Original article

Partially purified actinomycetes compounds enhance the intracellular damages in multi-drug resistant P. aeruginosa and K. pneumoniae

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Abstract

Based on the excellent nutrient level, the current study was focused on isolation and anti-bacterial activity of the actinomycetes from marine mangrove soil samples. As result, 10 different strains of actinomycetes strains were identified on actinomycetes isolation agar plates. The identified strains were shown with white, clear, uncontaminated well matured spore producing ability. Based on the initial observation, the isolated colonies were actinomycetes. The partially extracted crude compound shown excellent anti-bacterial activity against P. aeruginosa and K. pneumoniae with 15 mm and 13 mm zone of inhibitions were observed at 500 μL concentrations. The minimum inhibition concentration result was also confirmed the 500 μL concentration against both the tested concentration with high inhibition rate. Then, the intracellular damages, decreased cell growth of the crude actinomycetes extract treated bacterial strains were clearly observed by confocal laser scanning electron microscope. The extracellular damages of bacterial cell wall and shape of the both the pathogens were clearly shown by scanning electron microscope. Therefore, all the results were clearly supported to the partially extracted crude compound and it has excellent anti-bacterial activity against tested multi drug resistant bacteria.

1. Introduction

The intertidal region of tropical and sub-tropical ecosystem made by more saline content, having excellent reservoir of rich bioactive metabolites (Arunugam et al., 2017). It is a unique environment for the survival of microorganisms and its depends on the microbial diversity based on the supplied nutrient. This unique environment having punch of secondary metabolites that exhibited excellent biological properties against various infections. For synthesis of available bioactive metabolites, various factors are involved and contributed in the metabolic pathways that led to the biosynthesis of unique bioactive compounds (Dandan et al., 2018). Marine mangrove environment having diverse groups of microorganisms including bacteria, fungi, actinomycetes, micro, macro algae and protozoa (Gan Jun et al., 2010). Among the microbial community, bacteria is the most dominant microbes and available 90% and less than 1% of the marine microbes only identified and used for bio-metabolites identification (Kafilzadeh and Dehdari, 2015). Compared to terrestrial, less than 5% of the marine microbes are identified and reported till-date.

Among the microbial community, actinomycetes are a superior bacteria and it is a power of complex secondary metabolites. It is an aerobic, fungus like bacteria, rich GC content of genetic materials, aerobic, branched and unicellular (Hozzein et al., 2019). Among the actinomycetes, various genus are available with excellent biomedical characteristic nature including Streptomycyes, Nocardiosis, micromonaspora, Saccharopolyspora, Amycolatopsis, Actinomadura and Actinoplanes (Behera et al., 2017; Hames-Kocabas and Uzel, 2012). In particular, the genus Streptomycyes is most reported actinomycetes having excellent bioactivity molecules producing ability compared with others. Previously most of the
researchers are concentrated to synthesis of bioactive compounds from genus Streptomyces due to the excellent bioactive molecule producer (Sanjivkumar et al., 2020). It is great important in the bioactive metabolite synthesis, as they are numerous metabolic production rates and also played in the role of recycles organic matter. Also, it has more capability to degrade chitin, lignocellulose and other degrading materials. In addition, the unexplored mangrove region has excellent bioactive metabolites producing nature. It has potential advanced sources to improve the native secondary metabolites. The unpredictable environmental nutrients, saline, PH, temperature, fluctuated intertidal, carbon, nitrogen and other sources are enhance the secondary metabolites nature (Tian et al., 2017; Rajivgandhi et al., 2020).

There are 70–80% of the commercially available natural drugs are derived from Streptomyces and it is highly valuable (Mary et al., 2021). It produces countless chemical derivatives such as bioactive compounds, enzymes, vitamins, hormones and etc. Previously, more reports are available about mangrove actinomycetes and its secondary metabolites compounds against anti-bacterial, anti-fungal, anti-viral, larvicidal and anti-biofilm activities (Sun et al., 2018). Based on the above criteria, the current study was focused on unexplored environment of marine mangrove region, Muthupettai, Thiruvurur District, Tamil nadu, India for synthesis of novel bioactive compounds with intracellular damage activity against multi drug resistant bacteria.

2. Materials and methods

2.1. Isolation actinomycetes from mangrove soil

The soil samples were collected in 10 different places of Muthupettai mangrove Ecosystem, Thiruvurur District, Southeast Coast of Tamil Nadu, India. The clear soil of one gram was suspended into nine mL sterile saline water, and spins the mixture for 2 min. Then, the supernatant of the sample was diluted serially with 10^3–10^7, and it spread on Starch casein agar plate and allowed to maintain until 7 days for proper growth. After 7 days incubation, the original, well matured, spore forming ability of the actinomycetes were screened. All the emerged strains were picked and separately streaked on starch casein agar slant.

2.2. Characterization of actinomycetes

All the morphological, biochemical and phonotypical characterization of the isolated actinomycetes colonies were identified by light microscope and noted based on the observation (Venkata Raghava Rao and Raghava Rao, 2013). The smear of the selected actinomycetes strains were carried out with using Gram staining kit and then, reverse side pigment, melanoid pigment aerial, substrate mycelium, and spores formation was observed at 40× magnification.

2.3. Extraction and partial purification of the potential compound

The selected actinomycetes strains were inoculated into the starch casein broth and allowed to grow well until complete spores producing ability and extract the potential compounds by liquid-liquid extraction procedure as followed by Ramachandran et al. (Ramachandran et al., 2019). Briefly, well matured separated actinomycetes were inoculated into 2 L starch casein broth containing conical flask, and glucose as a nutrient supplements. Then, the conical flasks were allowed to maintain 1 month for mature the spores and metabolic production abilities of the strains. Next, the broth culture was taken and split into various falcon centrifuge tubes made 50 mL in each tubes. After, the tubes were centrifuged at 2500 rpm for 10 min and taken all the tubes without mix. Discord the pellet and collect the supernatant of all the tubes and make it for 1 L of final volume. Take a 2 L separating funnel, and mix 1 L of clear filtered supernatant and add equal volume of various polar and non-polar solvents. Then, the solution mixtures were shake vigorously until separate the both the layer with potential compound. Further, solvent phase and aqueous phase of the separating funnel containing samples were separated each other in respective fresh sterilized bottle. Finally, the ethyl acetate solvent phase of the sample was put into the hot air oven for evaporated the solvent without potential compounds. For this process, the temperature of 45 °C at 10 days was used for complete evaporation of the compound from ethyl acetate solvent phase. After complete dry of the solvent, the samples was put into the hot air oven, then collect the potential compound from the bottle and mixed with respective solvents for further studies.

2.4. Antimicrobial activity of potential compound

All the extracted compounds were performed against multi drug resistant P. aeruginosa and K. pneumoniae for detection of anti-bacterial ability of the extract and the procedure was following by previously reported method of Rajivgandhi et al. (Rajivgandhi et al., 2020). Shortly, the 24 h old pathogens were swabbed on freshly prepared muller hinton agar plates and make wells with 6 mm distance. To identify the zone of inhibition of extracted actinomycete compounds, various concentration of the actinomycete compounds (25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL were added in the wells, and the plates were incubated at room temperature for 12 hrs. Finally, the zone of inhibition around the concentration of the wells was noted and used that concentration for further study.

2.5. Minimum inhibition concentration (MIC)

The decreased quantity of bacterial growth shown at very lowest concentration was considered as minimum inhibition concentration. In this method, the micro broth dilutions were followed based on the previous reports of Sanjiv kumar (Sanjiv Kumar et al., 2016). Shortly, 12 h old bacterial culture plus 100–1000 µg/mL concentration of the extract was taken in muller hinton broth of 96-well plate. Then, no extract of the initial well served as a control. The plate was incubated in ordinary room atmosphere for 1 day. Then, the turbidity of the wells was monitored and consecutively taken the 600 nm O.D by spectrophotometer for detect the percentage of inhibition. The correlation of turbidity and highest inhibition percentages of the lowest concentration was identified and reported as minimum inhibition concentration. In percentage, the control O. D value was compared with tested values using previously reported reference of Zhang et al. (Zhang et al., 2020).

2.6. Intracellular damage of potential compound against bacteria

Weather the MIC was effective against bacteria in inside or not effective was successfully visualized by confocal laser scanning electron microscope after compare with live and dead cells using AO/EB stains. The intracellular damages of the bacteria detection by CLSM was followed by earlier reports of Govindarajan et al. (Govindarajan et al., 2014). Initially, 5 mil of well matured 12 h P. aeruginosa and K. pneumoniae growths were aliquot in sterilized test tubes. After, the sample tubes were centrifuged at 1000 rpm for 5 min and taken the pellet only. Then, 1% PBS was made and washed the pellet samples for adherences followed by centrifugation at same as before used rpm and time interval. Then, PBS mixed sample was removed and taken 5 µL of AO and EB together in both
the bacteria containing tubes. Finally, the needed amount of samples were taken in separate cover glass and closed by cover slip in dark room. Consequently, the samples were kept inverted position in CLSM and taken the result images at 40× magnification for detecting the live/dead cells (Olympus B × 43F).

2.7. SEM

The fixation method was performed against test and control bacteria for identify the external damage of the bacteria shape by SEM. This procedure was followed by earlier reported article of Alharbi et al. (Alharbi et al., 2020) with some modifications. Simply, 4% glutaraldehyde was taken and applied on the bacteria smeared cover slip for fixation. The solution was allowed 4 h to fixation in the slide. The complete fixation of the samples was taken after check properly, and then adds 100 µL of prepared ethanol in the serious of 10–100% for dehydration. Each and every dehydration process was done in 10 min time interval. Then, dehydrated samples were taken properly and add glutaraldehyde again and withstand for 2 h and followed dried the solution after removed. Finally, 1 mL of t-butanol was added on the dried slide containing bacterial samples and kept in deep freezer for over-night. Next, the samples were taken from deep freezer and cooled 2 h followed by seen in SEM at 40x magnification (Shimadzhou, Japan 2019).

3. Result

3.1. Isolation and identification of endophytic actinomycetes

The urgent needs of novel antibiotics from natural products were heightened more worldwide due to the antimicrobial resistance effect. So, researchers are focused on overlooked sources to detect the new compounds against these classes of bacteria and decrease their virulence without any toxicity. Positively, actinomycetes has the superior ability to produce excellent bioactive compounds that compete against drug resistant bacteria. As same as this research was focused on marine actinomycetes and their partially purified compounds were discovered for inhibition of bacteria. There are 10 strains of different actinomycetes were emerged on actinomycetes plate and it shown white, well matured, spore producing ability (Fig. 1). It was confirmed by previously reported evidences of Sudha Kalyani et al. (Sudha Kalyani et al., 2019); Thirumurugan et al. (Thirumurugan et al., 2018). Therefore, the isolation result was confirmed that the colonies were actinomycetes. They have the ability to inhibit multi drug resistant bacteria, because of the extreme environmental conditions such as pH, temperature, stress, carbon, nitrogen and other sources.

3.2. Extraction of anti-bacterial compounds

The 15 days fermented colonies were centrifuged continuously until the debris removal and then filtered using ethyl acetate as a solvent. Then, the filtrate using What man No. 1 filter paper for perform the anti-bacterial activity (Fig. 2). In anti-bacterial activity, the result was shown with 15 mm and 13 mm zone inhibitions were shown at 500 µL concentration (Fig. 3). This concentration was very low concentration compared with previous reports of actinomycetes extract Ramachandran et al. (Ramachandran et al., 2019). This concentration may be triggered by unpredictable marine environmental nature. Recently, the closed result was reported by Tang et al. (Tang et al., 2008); Bibi et al. (Bibi et al., 2020), and the marine mangrove actinomycetes has excellent anti-bacterial activity against multi drug resistant bacteria. Previously, Sudha et al. (Sudha and Masilamani, 2012) reported that the marine mangrove region mediated actinomycetes is an excellent antibiotic producer due to the unpredictable environmental parameters. Finally, the extraction and primary screening result was confirmed that the isolated actinomycetes strain has excellent anti-bacterial activity against multi drug resistant bacteria and that strain was named as GRG 10.

3.3. MIC and MBC

In minimum inhibition concentration, the result was identified with decrease bacterial growth due to the observance of decreased
turbidity. The turbidity was decreased gradually after 24 h of inhibition due to the influence of actinomycetes extract. The result was initially conveyed, that the actinomycetes extract has the inhibition ability at increasing concentration as same as concentration dependent inhibition activity. Because, initially, the 5% of inhibition was observed at 50 μL concentration and it extended to 50% of inhibition at 250 μL concentrations. Then, the 100% inhibition was observed at 500 μL concentration (Fig. 4). Also, the turbidity was equal to decreased percentages results. This resulted concentration of 500 μL was very low compared with others which have been reported previously. The MIC level of the extract was used to perform the further experiment for accurate results. So, in this study, 500 μL concentrations was fixed as a MIC.

3.4. CLSM

Based on the result of green and red color differentiation, the live and dead cells were easily identified, and it is more supported to the effect of compound. In this study, the extract has excellent inhibitory activity in inside of the bacteria. Because, the intracellular bacteria was collapsed totally due to the influence of actinomycetes extract (Fig. 5a, b). The normal rod shape of the bacteria was shown with irregular shape, and dispersed arrangement (Fig. 5c, d). When compared to control, the tightly attached, clear rod shape bacteria were shown. AO and EB are fluorescence dyes which have been used to identify the live/dead cells variation. Because, AO could be bind only in the live cells as same as EB could be bind only in the red cells. So, if the extract damaged the intracellular parts of the bacteria, the EB stain was attached on the damaged parts of the intracellular surface. So, it glowed with red color. In our result, the more number of red cells were observed in the treated cells. Also, normal control cells were exhibited with green color images. This evidence was proved that the extracted actinomycetes compound has the excellent anti-bacterial activity against tested bacteria.
3.5. SEM

Effective of actinomycetes compound in inside of the bacteria with complete damage was clearly observed in SEM images and it indicated that the partially purified actinomycetes compound has excellent anti-bacterial activity. The damaged images were shown with segregation of gene production parts, highly dead cells and dysfunction of the gene expressions (Fig. 6c, d). The actinomycetes extract damage the original rod shape in bacteria and it undergone the rough and unclear morphology. The extracellular and intracellular bacterial virulence materials were inactivated and dysfunction due to the complete damage of bacterial cells. Similarly, more number of death cells, highly condensed, complete necrotic formed cells in the treated cells, and the smooth, rod shape, original morphology was observed in control cells were observed respectively (Fig. 6a, b). Sometimes, the coiled shape with more leakages in inside of the bacterial body was clearly observed. Supportively, continuous belbling formation was observed in and around the places of cytoplasmic membrane. Additionally, the exopolysaccharide layers were completely arrested due to the effect of actinomycetes and finally the bacteria was not able to survive in live condition. Altogether, all the anti-microbial activity and MIC results were strongly evident that the extracted crude compound as excellent anti-bacterial compound.

4. Conclusion

Marine mangrove is an unpredictable atmospheric nature including pH, NaCl, temperature, carbon, nitrogen, nutrients and other sources. Sometimes, the new strains and novel compounds are emerged from marine sources with excellent bioactivity. Based on the advantages, the partially extracted actinomycetes exhibited 15 mm and 13 mm zone of inhibition against *P. aeruginosa* and *K. pneumonia* at 500 μL concentrations. It was very low concentration compared with terrestrial actinomycetes extract, and it may be influenced by mangrove nutrients and other chemical derivatives. The antimicrobial activity effect of the compound was confirmed by minimum inhibition concentration result, and it shown complete inhibition at 500 μL concentrations. At this MIC, the intracellular and extracellular cells were compromised and shown with irregular morphology. Altogether, all the anti-microbial activity and MIC results were strongly evident that the extracted crude compound as excellent anti-bacterial compound.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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