ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by Pharmascope Publications Journal Home Page: www.pharmascope.org/ijrps

Inhibition of AcrB efflux pump in multidrug-resistant gram-negative bacteria by herbal extracts

Florida Tilton*[1], Shanmughasundar D[1], Aneesh Nair[1], Bindu D[2], Chitralekha Saikumar[2], Johnson WMS[3]

[1]Biozone Research Technologies Pvt. Ltd., Chennai, Tamil Nadu, India
[2]Department of Microbiology, Sree Balaji Medical College and Hospital, Chennai, Tamil Nadu, India
[3]Research Centre for Cellular Genomics, Sree Balaji Medical College and Hospital, Chennai, Tamil Nadu, India

Article History:

Received on: 31.07.2018
Revised on: 20.09.2018
Accepted on: 22.09.2018

Keywords:

AcrAB,
MDR,
EPI,
E.coli

ABSTRACT

The emergence of multidrug-resistant gram-negative bacteria has increased drastically over the years contributing to therapeutic failures. Mechanisms which modify the membrane permeation processes, such as increased efflux pump activity or decreased influx of antibiotics, are the major contributors of multidrug resistance in bacterial phenotype. Many approaches from different perspectives are being developed and applied in clinical and research to overcome this threat. Antibiotic resistance can be encountered by introducing more powerful antibiotics that kill the bacterial cells more effectively, by using new therapeutic compounds or by eliminating one or more of the mechanisms of resistance by resistance modifying agents. Herbal plants synthesise a wide variety of compounds that are antibacterial or inhibit the activity of efflux pump (EPI) in nature. This study involves the analysis of efflux pump inhibitory activities of herbal extracts and comparison of their synergetic effect with antibiotics which would have a new approach to counter antibiotic resistance.

* Corresponding Author

Name: Dr. Florida Tilton
Phone: +91-9940027169
Email: florida1871@yahoo.com

ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v9i4.1701

Production and Hosted by

Pharmascope.org

INTRODUCTION

The emergence of multidrug-resistant gram-negative bacteria has increased drastically over the years contributing to therapeutic failures. Mechanisms which modify the membrane permeation processes, such as increased efflux pump activity or decreased influx of antibiotics, are the major contributors of multidrug resistance in bacterial phenotype (Piddock LJ, 2006; Alekshun MN and Levy SB.,2001; Davin-Regli A *et al*., 2008). Efflux pumps are found on the chromosomes or plasmids of all bacterial species. Based on the composition, the number of transmembrane spanning regions, energy sources, bacterial efflux pumps are classified into five families: the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), ATP (adenosine triphosphate)-binding cassette (ABC) superfamily, and the multidrug and toxic compound extrusion (MATE) family (K. Poole. 2007; L.J. Piddock, 2006; M. Putman et al., 2000). RND (AcrAB-TolC) superfamily is found only in Gram-negative bacteria. The other four families: MFS, ABC, SMR and MATE are found in both Gram-negative and positive bacteria (J. Handzlik *et al., 2013)*.

Escherichia coli and *Klebsiella pneumonia* are two major enteric pathogens which cause a wide range of hospital and community-based infections. Multidrug resistance is seen in both these bacteria. Efflux pump AcrAB, which contributes to resistance

Table 1: Selected Plants and the parts used

S.No.	Plant	Part of plant	Place of collection
1	*Zingiber officinale*	Rhizome	Kokkirakulam
2	*Terminalia chebula*	Seed	Nagercoil
3	*Azadirachta indica*	Leaf	Chennai
4	*Justicia tranqibalances*	Leaf	Tamirabarani river banks
5	*Bahinia racemosa*	Flower	Nagercoil
6	*Premna latifolia*	Leaf	Tamirabarani river banks
7	*Aerva lanata*	Leaf	Nagercoil
8	*Cadaba fruiticosa*	Leaf	Kokkirakulam
9	*Eclipta alba*	Leaf	Kanyakumari
10	*Tinspora cordofolia*	Leaf	Chennai
11	*Abutilon indicum*	Leaf	Kanyakumari
12	*Cassia auriculata*	Flower	Tirunelveli
13	*Centella asiatica*	Leaf	Chennai

to fluoroquinolone and other antibiotics, has been studied extensively in *K.pneumoniae* and *Escherichia coli* (Okusu H *et al., 1996;* Mazzariol A. *et al., 2002;* Padilla E *et al., 2010*). This literature review suggested the role of an efflux pump in resistance to antibiotics.

In order to combat the antibiotic resistance, efflux pump inhibitors (EPI), or resistance modifying agent, from natural sources can reintroduce the older antibiotics like ciprofloxacin and suppress the multidrug inhibitors (Michael Stavri Laura J *et al.,* 2007*)*. EPI does not exert pressure on the bacterial cells and hence it reduces the emergence and spread of antibiotic resistance (Kaatz GW. 2000).

Different plant products have been studied to increase the activity of antibiotics, fluoroquinolones along with oregano oil has been used to increase the activity against MDR E.coli (Si H *et al.,* 2008), gallotannin extracted from *Terminalia chebula* showed good EPI activity against MDR E.coli (Bag A and Chattopadhyay R R, 2014). A combination of flavonoids and antibiotics were tested against *Klebsiella pneumoniae* (Ozcelik B *et al., 2008)*. *Elettaria cardamomum* (Dried fruit), *Azadirachta indica* (leaf) and *Cassia fistula* (leaf) have shown good synergistic activity against *E.coli. Lawsonia inermis* (leaf), *Azadirachta indica* (leaf), *Catharanthus roseus* (leaf) and *Lawsonia inermis* (leaf) has shown good activity against *K.pneumoniae* (Leena Seasotiya and Sunita Dalal, 2014).

The current study aims to detect a novel inhibitor of efflux pump n clinical isolate of gram-negative bacteria from a natural source.

MATERIALS AND METHODS

Selection of plant material

Medicinal Plants belonging to different families were selected on the basis of traditional applications and pharmacological reports. The plant samples were collected from various places in Tamil Nadu, cleaned and transported to the laboratory in sterile conditions (Table 1).

Preparation of plant extracts:

Samples were washed under running tap water followed by sterile water and shade dried for 4-5 days. Dried plant materials were powdered and stored in airtight containers. Methanol was used as a solvent for extraction. 10 g of powdered sample was weighed and added to a conical flask containing 60ml of methanol. It was allowed to stand for 30 mins in a water bath (at 100°C) with occasional shaking followed by keeping all the flasks on a rotary shaker at 200 rpm for 24 h. Each preparation was filtered through a sterilized Whatman No. 1 filter paper and finally concentrated to dryness by placing the extracts on hot plates. This was finally dried under vacuum at 40°C using a rotary evaporator. The dried extract was stored at 4°C in the refrigerator (Aneja KR *et al., 2010*). The dried extracts were reconstituted to 10% in dimethylsulphoxide (DMSO) for the antibacterial analysis.

Microbial strains

The microorganisms (*Proteus mirabilis, Escherichia coli, Klebsiella pneumonia, Acinetobacter sp., Pseudomonas aeruginosa)* used in the present study were isolated clinical samples obtained from Sree Balaji Medical College and Hospital, Chrompet. All the isolates were resistant to methicillin and tetracycline.

The collected samples were stored in a sterile container and transported to the laboratory within one hour of collection and stored at 4°C, till the processing and media preparation. 100 µl of each bacterial sample was inoculated aseptically into 20 mL of LB broth (Hi-Media Pvt. Ltd. Mumbai) and incubated at 37°C for 24 hours.

Table 2: Absorbance values of Methicillin in cell lysis pellet, Cell lysis supernatant and Supernatant at various wavelengths

Wavelengths	Cell lysis pellet	Cell lysis supernatant	Supernatant
380	0.0473	0.069	0.63
390	0.0376	0.0647	0.7329
400	0.0561	0.0645	0.8094
410	0.0543	0.0535	0.8166
420	0.0372	0.0679	0.7644
430	0.0198	0.0554	0.7173
440	0.0186	0.0543	0.6489
450	0.0316	0.0675	0.5826

Antibiotic accumulation assay

Antibiotic accumulation assay is used to detect the accumulation of a particular antibiotic in a bacterial strain. Accumulation assay was done to detect the concentration of methicillin accumulated in the cells. 100 µl of E.coli from the broth culture was taken in 3 test tubes containing 6mL of LB broth. 5mcg of methicillin was added to each tube and incubated at 37°C for 24 hours. 3 mL of culture from each tube was transferred to an Eppendorf. One Eppendorf was centrifuged at 8000 rpm for 5 minutes at -4°C to obtain the cell-free supernatant. The second sample was centrifuged and the pellet was subjected to cell lysis by treating with 100µL of 10% SDS. The third sample was also subjected to cell lysis in the same manner, centrifuged and the supernatant was taken. Cell lysed samples were made up to 3 mL in Eppendorf tubes by PBS buffer. The three methicillin concentration in each of the sample was determined spectrophotometrically by reading at 380-450 nm. LB media with 5 mcg of methicillin was taken as blank for the first sample and PBS buffer was chosen as blank for the cell lysed samples.

Preparation of stock solutions

The stock solution of the plant extracts was prepared by dissolving 10 µg of each extract in 0.5 mL of methanol. This gave a concentration of 400µg/20µL

Estimation of efflux pump inhibitory properties

Disc diffusion assay

Disc diffusion assay using the Kirby-Bauer disk diffusion method (Bauer. *et al.* 1966) was performed. E.coli from the preserved slant culture was inoculated into 10mL Muller Hinton (MH) broth and incubated overnight at 37 ºC. E.coli from the broth was swabbed on thirteen MH agar plates. Three methicillin discs were placed on each plate which was loaded with 20 µL, 40 µL and 60 µL of a single plant extract respectively. From the stock solution loading of 20uL, 40uL and 60 uL extract gave a concentration of 400, 600 and 800 µg per disc. A sterile disc loaded with 20 µL of plant extract and a methicillin disc was also placed as a control. The plates were incubated at 37 ºC for 12 hours.

RESULTS

Accumulation assay

Antibiotic accumulation assay was performed and read at wavelengths 380, 390, 400, 410, 420, 430, 440 and 450 nm. The relatively low concentrations of Methicillin in cell lysis pellet and cell lysis supernatant indicate less accumulation of Methicillin inside the cells. Methicillin was transported out of the cell, which resulted in high levels of Methicillin in the supernatant. (Table 2)

The varying concentrations of Methicillin in and outside the cell was observed and documented in a graph. Less accumulation confirms the active efflux pump in E.coli. (Figure 1).

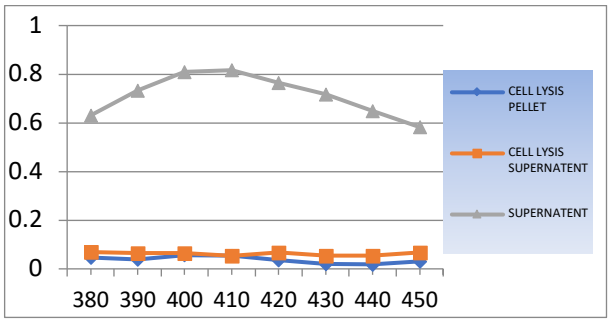

Figure 1: Graph representing the varying concentrations of Methicillin in Cell lysis pellet, cell lysis supernatant and Supernatant

Out of the 13 herbals extract tested, four herbals extract were found to inhibit the growth of bacteria when combined with an antibiotic. The herbal extracts were not antibacterial. The herbal extract was found to inhibit bacterial growth only when administered with an antibiotic. As the concentration of the herbal extract increases, so was the zone of inhibition. (Figure 2)

DISCUSSION

Antibiogram revealed that the culture was resistant to Methicillin. Antibiotic accumulation

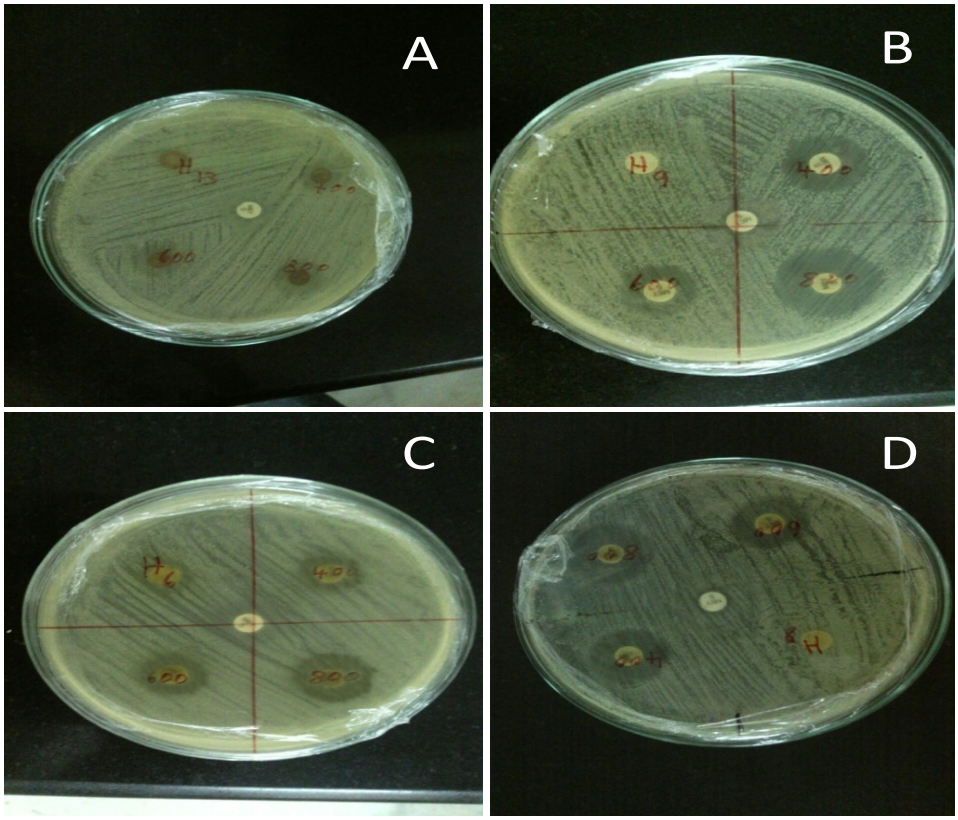

Figure 2: Comparison of the synergic effect of the four herbal extracts at a different concentration with antibiotic

assay proved that efflux pump is active in E.coli strain. Both the antibiotic and herbal extracts

individually were not able to inhibit the bacterial growth, the combination of antibiotic and herbal extract was found to inhibit bacterial growth. Furthermore, the increasing concentration of herbal extract increased the zone of inhibition with the antibiotic. This proves the EPI of herbal extracts.

It is evident that the herbal compounds act as Efflux pump inhibitors, increasing the drug accumulation. The disc diffusion assay suggests that the compounds present in the herbal extracts were responsible for the inhibition of the efflux pump. This resulted in increased drug accumulation in the bacterial cells, thus inhibiting the bacterial growth. The basis of the varying degree of sensitivity of bacteria may be due to varied efflux pump availability in different strains and the nature and combination of phytocompounds present in the crude extract. Out of the 13 plants studied, the methanol extracts of 4 plants were more effective in inhibiting drug efflux pumps. Hence, these extracts have a promising future for the development of effective EPIs which would augment the antibacterial activities of standard antibiotics. The identification of plants that can inhibit efflux pumps is important as they provide a potential lead optimization and future use with an existing antibacterial rendered ineffective due to MDR pumps in Gram-negative bacteria.

Therefore, in this study, we sought to identify medicinal plants that could provide compounds for further antimicrobial drug development. In addition, as there are many clinically licensed antibacterial agents for the treatment of infections by Gram-negative bacteria, but which are effluxed by the various pumps possessed by these bacteria, we sought to screen for activity that suggested efflux inhibition. One desirable property of a putative EPI is that it should synergize with antibiotics for bacteria. In this study, it is evident that the combination of antibiotic and the herbal extract is synergetic in effect. There are various studies (Si H *et al., 2008;* Bag A, and Chattopadhyay R R, 2014; Ozcelik B *et al.,* 2008. Leena Seasotiya and Sunita Dalal, 2014*)* of EPI from natural source against gram-negative bacteria. Further studies on the specific components present in herbal extract could lead to the development of Efflux pump Inhibitors that could be administered along with antibiotics for effective treatment against deadly bacterial diseases and infections.

CONCLUSION

Efflux pump is one major resistance mechanism recognized to cause resistance to many classes of antibiotics. A potential efflux pump inhibitor provides an approach to generate therapy by using the existing antibiotics, especially in MDR strains. The present study indicates some potential EPI from Indian medicinal plants. The activity of some of the

extracts is appreciable and warrant further investigation. This approach decreases the frequency of emergence of resistant strains. The extracts from four plants showed activity as efflux pump inhibitors. Identification and isolation of the lead compounds from these plant extracts can serve as templates for the production of new antibiotics as well as efflux pump inhibitors. Inhibition of MDR efflux pumps can restore the activities of antibacterial agents.

REFERENCES

Alek Shun MN, Levy SB, 2007. Molecular mechanisms of antibacterial multidrug resistance. Cell.2007, 128: 1037 – 1050.

Anja KR, Joshi R, Sharma C, 2010. The potency of Ballerina priorities L. bark extracts against oral diseases causing strains of bacteria and fungi of clinical origin. New York Sic J, 3:5-12.

Bag A, and Chattopadhyay R R, 2014. Efflux-pump inhibitory activity of a gallotannin from Terminalia chebula fruit against multidrug-resistant uropathogenic Escherichia coli. Nat Prod Res. 28(16), 1280-1283.

Davin-Regli A, Bolla JM, James CE, Lavigne JP, Chevalier J, Garnotel E, Molitor A, Pagès JM, 2008. Membrane permeability and regulation of drug 'influx and efflux' in enterobacterial pathogens. Curr Drug Targets. 9:750–759.

J. Handzlik, A. Matys, K. Kieć-Kononowicz, 2013. Recent advances in multi-drug resistance (MDR) efflux pump inhibitors of Gram-positive bacteria S. aureus. Antibiotics, 2, 28-45.

K. Poole, 2007. Efflux pumps as antimicrobial resistance mechanisms. Ann. Med, 39, 162-176.

Kaatz GW. Inhibition of bacterial efflux pumps, 2000. A new strategy to combat increasing antimicrobial agent resistance. Expert Opin Emerg Drugs., 7, 223-233.

L.J. Piddock, 2006. Multidrug-resistance efflux pumps? Not just for resistance. Nat. Rev. Microbiol., 4, 629-636.

Leena Seasotiya and Sunita Dalal, 2014. Screening of Indian medicinal plants as efflux pump inhibitors of fluoroquinolones. JPP, 3(1), 235-241

M. Putman, H.W. van Veen, W.N. Konings, 2000. Molecular properties of bacterial multidrug transporters. Microbiol. Mol. Biol. Rev., 64, 672-69

Mazzariol A Zuliani J Cornaglia G et al., 2002. AcrAB efflux system: expression and contribution to fluoroquinolone resistance in Klebsiella spp. Antimicrob Agents Chemother, 46, 3984- 3986

Michael Stavri Laura J. V. Piddock Simon Gibbons, 2007. Bacterial efflux pump inhibitors from natural sources. Journal of Antimicrobial Chemotherapy, 59 (6), 1247–1260.

Okusu H Ma D Nikaido H, 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multiple-antibiotic-resistance (Mar) mutants. J Bacteriol,178, 306-308

Ozcelik B, Orhan D D, Ozgen S, and Ergun F, 2008. Antimicrobial activity of flavonoids against extended-spectrum ß-lactamase (ESßL)-producing Klebsiellapneumoniae. Trop J Pharm Res. 7(4), 1151-1157.

Padilla E, Llobet E, Domenech-Sanchez A et al., 2010. Klebsiella pneumoniae AcrAB efflux pump contributes to antimicrobial resistance and virulence. Antimicrob Agents Chemother, 54, 177-183

Piddock LJ, 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev, 19: 382-402.

Si H, Hu J, Liu Z, Zeng Z L, 2008. Antibacterial effect of oregano essential oil alone and in combination with antibiotics against extended-spectrum beta-lactamase-producing Escherichia coli. FEMS Immunol Med Microbiol, 53, 190-194.