Abstract

The problem of antibiotic resistance, which has limited the use of cheap and old antibiotics, has necessitated the need for a continued search for new antimicrobial compounds. Understanding the mechanisms of resistance is important in the development of strategies to resolving the problem. *Pseudomonas aeruginosa* has become an important cause of morbidity and mortality, especially in patients with compromised host defence mechanisms. It is the most common pathogen isolated from patients who have been hospitalized longer than one week, and it is a frequent cause of nosocomial infections. *Pseudomonas* causes several complications which might be life-threatening. It causes various infection like endocarditis, pneumonia, skin and soft tissue infections, neoplastic diseases, urinary tract infections, respiratory tract infections, bone and joint infections, thermal burns, patients on mechanical ventilation, cystic fibrosis, gastrointestinal infections and systemic opportunistic infections, particularly in cancer and HIV/AIDS patients. Development of drug resistance in *P. aeruginosa* and spread of drug resistant strains is an alarming feature. Multi drug resistance in *P. aeruginosa* has been reported worldwide which is increasing every day. The increasing frequency of multi-drug-resistant *Pseudomonas aeruginosa* (MDRPA) strains is concerning as efficacious antimicrobial options are severely limited.

Drug resistance in *P. aeruginosa* has been mediated by several mechanism out of them efflux pump mediated drug resistance is one of the important mechanism of drug resistance. Active efflux of drugs, alteration of target sites and enzymatic degradations are the strategies by which pathogenic bacteria acquire or develop intrinsic resistance to antibiotics. Multidrug resistance MexAB-oprM efflux pumps capable of recognizing and expelling a variety of structurally unrelated compounds from the bacterial cell conferring resistance to a wide range of antibiotics in Gram negative bacteria *Pseudomonas aeruginosa*.

The ability of some chemical compounds (called MDR inhibitors or EPIs) to modify the resistance phenotype in bacteria by working synergistically with antibiotics in vitro has been observed. The search for such compounds which can be combined with antibiotics in the treatment of drug resistant infections may be an alternative to overcoming the problem of resistance in bacteria. Crude extracts of medicinal plants stand out as veritable sources of potential resistance modifying agents and the Indian biosphere promises to be a potential source
of such compounds owing to its rich plant species diversity. The aim of the present study was to identify the Efflux pump inhibitors for multidrug resistance Gram negative bacteria from plant sources and also study the synergistic effect of characterized EPI with resistant antibiotics in MDR strains of Gram negative bacteria *P. aeruginosa*.

A total of 100 clinical isolates of *P. aeruginosa* were collected from Gian Sagar Medical College and Hospital, Rajpura, Distt. Patiala, Punjab (India). Four strains of *P. aeruginosa* including, one wild type and three MexAB-oprM efflux pump knockouts/Overexpressing strains were procured from Dr. Thilo Kohler, University of Geneva, and Department of Microbiology and Molecular Medicine Genève, Switzerland. One control standard sensitive strain of *P. aeruginosa* MTCC-741 was obtained from IMTECH, Chandigarh. All clinical isolates of *P. aeruginosa* were cultured and characterized morphologically and Biochemically. Then all the 100 clinical isolates were processed for antibiotic susceptibility assay. The isolates were selected on the basis of Group A contains the antibiotics which are effluxed out by MexAB-oprM efflux pump of *P. aeruginosa*. Then the EtBr Agar Cartwheel assay was performed for determination of MDR phenotype. It confirms the presence or absence of efflux pumps in the bacterial strains. A total of 40 plants were screened for the presence of bioactive molecule with Efflux pump inhibitory activity. The EPI activity was evaluated by two methods; Berberine Potetiation assay and Ethidium bromide assay. The plants showing EPI activity with Berberine and Ethidium bromide assay were further explored for their synergistic effect with group A antibiotics (Ciprofloxacin, Tetracycline and Chloramphenicol). The methanolic fruit extract of *Terminalia chebula* and *Synzium cumini* has shown highest synergism in combination of selected group A antibiotics. It is further subjected to bioassay guided fractionation and column chromatography to isolate the bioactive molecule.

The fractions obtained from column chromatography were subjected for their synergistic activity. The fraction showing synergistic activity was then analysed for the identification of bioactive molecule by physical characteristics as well as its molecular weight by LCMS and structure was elucidated by NMR to know the exact bioactive molecule. Bioactive compound isolated from methanolic fruit extract of *T. chebula* and leaf extract of *S. cumini* was further analysed for its EPI activity by using EtBr assay and synergistic effect was also elucidated with Group A antibiotics.
All 100 clinical isolates have shown the characteristic features of *P. aeruginosa*. Out of 100, only 6 isolates were found multidrug resistant against more than two antibiotics. Out of 6 MDR clinical isolates 3 isolates were found resistant to group A antibiotics and sensitive to group B. These 3 isolates were selected for the further study. All sensitive strains have shown fluorescence as they accumulated EtBr due to the absence of active efflux pump while the fluorescence was not observed in drug resistant strains which again confirm the presence of efflux pump in MDR strains of *P. aeruginosa*. The maximum fluorescence was observed at 4°C in comparison to 37°C in sensitive strains. The MIC value of antibiotics was observed very high among these three MDR clinical isolates and five standard control strains containing MexAB-oprM efflux pump further confirms the presence of efflux pump in MDR isolates. While the sensitive control strains have shown very low MIC value. In the presence of control efflux pump inhibitor CCCP, a significant decrease was observed in MIC values of antibiotics for resistant strains, while the MIC of control sensitive strains without efflux pump was not changed and remains constant. The observations of the present study again suggested that the drug resistance in MDR clinical isolates is mediated by an active efflux pump which effluxed out the accumulated drugs.

Out of 40 methanolic plant extracts, 10 plants extracts (*T. chebula, S. cumini, M. koenigii, Z. officinale, C. pseudolimon, C. asiatica, P. granatum, G. glabra, C. longa and P. graveolens*) have shown efflux pump inhibitory activity by using Berberine potentiation assay and Ethidium Bromide efflux pump inhibition assay. The synergistic activity of these plants were evaluated in combination of group A antibiotics. Out of 10 plants only 2 methonolic plant extracts of *T. chebula* (Fruits) and *S. cumini* (leaves) has shown synergistic activity with three group A antibiotics (Ciprofloxacin, Tetracycline and Chloramphenicol). The bioactive compound was extracted from *T. chebula* and *S. cumini* by using TLC, bioassay guided fractionation and finally by column chromatography. The two bioactive compounds, Ethyl gallate and Gallic acid were extracted from *T. chebula* and *S. cumini* respectively.

The 1000 µg/mL dose of Ethyl gallate and Gallic acid has shown potent EPI activity and all resistant strains of *P. aeruginosa* have accumulated ETBr and shown EtBr fluorescence. Further, synergistic activity of Ethyl gallate and Gallic acid was elucidated used in combination with antibiotics and both the compounds, Ethyl gallate and Gallic acid have shown significantly very high synergistic activity with all three group A (Chloramphenicol, Tetracycline and
Ciprofloxacin) antibiotics against MDR strains of *P. aeruginosa* and effectively control the growth of bacteria in-vitro and significantly decreased the MIC of antibiotics. Ethyl gallate and Gallic acid may be used as future molecules as a putative EPI in combination to resistant antibiotics for the treatment of multi drug resistant strains of *P. aeruginosa*. As the MDR isolates used in this study was very less in number and large scale study is required to made any concrete conclusion but the data obtained in this study give a strong clue about the presence of bioactive molecules in plants which may control the worst antibiotic resistance in bacteria by inhibit the activity of efflux pumps in bacteria. These molecules may be an important molecule of future therapeutic regimes.

**Key words:** *Pseudomonas aeruginosa*, Efflux pumps, Multi drug resistance, Efflux pump inhibitors or Resistance modifying agents, Plant extracts.