtrate of all the strains were exhibited very low zone of inhibition against both the uropathogens. The variation of antibacterial activity was shown in Fig. 3b. Therefore, EA NMS 5 was chosen for this study.

3.4. Extraction and partial purification of active EA NMS 5 strain

The potential strain of NMS 5 was extracted with equal volume of ethyl acetate by liquid -liquid extraction method (Fig. 4a, b). After 20 days incubation, the dried crude compound was collected and diluted with ethyl acetate 0.1 mg/mL of the diluted crude compound was partially purified by TLC using the various mobile solvent system. Among the all solvent system, toluene-chloroform-methanol (5:8:3 v/v) was exhibited with 3 active spots. All the three spots were collected separately for further use. Among the three spots, spot 2 and spot 3 were showed 16, 11, 14, 12 mm of inhibitory activity against both the tested MDRs (Fig. 5a–c) and RF value was 0.28 and 0.26 respectively. Whereas spot 1 was not showed any inhibition activity against both the tested uropathogens. Based on the antibacterial activity, the spot 2 and spot 3 were merged each other and purified large quantity by preparative TLC used as same mobile phase (Fig. 4c). The result was confirmed that the active secondary metabolites against both the MDR

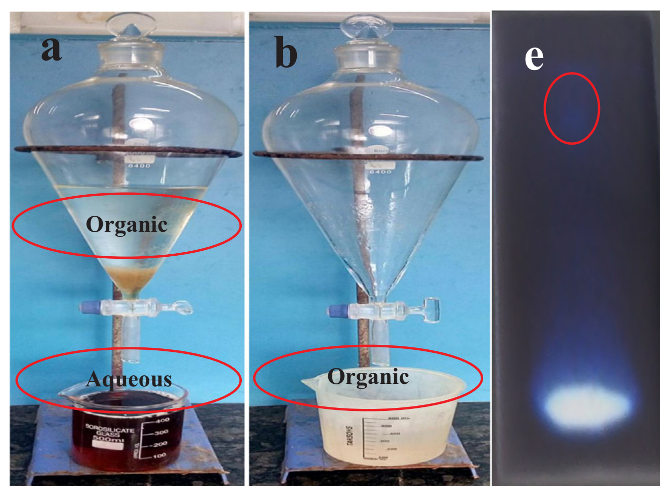

Fig. 4. Extraction of anti-bacterial metabolites from EA NMS 5 strain by ethyl acetate aqueous phase of the EA NMS strain (a), organic phase (b) and merged active TLC spot of EA NMS 5 extract (c).

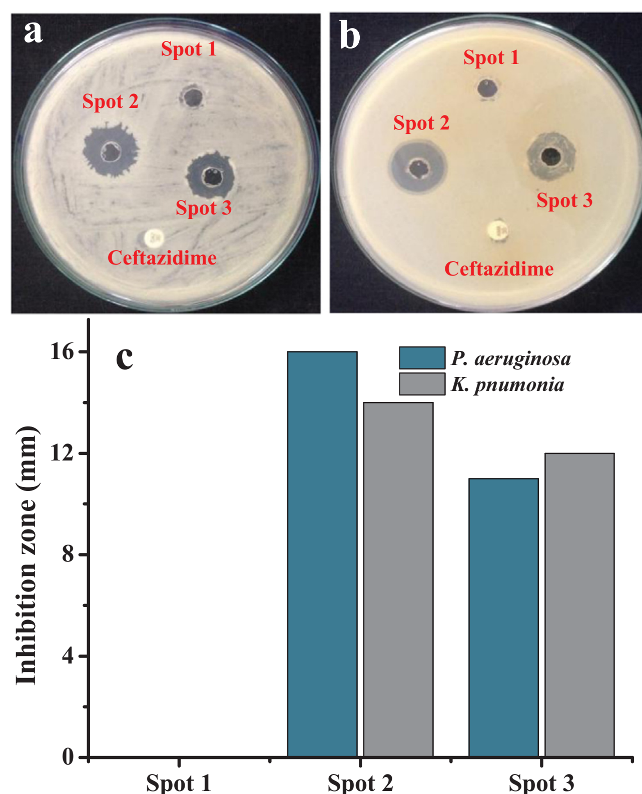

Fig. 5. Anti-bacterial activity of merged TLC spot against *P. aeruginosa* and *K. Pneumoniae* (a) and differentiation between all the 3 spots against both the uropathogens (b).

bacteria were present in the partially purified compound. In addition, some alkaloid and flavonoid contents of the chemical constituents of the NMS 5 crude compound was may influence the inhibition role in selected MDRs. Partially purified TLC extract has the ability to interfere the bacterial multiplication and metabolic pathway (Gos et al., 2017).

3.5. Minimum inhibition concentration of EA NMS 5

After 24 h incubation, the MIC assay of NMS 5 crude compound was consistently showed excellent antogonistic activity against both the uropathogens in increasing concentration. All the concentrations were showed and confirmed based on the turbidity. Based on the turbidity, the 120 µg/mL concentration was showed highest inhibition against both the uropathogens. Followed the growth of turbidity, all the inhibition concentration was also measured by spectrophotometer for detection of inhibition percentage. Initially, 57% and 58% of inhibition was observed at 70 µg/mL, and highest inhibition of 87% and 84% was observed at 120 µg/mL concentration (Fig. 6a). Therefore, the MIC of NMS 5 crude compound against both the uropathogens was 120 µg/mL concentration. This concentration was very low compared with previous report of algae derived actinomycetes (Djinni et al., 2014; Xia et al., 2014). Based on the inhibition concentration, previously reported marine endophytic actinomycetes against various bacteria was presented in Table 2. From the observed result of turbidity and percentage were reflected that at MIC, the crude compound of TLC was potential to fighting with both the uropathogens at very lowest concentration. The turbidity of the bacterial growth was indicated that the oxidation-reduction process performed due to the influence of NMS 5 crude compound (Passari et al., 2015). It also indicate the cell lysis and damage were showed in the turbidity wells. Even, the conformation of live or dead cells and morphological damage were observed in microscopical observations (Valle et al., 2015).

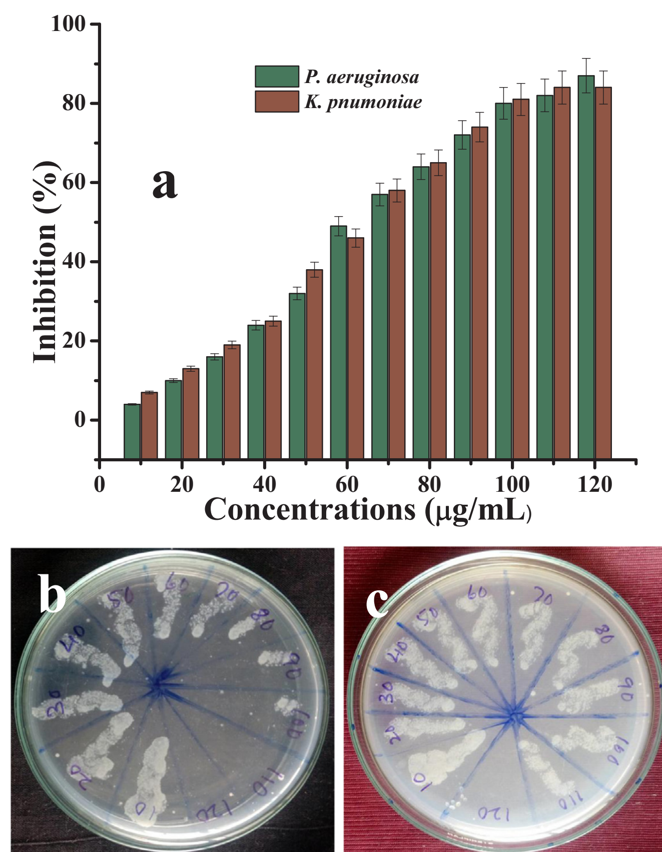

Fig. 6. Minimum inhibition concentration (a) and minimum bactericidal concentration of purified NMS 5 crude compound against *P. aeruginosa* (b), *K. Pneumoniae* (c).

3.6. Minimum bactericidal concentration of EA NMS 5

The MBC was also performed by inoculating the MIC dilutions for avoid of misinterpretation. In our result of MBC was expressed as same as MIC in both the bacteria (Fig. 6b, c). In the concentration of 120 µg/mL of MHA plates were showed no any bacterial growth. The MBC result was also supported the MIC and it proved that the NMS 5 crude compound had an antibacterial activity with concentration dependent. Hence, both the MIC and MBC result was confirmed at 120 µg/mL of NMS 5 crude compound fixed as an efficient concentration for further inhibition study.

3.7. Confocal laser scanning electron microscope analysis of EA NMS 5

The live /dead cells of *P. aeruginosa* and *K. pneumonia* against potential MIC of NMS 5 crude compound treatment was determined by CLSM. After 24 h treatment, the collapsed cells with loose arrangement of both treated bacteria were observed at MIC of 120 µg/mL. Whereas, smooth and clear morphology of control plate of the both bacteria were also observed. Further, the red color images of the cells clearly confirmed that the intensity of EB bind with damaged cell membrane and surface of unattached cells (Fig. 7e, f). Whereas, the untreated cells were up-taken AO stain and emitted green color, it proved that the cells were tightly packed and showed with closely associated morphology (Fig. 7a, b). The negative images of CLSM revealed the NMS 5 crude compound was strongly crashed the cell arrangement in treated bacteria (Li et al., 2015a, 2015b). Due to the dispersion of NMS 5 crude compound in the treated cells were revealed that the crude compound had potential antIbacterial agent against selected MDR uropathogens.

3.8. Scanning electron microscope analysis of EA NMS 5

The alteration of cell membrane integrity in treated or untreated bacterial cell were clearly showed by SEM. After 24 h incubation, the MIC of NMS 5 crude compound was exhibited damaged and disconnected cells in the surface membrane of treated bacteria. The continuous leakage of cellular material, distorted cell membrane and loosely aggregates cells were observed after dehydration of treated cells (Fig. 8b, d). While the untreated control cells were showed with tightly closed cell aggregates (Fig. 8a, c). Hence, the SEM result also more evidenced that the aggregation of both uropathogens was totally altered due to the MIC of NMS 5 crude compound (Li et al., 2013).

4. Conclusion

According to the WHO in 2017, antibiotic resistance due to the Enterobacteriaceae is increasing global thread for public health. All countries have focused on this problem, which is serious threat to modern medicine. In addition, the antibiotic resistant bacteria is considered as list of very “Critical” category, which are very sensitive to current antibiotics. To overcome this problem, we need to discover new classes of antibiotics form various unexploited sources. The marine environment is a alternative sources for these problems and it produced various pharmacological bio-potential for inhibition of various infections producing microorganisms. In this vast blender, marine EA are supreme secondary metabolites producer and purified antibiotics of EA was currently in preclinical and clinical evaluation, others show promising biological activities in vitro and in vivo assays, and others are making significant contributions to our understanding of cellular processes at the biochemical level. The result of the this study reinforce the importance of the analyzed marine actinomyctes as a potential source of novel bioactive compounds for the treatment of MDR *P. aeruginosa*, *K. pnumonia* and also related infectious diseases. Hence, the present result was justify, further chemical analysis should be performed on EA NMS 5 strain to detect their chemical composition and identify the exact phytocompounds responsible for anti-bacterial activity.

Table 2
Previous reports of marine algae derived actinomycetes against multi drug resistant bacteria.

Anti-bacterial activity of marine endophytic actinomycetes compound against various bacteria			
S. no.	**Bacteria/Others**	**MIC**	**Refs.**
1.	Methicillin resistant *Staphylococcus aureus* (MRSA)	3.12–12.5 µg/mL	Xu et al. (2018)
2.	*Aeromonas hydrophilia, Escherichia coli, Micrococcus luteus, Staphylococcus aureus, Vibrio arveyi, V. parahaemolyticus*	0.25–64 µg/mL	Meng et al. (2015)
3.	*Proteus mirabilis* and *Eschericia coli*	100 µg/mL	Rajivgandhi et al. (2018a, 2018b, 2018c, 2018d, 2018e, 2018f)
4.	Methicillin resistant *Staphylococcus aureus* (MRSA)	200 mg/mL	Djinni et al. (2014)
5	*Mycobacterium tuberculosis* protein tyrosine phosphatase B (MptpB)	8.70 µM	Xia et al. (2014)
6.	*Pseudomonas aeruginosa, Klebsiella pneumonia,*	120 µg/mL	Present Study

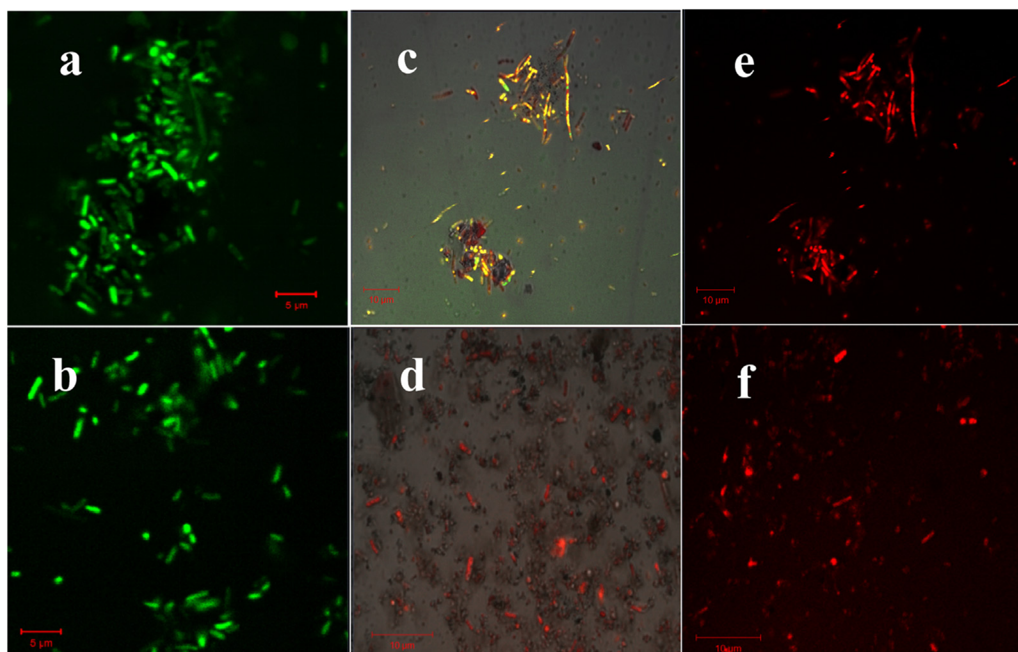

Fig. 7. CLSM images of controls (a, b) and treated (e, f) of *P. aeruginosa* and *K. Pneumoniae* by purified NMS 5 crude compound. Inhibition effect of both uropathogens in negative images (c, d).

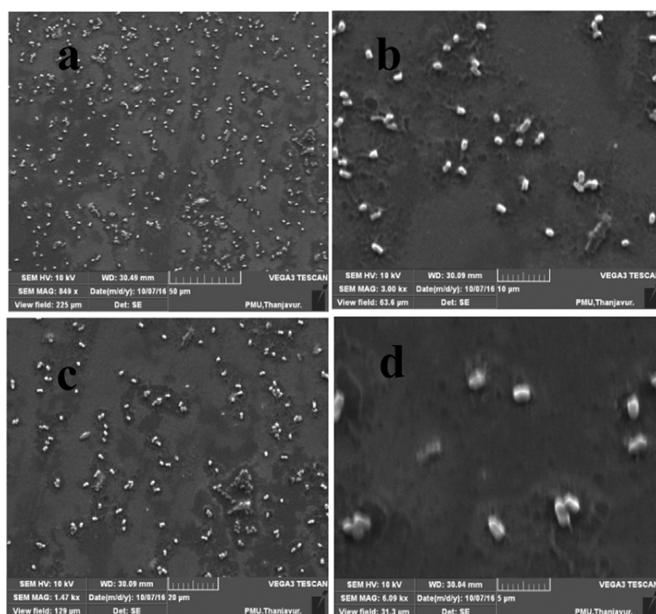

Fig. 8. SEM images of control (a, b) and treated (c, d) of *P. aeruginosa* and *K. pneumonia* by purified NMS 5 crude compound.

Acknowledgement

The authors would like to thank the Bharathidasan University for providing the University Research fellowship (Ref. No-05441/URF/K7/2013) for this work. The author also acknowledge the Bharathidasan University for utilize the facility of Confocal Laser Scanning Electron Microscope (CLSM) (DST-PURSE - Sanction Order No- SR/FT/LS-113/2009).

Ethical consideration

The samples were approved by the ethics review committee (S.No of IEC Management office: DM/2016/101/55) from the Department of Microbiology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. The Permission was sought from the hospital and laboratory authorities. The ethical principles of scientific research as well as related national laws and regulations were adhered to.

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