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Isolation and Identification of Antibacterial Compound from Marine Endophytic Actinomycetes against Multi Drug Resistant Bacteria

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Abstract

In present investigation, the antibacterial activity of Endophytic Actinomycetes (EA) isolated from mangrove plant of *Avicennia marina* in Muthupet Mangrove region, South East Coast of Tamil Nadu, India. The EA were isolated from Actnomycetes Isolation Agar (AIA) plate method after surface sterilization. After isolation, all the strains were detected their antibacterial activity against Multi Drug Resistant Uropathogens (MDRU) by conventional cross streak method. Further, the active strain was selected and crude extract was purified with various solvent by liquid-liquid extraction method. Then, the active solvent was chosen for further purification for antibacterial compound against selected MDRU including *Escherichia coli (E. coli), Proteus mirabilis* (*P. mirabilis*), *Pseudomonas aeruginosa* (*P. aeroginosa*), *K. Pnumoniae*(*K. pneumonia*) and *Enterobacter*sp. In result, the 100 strains of EA were showed white, creamy, powdery growth of the colony after surface steriliztation. The validation of surface sterilized water proved that the isolates were emerged from internal tissue of the plant, refereed as endophytes. Among the 100 strains, 10 strains were exhibited better antogonistic activity against selected MDRU strains. Based on the antogonistic activity, the excellent antibacterial activity of NM 5 was chosen for further study. The antibacterial potential of the NM 5 crude extract was purified by ethyl acetate extract and showed excellent inhibition activity 22, 17, 18, 20, and 16 mm against *E .coli, P. aeroginosa, K. pneumonia*and*Enterobacter*sp, were observed respectively. The Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the purified crude extract were showed excellent inhibitory activity at 100 µg/ml against selected uropathogens. This concentration was used for further studies like anti-fungal, anti-cancer, anti-biofilm and larvicidal activity.

Keywords: Entophytic actinomycetes; Multi drug resistant bacteria; Minimum inhibition concentration; Gram negative bacteria; Antibacterial activity

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Introduction

The antibiotic resistance among the uropathogens is emerging and spread rapidly over the past decade and it is alarmed that the incidence of MDRU development and infection are increasing [1]. Treatment of such MDRU infected patients are still another problem since the pathogens are almost resistant to all the available drugs and they easily develop resistance to the new drugs also [2]. Urinary Tract Infection (UTI) causing pathogens are one of the most important Multi Drug Resistant (MDR) bacteria and it developed resistance against almost all commertially available antibiotics including Ampicilin, amoxicilli, ciproflaxacin and all third generation chephalosporin due to the co-expressed resistance mechanisms; they lead to MDR effect in pathogens [3,4]. In particular Gram Negative Bacteria (GNB) play a important role and its MDR effect is the emerging study [5]. Many reports were published that the MDR relation in UTI pathogens acquired by excessive use of antibiotic, unprescribed format of antibiotic use and prior instrumentation to UTI bacteria [6]. It causes high mortality, morbidity, and increasing health care costs leads to death in worldwide. The diagnosis and treatment of the MDR effect in GNB are still controversial since association of related symptoms with various infections and sometimes asymptomatic indication [7]. The mechanism of MDR effect in GNB is also unclear and it acquired from various chemical, physical and biological sources [8]. Particularly, efflux pump, cell membrane permeability, ROS generation and some enzyme synthesis (ESBLs) [9]. In recent years, the MDR effect in GNB

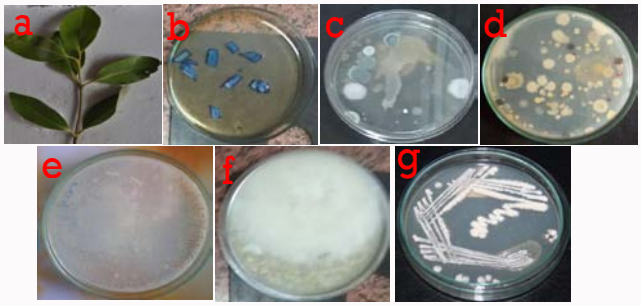

Figure 1: Isolation and identification of EA from Mangrove plant *Avicennia marina*.

Figure 2: Antogonistic activity of NM 5 against selected uropathogens.

significantly increased worldwide and show resistance to at least one of the drug most commonly used for eradication and some are resistant to all approved antibiotics. It most often inhabit in the UTIs of human and veterinary, and difficult to treat [10]. Therefore, it is necessary to find an alternative way to treat these types of MDRU without said problems.

The ocean covers 70 % of the earth has fabulous characteristics as synthesis of useful materials for human, animal and healthcare settings including pharmacological bio-potential to produce biologically active secondary metabolites which play a role in infectious diseases [11]. In this vast blender, actinobacteria is a Gram positive, flamentous, spore forming bacteria and the colony morphology is coarsely wrinkled or folded. It produce secondary metabolite extensively [12]. Among the all bacterial group, it is supreme secondary metabolites producer. An extensive source of antibiotic molecules from terrestrial actinobacteria was negligible to treat resistant pathogen by routine exploitation [13]. In the alternative study of novel drug discovery, some differences could be expected among the organisms for novel therapeutic approach to control and or the predominant EA which emerging from internal tissues of the plant, algae and synthesize new types of drugs from medical and infections [14]. It has nuclear therapeutic value and yet to afford attention to find new drugs. Previously, some of the natural compounds that reported earlier form marine endophytic actinomycetes including Alnumycin, munumbicins A to D [15], coronamycins and anthraquinones, lupinacidins A and B [16]. It has an excellent inhibition ability and communicate with structure of cells including cell wall, ROS and some metabolic pathways and lead to permeability, target achievement etc [17]. However, the limited research study was available on new compound from marine EA against MDRU. The discovery of new inhibitory compounds from natural is to overcome these resilient biofilm bacteria effectively. Hence, the current study was concentrated on isolation, identification, partial purification of antibacterial compounds from marine EA actinobacteria and its potential effect against MDRs uropathogens.

Materials Methods

Collection of Samples

The young leaf of plant *Avicennia marina* was collected from Muthupet Mangrove region, (Lat.10˚20'N & Long.79°35' E), South East Coast, Tamil Nadu, India. The collected plant was sealed with sterile plastic cover and kept in ice box. The sample was completely washed with tap water and followed by distilled water for removal of the free floating organisms. The surface sterilization of 70% ethanol was used for removal of epiphytes.

Bacteria maintenance

The MDR bacterial pathogens *P.mirabilis*, *K. pnumoniae, P. aeruginosa*, *K. pnumoniae* and *Enterobacter*sp. Were obtained from Department of Marine Science, Bharathidasan University, Tiruchirappli for used in this study. All the media and chemicals were purchased from Himedia Laboratory, India.

Isolation of endophyticactinomycetes

The endophytes was screened from surface sterilization of plant and followed by previous report of Kusari et al. [18]. The plant samples were thoroughly washed with tap water, and cut with small pices of leaves, twigs, and buds (approximately 10 mm length) by using sterile scale pale. Then, the small pieces of plant was surface sterilized by using 70% ethanol for 2 min and followed by 1.3 M sodium hypochlorite for 35 min, after washed with 70% ethanol for 1 min. After completion of chemical sterilization, these surface-sterilized pieces of plant were rinsed thoroughly in sterile, double-distilled water for 2 min, to remove unwanted surface sterilants after, all the tissue samples were air dried with 1 min. The surface sterilized tissues were gently placed on the Actinomycete Isolating Agar plates (AIA) sandwiched with streptomycin for removal of bacterial contamination, nystatin for removal of unwanted fungal growth, cycloheximide for increasing the growth of actinomycetes. All the plates were incubated at 28˚C for 6-7 days. For the proper sterilization, the parallel experiments were performed. The unsterilized tissue pieces of plant were placed on the AIA plate and incubated under the same conditions. The emerging colonies of actinomycetes without any contamination from surface treated tissue fragment was indicated as positive endophytes, fungal growth contamination of the unsterilized tissue of plant pieces as negative result were noted (differentiated morphologically by both macroscopic and microscopic evaluation) [12].

Primary Screening of actinomycetes

The primary screening of antibacterial activity of isolated EA was performed by cross streak method [19]. The bacterial pathogens *P.mirabilis*, *K. pnumoniae, P. aeruginosa*, *K. pnumoniae* and *Enterobacter*sp. Were used in this study. The isolated strains were streaked across the diameter on Mullerhinton Agar (MHA) plates. All the plates were incubated at 28°C for 6-7 days. After observing ribbon, white color growth of the strain, the 24h uropathogens were streaked perpendicular to the angle of central strip of the actinomycetes culture. All the plates were incubated at 37˚C for 24hrs. After incubation, the antagonistic activity of the positive strains were screened due to the inhibition effect of the pathogens and these strains were chosen for further study.

Extraction of Bioactive compounds from DMS 3

The antibacterial potential of active EA strain was extracted with active solvent of ethyl acetate by liquid- liquid extraction

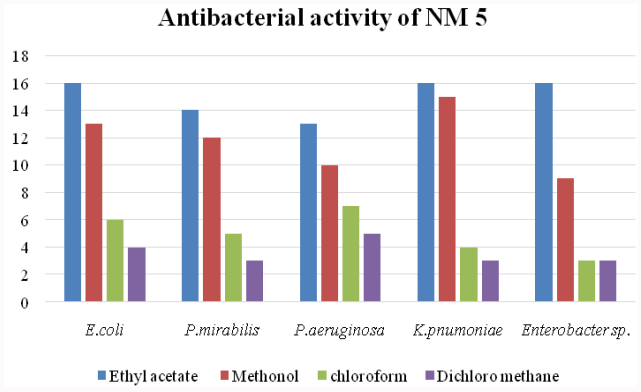

Figure 3: Antibacterial activity of selected uropathogens against NM 5 extract in various solvent.

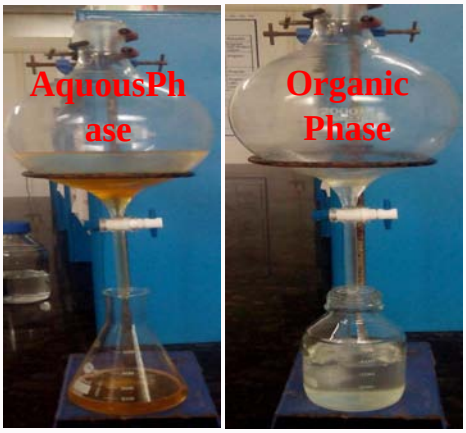

Figure 4a: Ethyl acetate extraction of isolated NM 5 strain extract.

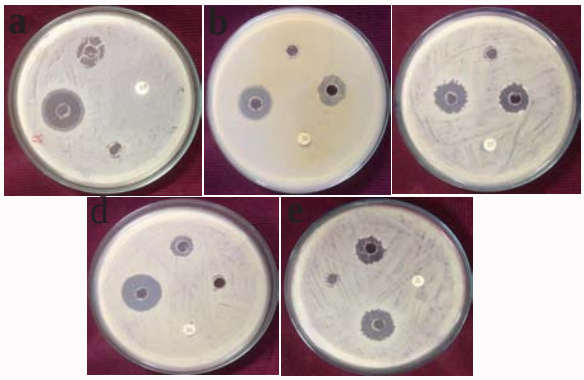

Figure 4b: Antibacterial activity of crude compound against selected uropathogens. A) *E. coli*, b) *P. mirabilis*, c) *P. aeruginosa*, d) *K. pnumoniae*, e) *Enterobacter* sp.

method [20]. The active strain was inoculated in Starch Casein broth (SCA) and the broth was incubated at 28°C for 15-20 days. After fermentation, the broth was centrifuged at 3000 rpm for 30 minutes. After centrifugation, the pellete was discarded and the supernatant was recovered by using filterate with Whattman No.1 filter paper. After filtering, The 1 L of ethyl acetate was mixed with 1 L of supernatant for antibacterial potential. The ratio was maintained at 1:1(w/v) and shaken vigorously 2 h for complete liquid extraction. From the extraction, the organic layer of the extract was separated from aqueous layer using separating funnel. The collected organic layer was incubator at 30 to 45°C for evaporation. After evaporation, the dried crude compounds were collected and determined the antibacterial activity against MDR uropathogens *P. mirabilis*, *K. pnumoniae, P. aeruginosa*, *K. pnumoniae* and *Enterobacter*sp by agar well diffusion method.

Secondary screening

The antibacterial ability of active strain was performed against MDRU at regular time intervals (24h, 48h, 96h) by well-diffusion method [21]. Briefly, the staleduropathogens cultures were inoculated on TSB and incubate at 37°C for 24 h. After 24 h, the bacterial cultures were spread on the MHA plates and consequetively the wells were cut using gel borer. After various concentration of the extract was added into the each well. The ethyl acetate extract acted as a control. All the plates were incubated at 37°C for 24 hrs. After 24hrs, the plates were observed for the zone of inhibition around the wells.

Determination of MIC and MBC

The MIC of antibacterial potential of active endophytic actinomycete strains was determined against selected MDRU (24) by micro broth dilution method [22] and the results of MIC was determined by spectrophotometer using 96 well microtiter plate. The various concentration (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 100µg/mL) of ethyl acetate extract of active actinomycetes extract was used to detect the MIC value against testeduropathogens. Briefly, 100µg/mL of TSB broth was added into the each well and 100µg/mL of test pathogens were added into all the wells. Finally, various concentration of selective actinomycetes extract was inoculated into the same wells and strile TSB broth was added into the wells for makeup to 300 µL. The final volume of the each well contained 300 µl. The without addition of bacterial culture containg wells acted as a control. Each plate was mixed well and then the plates were incubated at 37°C for24 h. After 24 h, no visible growth observed in the 96 well plate was noted and the lowest concentrations of the extract were indicated as MIC [9]. For the detection of MIC, the plates were read with UV spectrophotometer at 570 nm and percentage of inhibition was calculated by using the following formula

Percentage of inhibition:

(Control OD 570 nm - Test OD 570 nm)/ Control OD 570 nm X 100

Likewise, 10 µL of the all MIC concentration from treated bacteria broth was aliquot and streaked on MHA plates. All the plates were incubated at 37°C for 24 h. After incubation, the highest inhibition of the growth was observed from the minimum concentration of the MIC was indicated as MBC.

Result

Isolation and Identification of endophytica ctinomycetes

The young healthy green *Avicennia marina* leaves were collected from muthuppettai mangrove fields, Thiruvarur, Tamil Nau, India (Figure 1a). In the small pieces of the leafs were clearly surface sterilized by using sodium hypo chloride for EA isolation (Figure 1b) After surface sterilization, the AIA plates showed pure, fine, ribbons like powdery white color colonies (Figure 1c,d). Based on the bergey's manual, the 100 different types of actinomycetes were isolated. The isolated actinomycetes were endophytes or not were confirmed from the inoculation of surface sterilization or without surface sterilization plates. For the isolates were considered as endophytes, the last wash of the surface sterilized water was placed

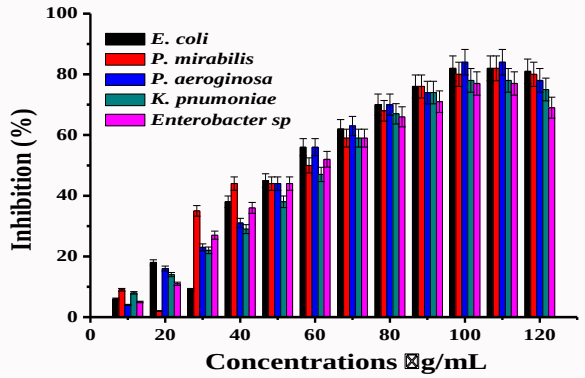

Figure 5: The MIC of NM 5 extract against selected uropathogens. a) *E. coli*, b) *P. mirabilis,* c) *P. aeruginosa*, d), *K. pnumoniae* and e) *Enterobacter* sp.

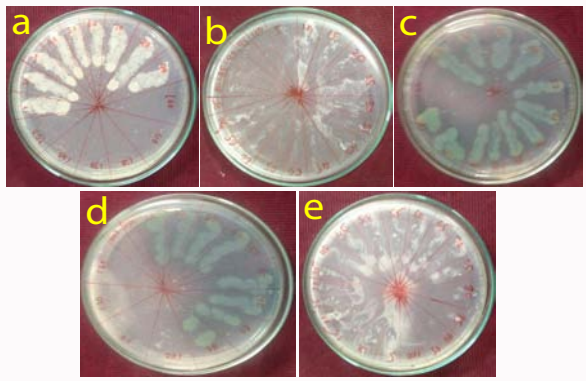

Figure 6: The MBC of NM 5 extract against selected uropathogens. a) *E. coli*, b) *P. mirabilis,* c) *P. aeruginosa*, d), *K. pnumoniae* and e) *Enterobacter* sp.

on AIA agar plates and it showed no any growth were observed in the plates (Figure 1e). Whereas the unsterilized plant wash water showed microbial contamination in their respective plates were also screened (Figure 1f). All the isolated endophyticactinomycete strains were taken separately and streaked on SCA agar plates for pure culture identification (Figure 1g). This isolated strains were indicated as endophytes and it confirmed, the isolated strains were emerged from internal tissue of the plants. Recently, Rajivgandhi et al. [12] reported that algae derived marine endophytic actinomycetes DMS 3 has better inhibitory activity against MDRs pathogen. Our result was accordance with earliest finding of Passari et al. [23] and reported that the NACl and sodium hypochlorite was the better choice for surface sterilization to plant and other endophyticactinomycetes.

Primary screening of isolated EA strains

The antogonistic activity of all the isolated EA strains were performed against selected uropathogens by conventional cross streaking method. After 24 h incubation, among the 100 EA strains, 10 strains were showed better antogonistic activity against selected uropathogens *E. coli*, *P. mirabilis, P. aeruginosa*, *K. pnumoniae*, *Enterobacter*sp. The screened strains were named as NM 1, NM 2, NM 3, NM 4, NM 5, NM 6, NM 7, NM 8, NM 9 and NM 10 (Table 1). They also showed minor discrepancy in relation to different strains and test organisms. Interestingly, NM5 has comperatively better the excellent antibacterial activity than other strains against ESBL producing bacteria was observed (Figure. 2). However, NM 5 was chosen for further study. The actinomycete was frequently identified from various marine sources and it produced new classes of antibiotics against various clinical disorders [24].The mangrove plant

Table 1: Identification of endophyticactinomycetes.

S. No	Strains	Antagonistic activity
1	NM 1	Good Activity
2	NM 2	Good Activity
3	NM 3	Good Activity
4	NM 4	Good Activity
5	NM 5	Excellent Activity
6	NM 6	Good Activity
7	NM 7	Good Activity
8	NM 8	Good Activity
9	NM 9	Good Activity
10	NM 10	Good Activity

Table 2: Various MIC concentration of EA NM 5 extract against selected uropathogens.

Various concentration of EA NM 5 extract												
Pathogens	10	20	30	40	50	60	70	80	90	100	110	120
E.coli	6	18	9	38	45	56	62	70	76	82	82	81
P.mirabilis	9	2	35	44	44	50	59	68	76	80	82	80
P.aeruginosa	4	16	23	31	44	56	63	70	74	84	84	78
K.pnumoniae	8	14	22	29	38	47	59	67	74	78	78	75
Enterobactersp	5	11	27	36	44	52	59	66	71	77	77	69

derived actinomycetes have antimicrobial activity and cytootxicity against MDR and A549 cell lines [25].

Extraction and antibacterial activity of bioactive compounds from NM 5

Based on the antogonistic activity, the potential strain of NM 5 strain was chosen and extracted with various solvent for recovery of potential antibacterial compound. Among the various solvent, ethyl acetate extract of NM 5 showed better inhibition activity than other solvents against selected uropathogens *E. coli*, *P. mirabilis, P. aeruginosa*, *K. pnumoniae and Enterobacter*sp (Figure 3). Hence, ethyl acetate extract of the NM 5 was chosen for further study.

Extraction and antibacterial activity of NM 5 strain

Based on the antibacterial activity, the excellent activity of the ethyl acetate extract of NM 5 showed better inhibition against selected uropathogens and this strain was chosen for further extraction (Figure 4a). After extraction, the organic phase was collected seperalty for further evoluation and aquous phase was stored in 4°C. Consecutively, the organic phase was maintained in the incubator at 40°C for complete evaporation. The ethyl acetate extract of aquous and organic phase of the NM 5 extract were shown in figure 4a. After complete evaporation, the dried crude compound were collected and weighed based on the amount of the solvent. The dried crude compounds were diluted in ethyl acetate for perform the various biological activity.

Further, the performed crude extract against selected uropathogens were showed excellent zone of inhibition and it showed 22, 17, 18, 20, and 16 mm were observed against *E .coli, P. aeroginosa, K. pneumonia, Enterobacter*sp, respectively (Figure 4b). The control wells exhibited no zone of inhibition in all the pathogens containing plates were also observed. The NM 5 extract along with the ceftazidime disc was also showed no zone of inhibition were observed. It was clearly confirmed that the selected uropathogens

were multi drug resistant. Hence, the result proved that the acetate extract of the NM 5 has more bactericidal effect against selected MDR uropathogens. Our result was most accordance with earliest study of Gorajana et al. [26] and reported that ethyl acetate was better solvent for production of active metabolites from actinomycetes strains. The supportive evidence of Zaitlin eat al. [27] also evidenced, marine derived actinomycetes compound has highest inhibition against Gram negative bacteria and 23, 25, 19 and 24 mm zone of inhibition against *S. aureus, E. coli, P. aeruginosa*and *B. subtilis* were observed.

MIC and MBC

The highest dilution or least concentration of the potential EA NM 5 extract was inhibit growth of the selected uropathogens were detected from MIC. It is a essential parameter that helps to discover the activity of newly discovered compounds against various types of pathogens. The ethyl acetate extract of the NM 5 extract exhibited 82, 80, 84, 78, 77 % of inhibition against *E. coli*, *P. mirabilis,P. aeruginosa*, *K. pnumoniae, Enterobacter*sp. Were observed at the concentration of 100 µg/ml (Figure 5). The inhibition effect of NM 5 was decreased the pathogenic effect in MDR bacteria by concentration dependent. However, the result revealed that the maximum inhibition was above 8o% against various uropathogens at the same concentration (100 mg/ml). Hence, 100 µg/ml was fixed as MIC.

For MBC, the misinterpretation of MIC was further detected due to turbidity of insoluble compounds into the broth of micro dilution plate, MBC was detected by culturing the MIC dilutions on the fresh MHA plates with different concentration (10-100µg/mL) of NM. In the MBC plates were exhibited no any visible growth at same MIC concentration was observed (Figure 6). The rate of inhibition effect was slightly differed from bacteria to bacteria. However, the result proved that the increasing concentration of NM 5 was decreased MDR bacteria growth effectively.

Both MIC and MBC results proved, the EA NM extract can be used to inhibit the development of growth in multi drug resistant bacteria which cause UTIs. Previous studies have reported those chemical constituents of actinomycetes extract have excellent antibacterial agents [28,29]. They have identified the active compounds from endophytic actinomycetes were may be alkaloids.

Conclusion

Our findings, mangrove actinomycetes is a major organisms for producing novel types of antibiotics against various types of uropathogens, mainly UTIs. Undefined antibiotic classes have emerged as a novel agent because of their living nature to volume ratio and unique chemical and physical properties. In particular, Mangrove Endophytic Actinomycete (MEA) is one of the important and relatively overlooked organisms that live within higher plants and recently gained more attention for the production of numerous antimicrobial compounds. It has excellent inhibition ability and interacts with cell wall lead to changes in permeability, some metabolic pathways, target achievement, generation of ROS, etc. Therefore, isolation and identification of NM 5 has been useful in discovery of novel compounds. The nature of mangrove environment. The nature of marine environment, low salinity, optimum pH, high temperature and carbon-nitrogen content influences NM 5 revealed tremendous antibacterial activity against selected uropathogens. This study helps future in designing the new drugs against multi drug resistant strains of urinary tract infections.

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