The need to combat microbial resistance to antibiotics is an increasing global concern. With the emergence of multidrug resistant organisms, combining the plants and antibiotics against resistance bacteria becomes necessary. Efflux related multidrug resistance (MDR) is a significant means by which bacteria can evade the effects of selected antimicrobial agents. *Pseudomonas aeruginosa* is not an obligate parasite, but the species of *Pseudomonad* commonly associated with human diseases. *P. aeruginosa* is gram-negative microorganism that have been shown to exhibit resistance to a wide range of commonly available antibiotics. 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*. The aim was achieved by using following objectives.

A total of 100 clinical isolates of *P. aeruginosa* were collected from Gian Sagar Medical College and Hospital, Rajpura, Distt. Patiala, Punjab (India). Four control 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 standard sensitive strain of *P. aeruginosa* MTCC-741 was obtained from IMTECH, Chandigarh. All the clinical isolates of *P. aeruginosa* were characterized by morphologically and biochemically. Further all the 100 clinical isolates were processed for antibiotic susceptibility assay. The antibiotics were divided into two groups based on the substrates and non- substrates of MexAB-oprM efflux pump of *P. aeruginosa*. Group A contains the antibiotics which are effluxed out by MexAB-oprM efflux pump of *P. aeruginosa* and group B contains the antibiotics which are not effluxed out by MexAB-oprM efflux pump of *P. aeruginosa*. The EtBr Agar Cartwheel assay was done for determination of MDR phenotype. This assay confirms the presence or absence of efflux pumps in the bacterial strains. A total of 40 medicinal plants were collected from University of Horticulture and Forestry Nauni, Solan (HP), Arya Vastu Bhandar Dehradoon (Uttarakhand) and Physical Garden of Shoolini University, Solan (HP). The plants collected from Arya vastu bhand and Shoolini University were authenticated by University of Horticulture and Forestry Nauni, Solan (HP).
All the 40 plants were selected for Efflux pump inhibitory activity. EPI activity was evaluated by Berberine Potetiation assay and Ethidium bromide assay. Berberine works as an efflux pump substrate and inhibits growth of bacteria in the presence of an EPI. Therefore, Berberine is used as a marker to find out the presence of an EPI in plant extracts and Ethidium Bromide is a fluorescent dye and gets accumulated in MDR efflux pump containing bacteria with an EPI and shows fluorescence after accumulation. Hence, it is also used as a marker for the detection of an EPI in plant extracts. Berberine Potetiation assay and Ethidium bromide assay was performed for methanolic extracts of all 37 plants and for oil of three plants. Out of 40 plants only 10 plant extracts (T. chebula, S. cumini, M. koenigii, Z. officinale, C.pseudolimon, C.asiatica, P.granatum, G.glabra, C. longa, P.graveolens) enhanced the accumulation of ethidium bromide at 1000 µg/ml concentration and inhibits of efflux pumps activity of the bacterial cells. In addition to this, Berberine control the bacterial growth with all 10 plants extracts. The observation of both assays, Berberine Potetiation assay and Ethidium bromide assay indicates that all these plants have potent EPI activity and a potential source of a bioactive molecule which has EPI activity. Further the synergistic activity for all 10 plants was evaluated in combination with Group-A antibiotics (Ciprofloxacin, Tetracycline and Chloramphenicol). The assay was determined that out of 10 plants only 2 plants extracts, T. chebula (Fruits) and S. cumini (leaves) shown synergistic activity in combination with group A antibiotics (Ciprofloxacin, Tetracycline and Chloramphenicol), and enhanced the sensitivity of these antibiotics used as a substrate for MexAB-oprM efflux pump for P. aeruginosa. Furthermore, T. chebula and S.cumini plant extracts were used to isolate bioactive compounds responsible for synergistic effect with Group A anitbotics.

Isolation and characterization of bioactive compound was done by extracting the powdered dry fruits of T. chebula and leaves of S. cumini with methanol and followed by chemical fractionation. Then column chromatography was performed on silica gel to extract the compound. Phytochemical analysis and TLC of T. chebula fruit extract and S. cumini leaf extract was done with various solvents depending on their polarity with different Standards. The fraction was then characterized to know the physical properties of bioactive molecule like colour, solubility, melting point and the molecular weight of bioactive molecule was...
determined by LCMS then the structure was elucidated by NMR. Further, EPI activity of the bioactive compound extracted from *T. chebula* and *S. cumini* was performed with two different concentrations (100 µg/ml and 1000 µg/ml) by EtBr assay. Bioactive compound isolated from methanolic fruit extract of *T. chebula* and *S. cumini* was further analyzed for its EPI activity by EtBr assay and was also evaluated for synergistic effect with the antibiotics.

All 100 clinical isolates of *P. aeruginosa* were collected and maintained in nutrient broth followed by culturing in nutrient agar. All the strains were found Gram negative bacilli with Slimy, opaque, irregular colonies with earthy smell on nutrient agar media and showing positive biochemical test of Catalase, Oxidase, Citrate, Nitrate, Motility and acid production in glucose. All the 100 clinical isolates have shown the characteristics features of *P. aeruginosa*. Drug susceptibility assay was performed for all the 100 isolates of *P. aeruginosa*. Nighty percent clinical isolates were found resistant at least for one antibiotic used for sensitivity assay. Out these 6 isolates were found multi drug resistant against more than two antibiotics. Out of 6 MDR strains 3 strains were found resistant to group A antibiotics even after repeated attempt and sensitive to group B antibiotics. All three clinical MDR isolates and three control strains including one wild type strain (R3-PAO1), two mexAB-oprM efflux pump overexpressing strain of *P. aeruginosa*, (R2-TETR and R4-PT629) has shown very high MIC value i.e 128 µg/mL. While the sensitive control strains have shown very low MIC value i.e 0.003 µg. The significant decrease was observed in MIC values of antibiotics up to 0.003 µg/mL in combination with control efflux pump inhibitor CCCP in all resistant strains, while the MIC of control sensitive strains (1 MTCC 741 and knockout strain, R1-TETR-T) without efflux pump was not changed and remain 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 drug.

Out of 40 plants extracts only 10 plant extracts (*T. chebula, S. cumini, M. koenigii, Z. officinale, C. pseudolimon, C. asiatica, P. granatum, G. glabra, C. longa, P. graveolens*) enhanced the accumulation of Ethidium bromide and inhibited its effluxs from cells. Furthermore, the observations of Berberine assay, again confirmed the presence of EPI compounds in all ten plants as all extracts have shown EPI activity with Berberine. The
observations of both assays Ethidium bromide as well as Berberine assay suggested that these plants have bioactive compounds which has EPI activity and may be proved as promising candidates bearing EPI compounds. All the 10 plants were subjected to evaluate their synergistic activity in combination with group A antibiotics. Out of 10 plants only the methonolic plant extracts of *T. chebula* (Fruits) and *S. cumini* (leaves) has shown synergistic activity with three antibiotics (Ciprofloxacin, Tetracycline and Chloramphenicol) belongs to group A, which are exclusively effluxed out by MexAB-oprM efflux pump against *P. aeruginosa*. The methonolic extracts of these two plants enhanced the sensitivity of *Ciprofloxacin, tetracycline and Chlormephenicol* upto lethal dose and control the growth of bacteria.

Then the bioactive compound was extracted from *T. chebula* and *S. cumini* by using TLC, Bioassay guided fractionation and finally by column chromatography. The bioactive compound extracted from *T. chebula* has molecular weight 198.17 and melting point 150° C with empirical formula C$_9$H$_{10}$O$_5$. Hence the compound was identified as Ethyl gallate. This phenolic compound has three OH groups. The bioactive compound extracted from *S. cumini* has molecular weight 170.12 and melting point 260° C with empirical formula C$_7$H$_6$O$_5$. Hence the compound was identified as Gallic acid. This phenolic compound has four OH groups.

EPI activity of Ethyl gallate and Gallic acid was performed by Ethidium bromide assay at two different concentrations i.e. 100µg/mL and 1000 µg/mL. The potent efflux pump inhibitory activity was observed on 1000 µg/mL. Further, Ethyl gallate and Gallic acid was used in combination with group A antibiotic to know the synergistic activity of the compound. Synergism was determined by FIC method. Both the compounds, Ethyl gallate and Gallic acid have shown significantly very high synergistic activity with all three (Chloramphenicol, Tetracycline and Ciprofloxacin) group A antibiotic against MDR strains of *P. aeruginosa* and effectively control the growth of bacteria in-vitro and significantly decreased the MIC of antibiotics.

The present study suggested that the Ethyl gallate and gallic acid may be used as a putative EPI against Gram negative bacteria and may be tried for clinical use. The emergence of multidrug resistance in Gram negative bacteria is a major challenge and the control of
these pathogenic bacteria is difficult by existing control measures. Ethyl gallate and Gallic acid may be used as a future molecule as a putative efflux pump inhibitor in combination with antibiotics for the treatment of multi drug resistant strains of \textit{P. aeruginosa}. The present study produced a preliminary data and the number of strains was small, the large number of MDR strains is required to make any concrete conclusion. But the controls used in this study were standard strains which were genetically confirmed for the activity of efflux pumps in bacteria. Therefore it may be assumed to a strong possibility of that the Ethyl gallate and Gallic acid is a potent efflux pump inhibitor against gram negative bacteria containing MexAB-oprM efflux pump which responsible to develop resistance against group A antibiotics in \textit{P. aeruginosa}. The previously reported EPIs have EPI activity against Gram positive bacteria the dose required for EPI activity is toxic, hence these efflux pump inhibitor may be found helpful to demolish the efflux pumps in Gram negative bacteria and to overcome the development of antibiotic resistance.