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EFFICACY OF SYNERGISTIC PLANTS AS EFFLUX PUMP INHIBITORS AGAINST EMERGING MULTIDRUG RESISTANCE IN *ESCHERICHIA COLI*

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ABSTRACT

Traditional antimicrobials are increasingly suffering from the emergence of multidrug resistance among pathogenic microorganisms. To overcome these deficiencies, a range of novel approaches to control microbial infections are under investigation as potential alternative treatments. Multidrug efflux is a key target of these efforts Multidrug resistance (MDR) has expanded dramatically in a broad range of organisms from bacteria to humans resulting in a global increase in life threatening infections and deaths. Multidrug resistance in bacteria may be generated by one of two mechanisms. First, multidrug resistance may occur by the increased expression of genes that code for multidrug efflux pumps, extruding a wide range of drugs. Second, these bacteria may accumulate multiple genes, each coding for resistance to a single drug, within a single cell. This accumulation occurs typically on resistance (R) plasmids. There is a high medical need to systematically explore the etiology and principles as well as to devise strategies leading to implementation of effective countermeasures. Efflux mechanisms are broadly recognized as major components of resistance to many classes of chemotherapeutic agents as well as antimicrobials. In recent years, there are many classes of efflux pump inhibitors has been reported. Some of these efflux pump inhibitors are synthetic while some of them are natural inhibitors. Some plants show potential efflux pump inhibitor activity along with some antibiotics and shows effect on many efflux pumps. In this review, we discuss our current knowledge on the molecular mechanisms and the new approaches for Multidrug resistance.

Keywords: Multidrug resistance, efflux pump inhibitors, antibiotics, *Escherichia coli*

INTRODUCTION

Escherichia coli (*E. coli*) is a gram negative, rod shaped bacterium that is commonly found in the lower intestine of warm blooded organisms. It is classified as part of the Enterobacteriaceae family of gamma-proteobacteria. [1] *E. coli* strains and its serotypes are pathogenic and can cause serious Urinary tract infections [2] *E. coli* constitute about 0.1% of gut flora, [3] and faecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Pathogenic *E.coli* strains can be categorized based on elements that can elicit an immune response in mammals, namely: 1. O Antigen: part of lipopolysaccharide layer. The O antigen is used for serotyping *E. coli* and these O group designations go from O1 to O181, [4] 2. K Antigen: capsule K antigen: the acidic capsular polysaccharide (CPS) is a thick, mucous like, layer of polysaccharide that surrounds some pathogen *E. coli*.3. H antigen: flagellin. The H antigen is a major component of flagella, involved in *E. coli* movement. There are 53 identified H antigens, numbered from H1 to H56 (H13 and H22 are not *E. coli* antigens. [5] *E. coli* is a common pathogen linked with community-associated as well as nosocomial infections [6, 7]. In the last few years, the emergence and wide dissemination of *E. coli* strains showing resistance to broad-spectrum of antimicrobial agents has been reported. [6, 8, 9] Emergence of resistance to multiple antimicrobial agents in pathogenic bacteria has become a significant public health threat as there are fewer, or even sometimes no, effective antimicrobial agents available for infections caused by these bacteria. [10] According to the European Centre for Disease Prevention and Control (ECDC) and the Centres for Disease Control and Prevention (CDC), multi-drug resistant (MDR) is defined as non-susceptibility to at least one agent in three or more antimicrobial categories. [11] MDR bacteria are the principal cause of failure in the treatment of infectious diseases, resulting in increases in the term and magnitude of morbidity, higher rates of mortality, and a greater health cost burden. [12]

CLINICAL MANIFESTATION OF *Escherichia coli*

The bacteria can cause number of infections in human body. Acute bacterial meningitis new-borns with *E.coli* meningitis present with fever and failure to thrive or abnormal neurologic signs. Other diseases in neonates include jaundice, decreased feeding, periods of apnea, and listlessness. Patients younger than 1 month

present with irritability, lethargy, vomiting, lack of appetite, and seizures. [13] Patients with *E coli* dysentery caused by entero invasive *E. coli* (EIEC) orenterohemorrhagic *E. coli* (EHEC) have fever, bloody diarrhoea, and dehydration [14]. Cholangitis manifests with fever (>102°F), shaking chills, right quadrant pain and can be complicated by hepatic abscess [15]. The study was conducted in M.SRamaiah hospital Bangalore from January to December 2008. *E.coli* (66.9%) was the most common organism causing community-acquired urinary tract infection. [16] This toxin attacks small blood vessels, killing intestinal cells, and cause haemolytic uremic syndrome (HUS), a potentially deadly condition that can involve widespread clots in capillaries and haemolytic anaemia, thrombocytopenia, and renal failure. [17]

DRUG RESISTANCE

Multidrug resistance (MDR) refers to the capability of bacterial pathogens to withstand lethal doses of structurally diverse drugs which are capable of eradicating non-resistant strains [26]. Antibiotic resistance rates in *E.coli* are rapidly rising, especially with regard to fluoroquinolonescephalosporin.

Surprisingly, most of these multidrug-resistant strains are acquired in the community rather than in healthcare settings. [19] The details of *E. coli* grown from urine sample of patients of Indira Gandhi medical college & research institute (IGMC) Puducherry India. Of these *E. coli* isolates, (76.51%) were multi drug resistant (MDR). The isolates showed high levels of resistance to ampicillin (88.4%), norfloxacin (74.2%), cefuroxime (72.2%), ceftriaxone (71.4%) and co-trimoxazole (64.2%). The isolates were sensitive to amikacin, piperacillin-tazobactum and imipenem [20]. In May, June, and July, 2011 an outbreak of gastroenteritis caused by Shigatoxin producing *E. coli* was seen in Germany. The majority of patients were adults and 22% of the cases developed haemolytic–uremic syndrome [19]. A study was done at Aligarh Muslim University, Aligarh, India followed by isolation and identification of *E. coli* strains. Antibiotic sensitivity and resistance analysis was performed by the disc diffusion method [21]. The isolation shows resistance towards Ampicillin (90%), chloramphenicol (60%), erythromycin (70%), rifampicin (60%), tetracycline (79%). Study revealed that majority of the isolates showed the UTI infections. Studies also showed that 60-79% isolates were resistant against chloramphenicol, erythromycin, rifampicin, and tetracycline. *E. coli* isolates from children were found

more resistant to fluoroquinolones. [24] The sensitivity pattern of *E. coli* to antibiotics in UTI were Nitrofurantoin (85.19%), Co-trimoxazole (31.31%), Gentamycin (26.90%), Ceftazidime (26.69%) Cefotaxime (22.30%), Ceftriaxone (17.47%) [25].

EFFLUX PUMPS

Multidrug resistance (MDR) refers to the capability of bacterial pathogens to withstandlethal doses of structurally diverse drugs which are capable of eradicating non-resistant strains. [26] MDR has been identified as a major threat to the public health of human being bythe World Health Organization (WHO). [26] Among the four general mechanisms that cause antibiotic resistance includes [27]

1. Efflux Pumps
2. Drug inactivation
3. By-passing of the target
4. Target alteration

Efflux pumps are transport proteins involved in the extrusion of toxic substrates from within cells into the external environment [28]. Production of this altered structure requires the participation of several imported genes and increased efflux, drug extrusion by the multidrug efflux pumps serves as an important mechanism of MDR [26]. Drug and multidrug resistant bacteria harbour several distinct molecular mechanisms for resistance. Bacterial antimicrobial agent efflux pumps represent a major mechanism of clinical resistance [29]. Their actions reduce the intracellular concentrations of antibiotics to sub lethal levels leading to the development of specific modes of resistance via gene mutations or antibiotic degradation [30, 31]. Efflux pumps not only can expel a broad range of antibiotics owing to their polysubstrate specificity, but also drive the acquisition of additional resistance mechanisms by lowering intracellular antibiotic concentration and promoting mutation accumulation. Over expression of multidrug efflux pumps have been increasingly found to be associated with clinically relevant drug resistance. The carriage of efflux pump genes on the chromosome gives the bacterium an intrinsic mechanism that allows survival in a hostile environment (e.g. the presence of antibiotics), and so mutant bacteria that over-express efflux pump genes can be selected without the acquisition of new genetic material. It is probable that these pumps arose so that noxious substances could be transported out of the bacterium, allowing survival. Indeed it is now widely accepted that the ‘intrinsic resistance’ of Gram-negative bacteria to certain antibiotics relative to Gram-positive bacteria is a result of the activity of efflux systems [34]. Phylogenetically bacterial

efflux pumps are classified into five families: the resistance nodulation division (RND) family, the major facilitator super family (MFS), the ATP(adenosine triphosphate) binding cassette (ABC) super family, the small multidrug resistance (SMR) family [a member of the much larger drug/metabolite transporter (DMT) superfamily], and the multidrug and toxic compound extrusion (MATE) family [33, 36]. As many efflux pumps possess significant structural homology, it is hoped that one inhibitor compound will be active against a range of pumps from different bacterial species. Following the recognition that the housekeeping efflux pump AcrAB-TolC serves as an important antibiotic resistance determinant and plays a major role in the MDR phenotype of *E. coli* clinical isolates [37, 38]. Different types of bacterial multidrug efflux pumps with their superfamily shown in table-1

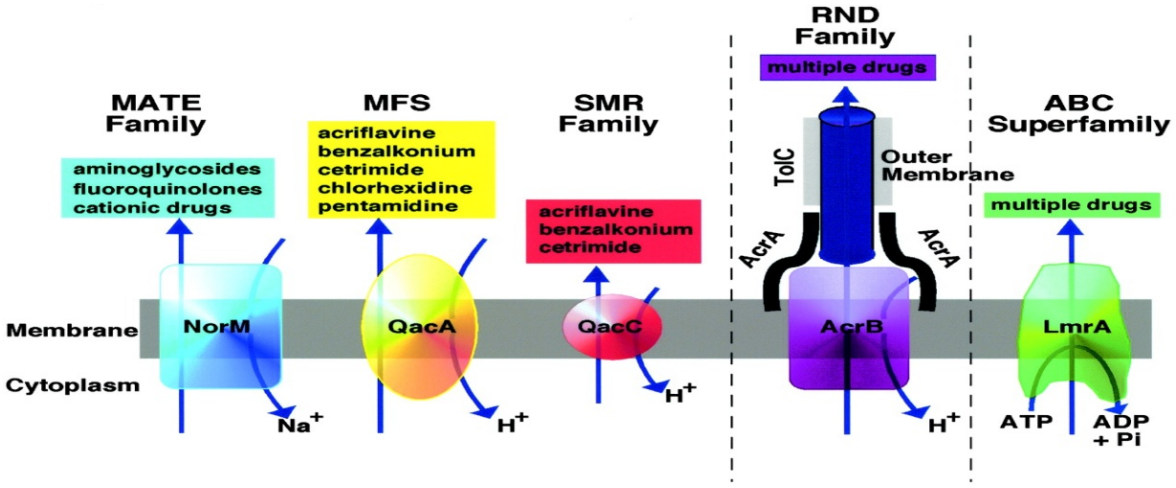

Fig 1: Diagrammatic representation offivesuper families of Multi Drug Resistance Efflux pump

Table 1: BacterialMultidrug Efflux Pumps with their Super families

Organism	Efflux pump	Super family	Ref. No.
Escherichia coli	AcrAB	RND	[39]
	AcrD	RND	[40]
	AcrEF	RND	[41]
	EmrAB	MFS	[42]
	EmrKY	MFS	[43]
	MdtABC	RND	[44]
	MdtEF	RND	[45]
	MacAB-TolC	ABC	[45]
	EmrAB-TolC	MFS	[45]
	Fsr		[45]
Psedomonasaeruginosa	Tet A, C, E	MFS	[45]
	MexAB-OprM	RND	[46]
	MexCD-OprJ		
	MexEF-OprN		
	MexJK-OprM		
	MexXY-MOpr		
Enterobacteraerogenes	CmlB	MFS	[47]
	AcrAB-TolC	RND	[47]

EFFLUX PUMP INHIBITOR

Efflux pump inhibitors (EPIs) are the molecules which interfere with the process of removing toxic substances and antibiotics from the bacterial cell. EPIs are divided into two categories Synthetic EPIs And Natural EPI's. The synthetic EPI's are classified into peptide mimetics and pyridopyrimidines, [48] Phenyl arginine betanaphthylamide (PAβN) (MC207,110) was the first identified Efflux Pump Inhibitor PAβN also has an activity against the AcrAB-TolCin a variety of Gram-negative pathogens *E. coli*its inhibition mechanism was proposed to be acting as an RND substrate [49]. Another group of EPIs is called the quinoline derivatives, because of their structural similarity with quinolones [51] In addition to the structurally modified synthetic compounds that display efflux pump inhibition activities, high through put assays have also used to screen compounds that might be putative EPI's. In a high through put screening for putative inhibitor of *E. coli* in the presence of novobiocin, a 3arylpiperazinederivative was identified to be able to increase the intracellular concentration of novobiocin and another antibiotic linezolid [52]. Another screening of an N-heterocyclic organic compound library was conducted to identify putative EPIs that can reverse multidrug resistance in *E. coli* that overexpresses AcrAB and AcrEF efflux pumps [53]. Among the compounds tested naphthyl piperazines (NMP) was the most potent arylpiperazines that has been shown to increase the intracellular accumulation of several antibiotics, such as fluoroquinolones, chloramphenicol, and linezolid. However, these compounds seem to be too toxic for clinical usage because of "serotonin agonist" properties [54]. Natural products (NP) that have been implicated in efflux inhibition have also attracted intensive attention since these compounds are often less toxic than synthetic compound pheophorbide*a* extracted from *Berberisaetnensis* was shown to lower the MIC of ciprofloxacin against *E. coli.* [55, 56] Future research will focus on the development of extensive biological assays towards the application of EPIs in clinics, such as fitness and in vivo modelling studies [49]. In addition, expanded EPI efficacy assays beyond the several model microorganisms, such as *P. aeruginosa* and *E.coli* should also be developed. These are various natural EPIs that derived from natural sources are mentioned in the Table no. 2.

Table 2: List of Natural Efflux Pump Inhibitors with their plant sources against specific efflux pump

Bacteria	Efflux pump inhibitor	Plant source	Efflux pump	Ref. No.
Escherichia coli	Baicalein	*Thymus vulgaris*	TetK	[57]
Klebsiellapneumonia	Theobromine	*Theobroma cacao*	AcrABTolC	[58]
Salmonella Typhimurium	Theobromine	*Theobroma cacao*	AcrABTolC	[58]
Salmonella typhimurium	Cathinone	*Catha edulis*	AcrABTolC	[58]
Pseudomonas arugenosa	Pheophorbide	*Berberisaetnensis*	MexAB-OprM	[59]
Enterobacter cloacae	Theobromine	*Theobroma cacao*	AcrABTolC	[58]
Enterococcus faecalis	Caffeoylquinic acid	*Artemisia absinthium*	NorA	[60]
Mycobacterium spp	Fernasol	*Cymbopogoncitratus*	TetK	[61]
Mycobacterium spp	Isorhamnetin	*Tageteslucida*	TetK	[62]
Mycobacterium spp	Epicatechin	*Camellia sinensis*	TetK	[63]
Mycobacterium spp	Resveratrol	*Fallopia japonica*	TetK	[64]
Bacillus cereus	Chalcone	*Nicotianatabacum*	NorA	[65]
Streptococcus pneumonia	Reserpine	*Rauwolfiavomitoria*	NorA	[66]
Staphylococcus aureus	Porphyrin,Pheophorbide	*Berberisaetnensis*	NorA	[67]
Staphylococcus aureus	Carnosic acid, carnosol	*Rosmarinusofficinalis*	MsrA	[58]
Staphylococcus aureus	Piperine	*Piper nigrum, Piper longum*	MdeA and NorA	[68]
Staphylococcus aureus	Reserpine	*Rauwolfia vomitoria*	TetK, NorA	[66]
Staphylococcus aureus	Porphyrin	*Berberis aetnensis*	NorA	[67]
Staphylococcus aureus	Murucoidins	*Ipomoea murucoides*	NorA	[69]
Mycobacterium spp	Sandaracopimaric acid	*Juniperusprocera*	TetK	[63]

RATIONAL FOR USING PLANTS AGAINST BACTERIA

Antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them produce toxic reactions, but also due to emergence of drug-resistant bacteria. It is essential to investigate newer drugs with lesser resistance. Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. In many developing countries, traditional medicine is one of the primary healthcare systems. [70, 71]. Herbs are widely exploited in the traditional medicine and their curative potentials are well documented [72]. Natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanisms of action [73, 74]. The World Health Organization estimates that plant extracts or their active constituents are used

as folk medicine in traditional therapies of 80% of the world's population [75]. With the continuous use of antibiotics microorganism have become resistant. I addition to this problem, antibiotics are sometimes associated with adverse effects on host which include hypersensitivity, immunosuppressant and allergic reactions [76]. Baicalein, a well-known efflux pump inhibitor which shows the activity against efflux pump of *E.coli*. Baicalein is isolated from Thymus vulgaris [57]. Derivatives of isopimarane shows efflux pump inhibitory activity against efflux pumps of *Enterobacter aerogenes* [77]. The obromine which is a bitter alkaloid which shows synergistic activity with ciprofloxacin against RND efflux pump of different gram– negative bacteria such as, *Klebsiella pneumoniae, Salmonella typhimurium* and *Pseudomonas aeruginosa.* The extract of Berberisa etnensis along with ciprofloxacin shows efflux pump inhibitory activity against *E. coli.* The ethanolic extracts of Vernoniaadoensis, Mangiferaindica and Callistemon citrinus shows efflux pump inhibitory activity against *Pseudomonas aeruginosa* and *E. coli*. [78]

Table 3: List of Synthetic Efflux Inhibitorswith their sources against specific efflux pump

Organism	Compound	Source	Efflux pump	Ref. No.
Escherichia coli	**3-amino-6-carboxyl-indole, 3-nitro-6-aminoindole**	**Indole derivative**	**AcrAB-TolC**	**[79]**
Pseudomonas aeruginosa	**Phe-Arg- β-naphthylamide (PAβN; MC207,110)**	**Dipeptide amide compound**	**MexAB-OprM, MexCD-OprJ, MexEF-OprN**	**[80]**
Escherichiacoli P.aeruginosa	**4(3-morpholinopropylamino) Quinazoline**	**4alkylaminoquinazoline Derivatives**	**AcrAB-TolC MexAB-OprM**	**[81]**
Enterobacter Aerogenes	**7nitro8methyl4[2′(piperidino)ethyl] Aminoquinoline**	**Alkyl amino quinolines**	**AcrABTolC**	**[82]**
Klebsiella Pneumonia	**2,8dimethyl4(2′pyrrolidinoethyl) Oxyquinoline**	**Alkoxyquinoline Derivative**	**NorA**	**[83]**
Acinetobacter Baumannii	**2 substituted benzothiazoles**	**Synthetic**	**AdeABC**	**[84]**
Escherichia coli	**1(1Naphthylmethyl) piperazine (NMP)**	**Arypiperazine**	**AcrAB, AcrEF**	**[85]**
Enterobacteraerogenes	**New chloroquinoline Derivatives**	**Fluoroquinolones**	**AcrAB-TolC**	**[86]**
Escherichia coli	**MBX2319**	**Synthetic Pyranopyrimidine**	**AcrB**	**[87]**

DISCUSSION

The study on efflux Pump Inhibitor(s) evaluation assay studies suggest that some moderate or strong polar components play a role in inhibiting bacteria. The basis of varying degree of sensitivity of bacteria may be due to varied efflux pump availability in different strains and the nature and combination of phytochemical

compounds present in crude extract. The methanol extracts of plants were more effective in blocking drug efflux pumps. A possible reason for this would be the presence of abundant chemical compounds in the extracts with efflux pump inhibitory activity. Hence, these extracts have a promising future for the development of effective drugs against *E. coli* EPIs which would augment the antibacterial activities of standard antibiotics. The identification of plants able to 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 both Gram-positive and Gram-negative bacteria. Therefore, in this review 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 pump possessed by these bacteria, we sought to screen for activity that suggested efflux inhibition. The search of potential efflux pump inhibitors provides an approach to generate therapy by interaction between different mechanisms of resistance. The present study indicates a high potential of EPI in Indian medicinal plants. The activity of some of the extracts is appreciable and particularly in *Staphylococcus* strain. Tariro *et al*., reported ethanolic extracts of *M. indica is a* potent EPI against *P. aeruginosa*. Plant extracts provide lead compounds for further exploration and development as antimicrobial agents, as agents to inhibit efflux or for combination with licensed antimicrobial.

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