

Antimicrobial Compounds of Plant Origin as Efflux Pump Inhibitors: New Avenues for Controlling Multidrug Resistant Pathogens

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Abstract

The emergence of multiple drug resistant pathogenic bacteria has severely constricted the antimicrobial options of treating infectious diseases. One of the powerful mechanisms of antibacterial resistance employed by the antibiotic resistant bacteria is the active extrusion of antimicrobials with the help of membrane transporters known as efflux pumps. Efflux pumps effectively reduce the intracellular concentrations of antimicrobials by their antiporter activity in which antimicrobials are extruded outside the bacterial cell using energy derived from ionic gradient across the cell membrane. While a few efflux pumps are capable of conferring clinical levels of resistance to antibiotics, while many others only marginally increase the minimum inhibitory concentrations (MIC) of antibiotics. However, the role of efflux pumps in gradual development of antibiotic resistance by pathogenic bacteria due to mutations and other molecular mechanisms during the course of antimicrobial therapy is well recognized. The inhibition of active efflux can result in maintenance of an intracellular concentration of antibiotics necessary to inhibit or kill bacteria. Plant-derived compounds have historically been recognized as effective antimicrobial agents. Advances in analytical techniques have enabled purification of natural compounds responsible for efflux pump inhibition and these compounds and their derivatives can act as lead compounds for designing more effective efflux pump inhibitors. Efflux pump inhibition is promising as an effective method of confronting bacterial resistance to antimicrobials either alone or as adjuvants with antibiotics, and thereby restoring the antibacterial efficacy of antibiotics.

Keywords: Efflux pump inhibition; Antibiotic resistance; Plant compounds; Antibiotic potentiation; Antiporter; Secondary active transporter

Introduction

For over seven decades after the introduction of the first antibiotic penicillin for treatment of infectious diseases, antibiotics have remained as sole weapons against pathogenic bacteria. However, the power of antibiotics is fast waning due to the rapid development of resistance by the pathogenic bacteria [1]. Apart from their use in the treatment of human infections, antibiotic have been used extensively in food animals, agriculture and aquaculture. A large proportion (>70%) of globally produced antibiotics are used in animals for purposes of growth promotion, prophylaxis, improvement of feed efficiency and the treatment of infections [2]. The antibiotic overuse has gradually led to the development of resistance among human pathogenic bacteria. methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus faecalis* (VRE), extremely drug resistant *Mycobacterium tuberculosis*, carbapenem resistant Enterobacteriaceae (CRE), New Delhi metallo- β -lactamase (NDM)-producing *Klebsiella pneumoniae* have all challenged the efficacy of antimicrobial chemotherapy [3-5]. Considerable research efforts are being focused on the development of new and effective chemotherapeutic agents. However, in the context of fast evolving antibiotic resistance mechanisms, it is critical to preserve the efficacy of available antibiotics by prudent and judicious use.

Overview of bacterial resistance mechanisms

Bacteria have evolved diverse mechanisms to overcome antibiotic pressure. Although exposure to antibiotics is a key reason for the development of antibiotic resistance, bacteria may also be intrinsically resistant to certain antimicrobials [6]. The common mechanisms of bacterial resistance to antibiotics include i) Enzymatic degradation of the antibiotics with the help of enzymes which hydrolyze antibiotics into ineffective compounds, (ii) Enzymatic modification of cellular targets of antibiotics such as the ribosomal protein subunits, nucleic acids, metabolic enzymes and the bacterial cell wall components so that the antibiotic is unable to effectively bind to its intended target, (iii) modification of cell membrane leading to reduced permeability of the antibiotic into the cytoplasm of the bacterial cell, (iv) active extrusion of the antibiotics by membrane efflux pumps thereby reducing the cellular concentrations of antibiotics to levels insufficient to kill or halt the growth of bacteria [6,7]. Bacteria may be single drug or multidrug resistant (MDR). MDR bacteria possess multiple mechanisms of antibiotic resistance and such abilities are encoded on mobile genetic elements such as the transposons and plasmids [8]. Horizontal transmission of the genetic elements such as plasmid and transposons through molecular mechanisms such as transformation, conjugation or phage-mediated transduction are responsible for the acquisition of multiple mechanisms of drug resistance [7,9]. On the other hand, many bacteria are intrinsically resistant to certain antibiotics. A classic example of intrinsic resistance is the antimicrobial resistance conferred by the chromosomally encoded marRAB locus of *Escherichia coli* [10]. This genetic element is responsible for the

intrinsic resistance of *Escherichia coli* to diverse antibiotics including penicillins, cephalosporins, fluorquinolones, tetracyclines, chloramphenicol, nalidixic acid, rifampin etc. [11]. The AcrAB efflux pump located on the mar locus is responsible for the antibiotic resistance phenotype of the Gram-negative bacteria harboring marRAB locus [11,12].

Secondary active transporters

Bacterial mechanism of transporting structurally diverse molecules across the membrane involves versatile membrane transporters classified as primary and secondary active transporters. The primary active transporter proteins use the energy derived from the hydrolysis of ATP, while the secondary active transporters make use of an ionic gradient across the membrane to energize the transport of solutes against a concentration gradient [13]. Secondary active transporters utilize the energy gradient created by H^+ or Na^+ across the membrane and transport diverse molecules such as salts, sugars, vitamins, fatty acids, amino acids, Krebs's cycle intermediates and toxic metabolites [14]. Secondary active transporters are of two types; symporters, which transport the substrate and the ions in the same direction, and the antiporters, which transport ions and the substrates in opposite directions [15,16]. Based on the sequence homology, secondary active transporter proteins responsible for antimicrobial efflux are classified into 4 major families namely i) the major facilitator super family (MFS), ii) the resistance-nodulation-cell division transporter super family (RND), iii) the small multidrug resistant transporter super family (SMR), and iv) the multiple antimicrobial extrusion protein super family (MATE). MFS, RND and SMR groups of efflux pumps are H^+ /drug antiporters, while the MATE group of efflux proteins are Na^+ /drug antiporters [17,18]. Apart from these secondary active efflux proteins, bacteria also harbor ATP-binding cassette (ABC) transporters which require hydrolysis of ATP to drive the extrusion of antimicrobial agents from the bacterial cell [19].

Efflux pumps and the antibiotic resistance

Efflux pumps are widely distributed in Gram-positive and Gram-negative bacteria. The genome of *Escherichia coli* has about 39 putative efflux pumps, while the corresponding number in the genome of *Staphylococcus aureus* is 31 [20,21], although the number of efflux pumps need not necessarily determine the extent of antibiotic resistance [22]. The RND family of efflux pumps is specific to Gram-negative bacteria with AcrAB of *Escherichia coli* being one of the earliest and well characterized RND efflux pumps. Similarly, MFS efflux pumps are prominent in Gram-positive bacteria. NorA of *Staphylococcus aureus* is an extensively studied MFS efflux pump [23]. With the realization of the significance of efflux pumps in the resistance of bacteria to antimicrobial compounds and the availability of whole genome sequences of bacteria, several new efflux pumps have been identified and characterized, some of which are clinically very important [24,25]. Due to inherent problems in the crystallization of membrane proteins for X-ray crystallography, researchers have largely relied on in silico prediction of the efflux pump structures to understand their structure function relationships [26]. In the context of pathogenic bacteria rapidly gaining antimicrobial resistance, inhibition of efflux activity of MDR efflux pumps is an attractive approach towards restoring the efficacy of antibiotics [27]. Natural compounds such as the plant extracts are preferred owing to their generally non-toxic nature.

Plant-based compounds as potential efflux pump inhibitors

Before the discovery of antibiotics, treatment of microbial infections relied extensively on herbal medicine, especially in the Asian subcontinent. Plants produce potent antimicrobials as self-defense to overcome bacterial and fungal infections and these substances may possess bactericidal or bacteriostatic properties. Plant-derived compounds are promising as efflux pump inhibitors (EPIs) which can potentially restore the efficacy of antibiotics by blocking the activities of efflux pumps and thus allowing the antibiotics to reach the minimum bactericidal concentration inside the bacterial cell [28]. Since numerous plant-based preparations are known to be antibacterial, analysis of such preparations using modern analytical tools such as the High Performance Liquid Chromatography (HPLC) and Tandem Mass Spectrophotometry (MS/MS) will help to identify the compounds responsible for antimicrobial activities. Several recent reviews have elaborated on the usefulness of plant-derived compounds as potent efflux pump inhibitors [29,30]. EPIs can inhibit efflux pumps by any of the mechanisms depicted in Figure 1. *In vitro* studies have shown that EPIs can restore the efficacy of an antibiotic by rendering a resistant bacteria susceptible to the antibiotic, reduce the Minimum Inhibitory Concentrations (MICs) of antibiotics and reduce the chances of resistance development when used in combination with the antibiotics [30,31].

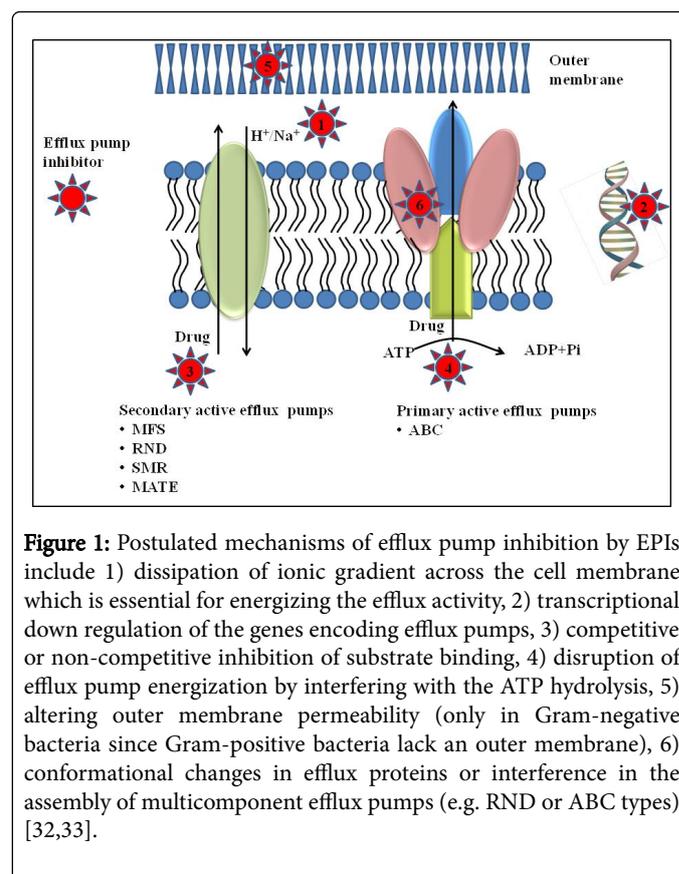


Figure 1: Postulated mechanisms of efflux pump inhibition by EPIs include 1) dissipation of ionic gradient across the cell membrane which is essential for energizing the efflux activity, 2) transcriptional down regulation of the genes encoding efflux pumps, 3) competitive or non-competitive inhibition of substrate binding, 4) disruption of efflux pump energization by interfering with the ATP hydrolysis, 5) altering outer membrane permeability (only in Gram-negative bacteria since Gram-positive bacteria lack an outer membrane), 6) conformational changes in efflux proteins or interference in the assembly of multicomponent efflux pumps (e.g. RND or ABC types) [32,33].

One of the earliest EPI discovered from plant sources is the plant alkaloid reserpine which was shown to be an effective inhibitor of Bmr efflux pump of *Bacillus subtilis* [34]. However, due to the toxicity of reserpine to humans, its application is restricted to *in vitro* studies involving efflux pump inhibitions. Belofsky et al. [35] found that a flavonoid and phenolic compounds extracted from the plant *Dalea*

versicolor potentiated the activities of berberine and some antibiotics possibly due to efflux pump inhibition. Similarly, isoflavones extracted from *Lupinus argenteus* was found to potentiate the antibacterial activities of alpha-linolenic acid found in the same plant, as well as the plant antimicrobial berberine and the antibiotic fluoroquinolone (Table 1) [36].

Baicalein derived from the Chinese medicinal plant *Scutellaria baicalensis* Georgi is known to significantly enhance the activities of β -lactam antibiotics, tetracycline and tetracycline against MRSA [37]. Diterpenes such as ferruginol extracted from the cones of *Chamaecyparis lawsoniana* exhibited efflux modulatory activities in methicillin-resistant *Staphylococcus aureus* and also potentiated oxacillin activity against MRSA [38]. Ferruginol could result in 40% reduction in ethidium bromide efflux by a MDR strain of *Staphylococcus aureus* harboring NorA efflux pump [38]. Another study evaluated 13 different phytoalkaloids as efflux inhibitors and found that alkaloids such as quinine, piperine and harmaline exhibited significant efflux inhibitory activities against methicillin resistant *Staphylococcus aureus* [39]. Similarly, plant alkaloids ellagic and tannic acids not only potentiated the antibacterial activities of novobiocin, coumermycin, chlorobiocin, rifampicin and fusidic acid against *Acinetobacter baumannii*, but also resulted in the inhibition of efflux activities [40]. Indirubin, extracted from the leaves of *Wrightia tinctoria* exhibited antibacterial activity against MDR *Staphylococcus aureus* and *S. epidermidis*, and also inhibited efflux activity similar to reserpine. Although not experimentally determined, the ciprofloxacin potentiation activity of indirubin suggested possible inhibition of NorA efflux pump [41]. Cucurbitane-type triterpenoids extracted from aerial parts of *Momordica balsamina* significantly inhibited efflux of EtBr by a MRSA strain and *Enterococcus faecalis* [42]. Coumarins derived from *Mesua ferrea* have also been found to be potentiators of norfloxacin against clinical strains of *Staphylococcus aureus* as well as MRSA [43]. These compounds also significantly inhibited efflux activity in a NorA overexpressing strain of *Staphylococcus aureus* [43]. In a study by Dwivedi et al. [44] 4-hydroxy- α -tetralone (1) isolated from *Ammannia spp.* along with its semi-synthetic derivatives (1a-1e) was found to reduce the MIC of tetracycline to multidrug resistant *Escherichia coli*. Using Rhodamine 6 G accumulation assay, it has been shown that the ethanolic leaf extracts of two plant species *Callistemon citrinus* and *Vernonia adoensis* from Zimbabwe were inhibitors of efflux pumps of *Staphylococcus aureus* [45]. In continuation of this finding, it was shown that alkaloids from *Callistemon citrinus* were more effective in inhibiting the efflux activities in *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The mechanism of efflux

inhibition involved blocking of ATP-dependent transport of solutes across the membrane [46]. In addition, these extracts increased the permeability of cell membranes of *Staphylococcus aureus* and *Pseudomonas aeruginosa* which presumably enhanced the movement of antibiotic across the membrane [45]. Of the several extracts from the roots of the African medicinal plant *Zanthoxylum capense* Thunb. (Rutaceae), the phenylpropanoid (+)-ailanthoidiol (6) and its ester derivatives exhibited efflux pump inhibitory activities in *Staphylococcus aureus* and enhance the ciprofloxacin activity against the MRSA strain [47]. Aromatic plant-derived monoterpene phenols thymol and carvacrol have been reported to be efflux pump inhibitors in food-borne pathogens such as *Salmonella enteritidis*. Thymol was found to be more effective in reducing the MICs of tetracycline and benzalkonium chloride [48]. Extracts of *Anadenanthera colubrina* var. *cebil* enhanced the antimicrobial activities of aminoglycosides against MDR *Staphylococcus aureus* [49]. Essential oils (EOs) from *Salvia fruticosa* could reduce the MICs of tetracycline by inhibition of efflux pumps and decrease the expression of *tet(K)* gene in tetracycline resistant clinical isolates of *Salmonella enteritidis* [50]. A gallotannin 1,2,6-tri-O-galloyl- β -D-glucopyranose extracted from *Terminalia chebula* inhibited efflux pump activity-dependent ethidium bromide extrusion in a multidrug resistant *Escherichia coli* [51]. A recent reported the LmrS efflux pump inhibitory activity of the spice cumin in a dose dependent manner [52]. Conessine, a steroidal alkaloid compound derived from the plant *Holarrhena antidysenterica*, exhibited synergistic activities with cefotaxime, levofloxacin, and tetracycline resulting in their significantly reduced MICs to *Pseudomonas aeruginosa* by inhibiting MexAB-OprM efflux pump [54].

The real time gene expression suggested significant down regulation of ABC-type efflux pump gene *yojI* and the affinity of the compounds 1 and 1e with *YojI* was confirmed by molecular docking studies [44]. While the inhibition of efflux activity and potentiation of antibiotics could be ascertained by MIC and ethidium bromide efflux assays, it is relatively difficult to determine if one or more efflux pumps are inhibited, and to identify the target efflux pump. The real time PCR would help to determine if known efflux pumps are under expressed due to the activities of inhibitory compounds. For example, Aghayan et al. [54] investigated the effect of two EPIs Berberine and Palmatine on the expression patterns of *mexA*, *mexB*, *mexC*, *mexD*, *mexE*, *mexF* and *mexX* in *Pseudomonas aeruginosa* isolated from burn infections. This study revealed that the effect of these EPIs was more on the MexAB-OprM operon [53].

Plant source	Active compound	Target bacterium	Target efflux pump	Reference
<i>Ammannia spp.</i>	4-hydroxy- α -tetralone (1) & semisynthetic derivatives	<i>Escherichia coli</i>	YojI	[44]
<i>Anadenanthera colubrina</i>	ND	<i>Staphylococcus aureus</i>	ND	[49]
<i>Berberis aetnensis</i>	Phosphoribide	<i>Staphylococcus aureus</i>	Multiple efflux pumps	[54]
<i>Berberis vulgaris</i>	Berberine and Palmatine	<i>Pseudomonas aeruginosa</i>	MexAB-OprM	[55]
<i>Callistemon citrinus</i> and <i>Vernonia adoensis</i>	Ethanolic leaf extracts	<i>Staphylococcus aureus</i>	ATP-dependent efflux pump	[45]
<i>Capsicum spp.</i>	Capsaicin (8-methyl-N-vanillyl-6-nonenamide)	<i>Staphylococcus aureus</i>	NorA	[56]
<i>Chamaecyparis lawsoniana</i>	Diterpenes (Ferruginol)	<i>Staphylococcus aureus</i>	NorA	[38]

<i>Chamaecyparis nootkatensis</i>	phenolic diterpene totarol	<i>Staphylococcus aureus</i>	NorA	[57]
<i>Dalea versicolor</i>	Flavanoid, phenolic compounds	<i>Staphylococcus aureus</i> , <i>Bacillus cereus</i>	NorA	[36]
<i>Holarrhena antidysenterica</i>	Conessine	<i>Pseudomonas aeruginosa</i>	MexAB-OprM	[53]
<i>Hypericum olympicum</i>	Compound 1	<i>Staphylococcus aureus</i>	NorA	[58]
<i>Lupinus argenteus</i>	Isoflavones	<i>Staphylococcus aureus</i>	NorA	[36]
<i>Mesua ferrea</i>	Coumarins	<i>Staphylococcus aureus</i>	NorA	[43]
<i>Momordica balsamina</i>	Cucurbitane-type triterpenoids	<i>Staphylococcus aureus</i> , <i>Enterococcus faecalis</i>	-	[42]
<i>Persea lingue</i>	Kaempferol rhamnoside	<i>Staphylococcus aureus</i>	NorA	[59]
<i>Rosmarinus officinalis</i>	Abietane diterpenes	<i>Staphylococcus epidermis</i>	Msr(A), Tet(K)	[60]
<i>Salvia fruticosa</i>	Essential oils	<i>Staphylococcus aureus</i>	TetK	[50]
<i>Scutellaria baicalensis Georgi</i>	Baicalein	<i>Salmonella enteritidis</i>	NorA	[37]
<i>Terminalia chebula</i>	Gallotannin 1,2,6-tri-O-galloyl- β -D-glucopyranose	<i>Escherichia coli</i>	ND	[51]
<i>Various aromatic plants</i>	Thymol and carvacrol	Food-borne pathogens	ND	[48]
<i>Wrightia tinctoria</i>	Indirubin	<i>Staphylococcus aureus</i> , <i>Staphylococcus epidermidis</i>	NorA	[41]
<i>Zanthoxylum capense Thunb.</i>	Phenylpropanoid (+)-ailanthoidiol (6)	<i>Staphylococcus aureus</i>	ND	[47]

Table 1: Efflux pump inhibitor compounds from plant sources, their target efflux proteins and the bacterial pathogens inhibited.

The gene expression analysis approach is confounded by the fact that the effect of many efflux inhibitors could be at the protein level or they may be simply acting by diffusing the ionic gradient required for driving the ant port activity [61]. In addition, a bacterium may possess many efflux pumps including several uncharacterized or even unidentified efflux pumps. Microarray is an alternative approach which can help to identify unknown efflux pump genes modulated by the activities of inhibitory compounds. Further, molecular docking is a powerful tool to understand the interactions of natural compounds with the efflux pumps and to identify the actual target sites of inhibitory compounds [62]. In a recent study, 328 secondary plant metabolites were screened for their inhibitory activity against cytoplasmic exporter protein MexB of MexAB-OprM efflux pump of *Pseudomonas aeruginosa*. *In vitro* studies showed that p-coumaric acid was able to potentiate the antibacterial activity of ciprofloxacin in MexAB-OprM overexpressing strain of *Pseudomonas aeruginosa*. Further, in silico studies showed that a p-coumaric acid derivative 4-(4-((Z)-2-carboxy-2-((Z)-2,3-dihydrobenzo[e][1,4]diazepin-1-yl)-1-(4-hydroxyphenyl)vinylamino) phenylsulfonamido)-2-hydroxybenzoic acid was able to dock more efficiently than the parent compound and could act as a more potent inhibitor of RNA efflux pumps [63].

Conclusions

The potential role of EPIs in overcoming efflux mediated antibiotic resistance in pathogenic bacteria is increasingly being recognized. EPIs can restore the activities of antibiotics wherever efflux is the sole mechanism of antibiotic resistance. Medicinal plants with known antimicrobial activities can make good candidates to search for

potential EPIs. High throughput screening methods have enabled testing of large number of compounds, both natural as well as their semisynthetic or synthetic derivatives. EPIs identified by *in vitro* approach can be subjected to structural elucidation followed by in silico docking analysis to identify the molecular interactions between the EPIs and their targets. Understanding the molecular interactions will help in modifying EPIs for better binding with their target efflux pumps and hence, more efficient inhibition of efflux pumps. Thus, EPIs are highly promising as potential adjuvants to maintain the efficacy of antibiotics in the treatment of infectious diseases.

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