CHAPTER 7

CONCLUSION

7.1 Conclusion

The treatment of infectious MDR bacterium is a major challenge faced by the medical community in day to day life. Emergence of MDR to XDR bacteria is a boundless threat, with all drug resistance potential virulence factors, the bacterium evolves new strategies to become resistant to commercially available antimicrobial agents. This study first focused on the screening for fluoroquinolone resistance in ESβL isolates of *K. pneumoniae* from Chennai collected in October 2009 which showed that 61% isolates were resistant to ciprofloxacin and 52% to levofloxacin. The frequency of *qnr* genes in this study was found to be 69.5% for *qnrA* and 47.8% for *qnrB* and none of the strains showed *qnrS*. The plasmid borne nature of the quinolone resistance was shown for the isolates P12 and P13. Plasmids from the isolates P12 and P13 gave a very high MIC 240 μg/mL to ciprofloxacin when transformed into the *E. coli JM109* strain. Our analysis of the isolates revealed that 13 isolates out of 19 tested harboured the *aac(6’)-1b* gene encoding aminoglycoside 6’-N-acetyltransferase, which confers resistance to amikacin, kanamycin and tobramycin and found to be broadly distributed geographically and present in many clinically important Gram-negative rods.

Our results show the presence of the *aac(6’)-1b* gene in the *Klebsiella* isolates from Chennai and the presence of the cr variant in at least one of the isolates. This report is significant since this is the first report showing that PMQR is prevalent in *K. pneumoniae* in Chennai. Our earlier analysis on these isolates had shown the presence of the CTX-M gene and the IncF1C replicon in the plasmid pKNMGR13 (Magesh et al 2011).

The second objective of the present study was to identify natural compounds which can inhibit biofilm formation in clinical isolates of *K. pneumoniae*. Towards this, 35 clinical isolates were screened; out of which 7 strong biofilm producers were identified.
effects of eugenol, linoleic acid, chitosan, curcumin and efflux pump inhibitors berberine and reserpine were tested for their inhibitory effects on bacterial growth. The results showed that reserpine followed by linoleic acid, were the most potent biofilm inhibitors. Reserpine, an efflux pump inhibitor was effective at biofilm inhibition at a concentration of 0.0156 mg/mL, 64-fold lower concentration than its MIC. Linoleic acid, an essential fatty acid was effective as a biofilm inhibitor at 0.0312 mg/mL, which is 32-fold lower than its MIC. Berberine, another plant derived antimicrobial, chitosan and eugenol had an MBIC value of 0.0635 mg/mL. Curcumin, a natural phenolic compound was effective at biofilm inhibition at a concentration of 0.25 mg/mL, which is 50 fold less than its MIC. Notably, the MIC and MBIC data on these 6 natural compounds was reproducible in all seven high biofilm forming isolates of *K. pneumoniae*. The present report is a comprehensive comparative analysis of the dose dependent inhibition of various natural compounds on biofilm formation in *K. pneumoniae* (Magesh et al 2013).

The MDR strain *K. pneumoniae* U25, which is used in the present study was previously reported to possess high biofilm forming ability causing urinary tract infection, plasmid carrying ESβL gene (bla*OXA*, *bla*TEM) (Kamatchi C et al 2009), Plasmid- Mediated Quinolone Resistance (PMQR) resistant gene (*qnrA*, *qnrB* and *aac(6’)-1b* gene) (Magesh et al 2011) and also NDM positive, repicon type (Inc/rep type L/M,X,FIA,W,FIC) (Kamatchi C et al 2011). From the present study, data showed that the strain possesses an additional resistance mechanism of efflux pumps. MDR bacterium with these many resistance mechanisms are difficult to treat. Hence, to overcome these problems, it is necessary to bring back the older antibiotics which are no longer potent by modifying their activity. Therefore, we analyzed ethanolic extract of 5 medicinal plants showing antibacterial and synergistic activity with ciprofloxacin. It was found that norfloxacin accumulation was seen with the ethanolic extract of *Punica granatum* and *Tectona grandis* and also from their sequential n-hexane and chloroform fractions of *Punica granatum* and n-hexane fraction of *Tectona grandis*, respectively. Thus, indicating that they might act as efflux pump inhibitors for *K. pneumoniae* U25. Time-kill results of *K. pneumoniae* U25 proved that the concentration of antibiotic and the plant extract required was the sub-inhibitory concentration, which is 8-16 fold reduction from their actual MIC.
The efflux pump inhibition activity of *Punica granatum* and *Tectona grandis* were positive for *K. pneumoniae* U25, therefore we decided to analyse the MDR strains *Pseudomonas aeruginosa* P3 and *Escherichia coli* E6 which yielded positive results as well. These results suggested that ethanolic extracts of *Punica granatum* and *Tectona grandis*, not only showed synergistic interaction of drug-herb in a sub-inhibitory concentration levels for MDR *K. pneumoniae* U25; they also showed activity for other MDR pathogens like *E.coli* E6 and *Pseudomonas aeruginosa* P3.

We have also tested the efflux inhibition activity of natural efflux pump inhibitors berberine and reserpine on MDR strain *K. pneumoniae* (U25 & U6) and *E.coli* (E6) and only reserpine was found to be possessing norfloxacain accumulation ability.

EPIs from natural sources have also shown antibiofilm activity. Thus, the natural EPI caffeoylquinic acid (CQA) from *Artemisa absinthium* reduces biofilm viability in combination with subinhibitory concentrations of ethidium bromide and moxifloxacin in *S. aureus* and *Enterococcus faecalis*. This EPI acts by enhancing the killing effect of these compounds (Fiamegos et al 2011). Therefore, due to their characteristics, EPIs could be used as enhancers of the antibiotics and used in the treatment of biofilm (Kvist et al 2008).

**7.2 Summary**

This study involves screening for fluoroquinolone resistance in ESβL isolates of *K. pneumoniae* from Chennai. The present study was carried out to screen *qnr* genes in 23 ESβL isolates of *K. pneumoniae* collected in October 2009. Our results show the presence of the *aac(6')-1b* gene in the *Klebsiella* isolates from Chennai and the presence of the *cr* variant in at least one of the isolates. This report is significant since this is the first report from India demonstrating plasmid mediated quinoline resistance (PMQR) mediated by *qnr* genes and the *aac(6')-1b-cr* allele in *K. pneumoniae*. This was proved by transforming resistance capability to *E.coli* JM109.

The present report is a comprehensive comparative analysis of the effects of various natural compounds on MIC and biofilm inhibition in strong biofilm forming isolates of *K. pneumoniae*. The results showed that reserpine, followed by linoleic acid are the most potent as biofilm inhibitors. While reserpine at higher concentrations can be a neurotoxin, it can be used at low concentrations in combination with antibiotics. Linoleic acid as a
natural fatty acid with biofilm inhibitory activity has much promise for use in topical application formulations and coating of indwelling implants.

*Punica granatum* and *Tectona grandis* which were reported to have antibacterial activity were taken and in this present study, it was proved that the mode of action of *Tectona grandis* and *Punica granatum* ethanolic extracts was by efflux pump inhibition in the MDR *K. pneumoniae U25*, *Escherichia coli E6* and *Pseudomonas aeruginosa P3*. Thus, results in synergism by increasing the intercellular concentration of antibiotic and the optimal MIC were achieved by inactivation of the efflux pumps in the MDR bacteria. This report is significant since this is the first report from India demonstrating ciprofloxacin synergism and the norfloxacin accumulation in the MDR bacteria by using medicinal plant extracts.

In summary, there is currently insufficient knowledge as to the role of efflux pumps in biofilm resistance and, thus, further studies are needed to elucidate the role of these systems in bacterial biofilms. However, the existing studies show that efflux pumps could be an attractive target for antibiofilm drug development (Soto, 2013).

**7.3 Future Scope**

To date, no efflux pump inhibitor has been licensed for use in the treatment of bacterial infections in human or veterinary medicine, and it is clear that this gap in our antimicrobial armamentarium must stimulate research that leads to the development of new EPI molecules. The major benefits derived from developing efficient efflux pump inhibitors will be the ability to re-use various antibiotics affected by the efflux pumps as well as the control of the emergence and the dissemination of MDR efflux strains. Until now, the majority of the known inhibitors have been obtained from screening libraries of synthetic compounds, from purification of natural compounds or ‘naive’ chemical modification of existing molecules.

With this new generation of designed antibacterial compounds that are able to restore the antibiotic susceptibility by selectively blocking membrane transport, novel uses of transport inhibitors will emerge in order to

(i) Compete with the diffusion of quorum sensing molecules, or

(ii) Block the secretion of virulence factors.
Thus, combination therapy or synergistic therapy against resistant microorganisms may lead to new ways of treating infectious diseases and probably this represents a potential area for further future investigations.

There is a need for more studies concerning the molecular basis of synergistic interactions, to understand the synergistic mechanism which is fundamental to the development of pharmacological agents to treat bacterial infections using medicinal plants. Hence, research should be focused towards this direction to identify more medicinal plants which exhibit synergistic action.